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CPT and Zeocin Induce Increased Levels of p53 Independent of Cell Cycle Stage

by

Sandra D. Houser

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

2003

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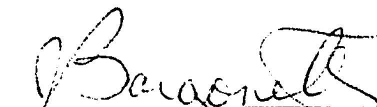

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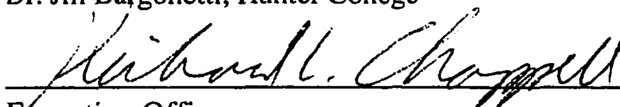

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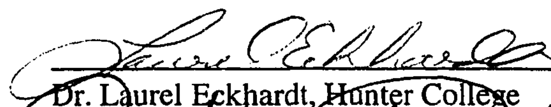

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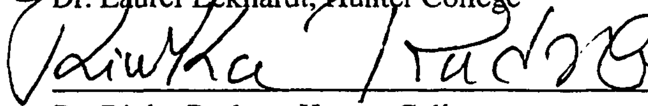

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ABSTRACT

CPT and Zeocin Induce Increased Levels of p53

Independent of Cell Cycle Stage

by

Sandra D. Houser

Advisor: Professor Jill Bargonetti

We investigated how DNA damage activates the tumor suppressor p53 during the cell cycle. Centrifugal elutriation was utilized to separate exponentially growing ML-1 cells (containing wild-type p53). Distinct fractions were subjected to the DNA damaging agents Camptothecin (Cpt) (topoisomerase I inhibitor) (Hisang, Y., *et al,* 1989) and Zeocin (bleomycin/phleomycin family of antibiotics) (Burger, R. M., 1998, *et al.*, 1994,) and analyzed for p53 levels, p53 DNA binding properties to p53-targeted genes, phosphorylation at serine 392 (a phosphoserine site located in the C-terminus of the protein) and cleavage states of p53.

By using gel shift and Western blot analysis we observed that both drugs induced DNA binding p53. However only the Cpt-induced p53 was phosphorylated at serine-392, while cells treated with Zeocin-induced a form of p53 phosphorylated at serine-15. Phosphorylation at serine-392 occurred in every cell cycle stage and this phosphorylated form of p53 (approximately 40 kDa) migrated more rapidly on the SDS-PAGE gel. Apoptosis occurred in every cell cycle fraction, suggesting that all the machinery to

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initiate the apoptotic response as well as the increase in p53 is present throughout the cell cycle (Houser, S., et al., 2001).

By using Western blot analysis with different p53 specific antibodies, we discovered that p53 was not induced when DNA replication was blocked for a short time with aphidicolin (APH) alone but, transcriptionally active p53 levels were stabilized in the presence of Zeocin and Cpt. This suggests that Zeocin and Cpt both activated p53 and Cpt, whose induction of p53 is said to require the replication process (Hisang, Y., *et al.*, 1989), can signal p53 to be activated when DNA synthesis is blocked. Non-cycling cells treated with higher doses of both drugs incorporated bromodeoxyuridine (BrdU). This incorporation of BrdU is the result of cells utilizing deoxynucleotides (dNTPs) in DNA repair synthesis, which is necessary throughout the cell cycle. We and others have found that the stabilization of p53 is not cell cycle-dependent and that different types of DNA damage (induced by Cpt and Zeocin) can signal for increased levels of differentially modified p53.

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To my colleagues, thank you for all your help. A special thanks to a friend, Angela Dixion, who showed me inner strength.

To my family: my great grandmother, Betty Rebecca, who taught her daughter, Amanda Fair, the grace and dignity she taught my mother, Rita Houser. Because a force so caring, strong and righteous has to grow over generations. The gift that they have given me they will never know. True teachers.

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DEDICATION

This thesis is dedicated to my mother, Okarita Houser. No words can express what you have taught me. You are a powerful guiding force in my life, still. A true teacher is not characterized by the knowledge she or he knows but the knowledge that she or he is able to teach to others and how she or he teaches that knowledge. To my Father, Roland Houser II, my brothers Roland and William Houser and my sister Lessie Houser.

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Chapter 1

Introduction

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Introduction

The human p53 gene that includes approximately 20 kb of genomic DNA and contains 11 exons has been localized to the short arm of chromosome 17 (17p13) (Oren, M., *et al,* 1985; Soussi, T., *et al,* 1990; Benchimol, S., *et al,* 1985). By cross-species comparison of amino acid sequences and nucleotide analysis, five evolutionary conserved regions within amino acid residues 13-23, 117-142, 171-181, 234-250 and 270-286 were discovered. Humans, mice, frogs and drosophila contain these conserved sequences in exons 1, 4, 5, and 8. These regions termed domains I-V are thought to be essential for the normal functioning of p53 (Soussi, T., *et al,* 1990; Soussi and May, *et al,* 1996). The p53 protein was discovered in SV-40 transformed cells when it was seen to coimmunoprecipitate with the SV-40 large T antigen (Lane, D.P. and Crawford, L. V., *et al,* 1979; Linzer, D. L. H. and Levine, A. J., *et al,* 1979) leading to its mistaken categorization as a tumor antigen. It was then considered an oncogene because of its ability to immortalize cells by itself or in the company of the Ras oncogene (Jenkins, J. R., *et al.*, 1984; Rovinski, B., and Benchimol, S., *et al.*, 1988). However, with growing evidence such as tumor cell lines transfected with wild-type p53 showing reduced or terminated cell growth and division (Mercer, W. E. *et al.*, 1990a ; Chen, P. L., *et al.*, 1990; Mercer, W. E., *et al.*, 1990b) and p53-deficient mice, with two p53 null alleles, being more prone to tumors at a young age (Donehower, L. A., *et al.*, 1992) pointing to a tumor suppressive activity, p53 was therefore reclassified as a tumor suppressor gene in 1990.

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What thrust p53 into the spotlight once it was discovered, was that the most commonly seen genetic events in human cancers were alterations of the p53 gene and the protein that it encoded. It was found to be the most commonly mutated gene in about 60 to 70 % of most types of human cancers (Levine, A. J., *et al,* 1991; Hollstein *et al.*, 1991; Caron de Fromentel, C., *et al,* 1992). The majority of mutations were amino acid substitutions such as missense mutations in amino acid regions (codons 175, 249, 273 and 282) critical for DNA binding (Greenblatt, M. S., *et al.*, 1994; Cho, Y., *et al.*, 1994). These mutations usually altered the conformation of the p53 protein causing nuclear accumulation of a protein that exhibited characteristics of a dominant oncogene. In its wild-type or non-mutated form, p53 has the characteristics of a recessive tumor suppressor gene and behaves clearly as an anti-oncogene. This anti-oncogenic product functions as a negative regulator of cell proliferation and division (Sarnow, P., *et al.*, 1982; Werness, B. A., *et al.*, 1990).

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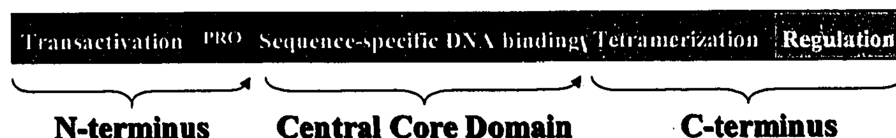
What is p53?



- **Tumor suppressor gene**
- **Encodes for a 53 kDa nuclear phosphoprotein**
- **Has three structural and five functional domains: N-terminal domain, Central domain and C- terminal domain**
- **Functions as a transcription factor**

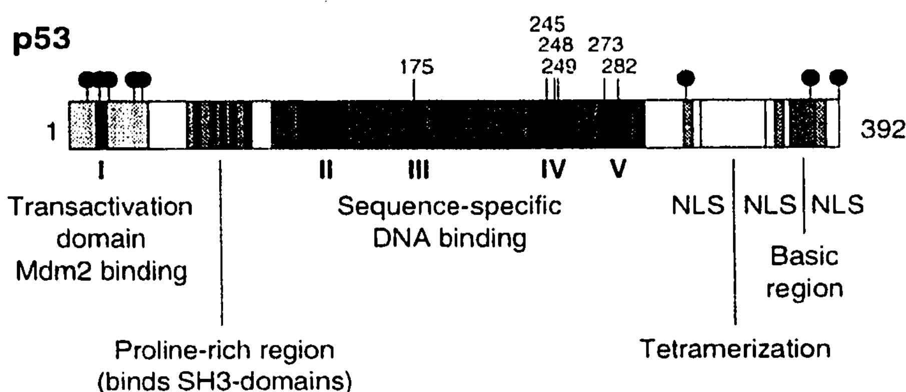


Figure 1: Schematics of p53 protein. Conserved sequences I-V in the central domain and mutations of residues within these regions and the structural and functional domains of p53 are shown. NLS stands for nuclear localization signal (Kubbutant, M. H. G., and Vousden, K., et al, 1998) and PRO stands for proline.

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The mRNA transcript from the p53 gene is approximately 2.8 kb in length. It encodes a 53 kd nuclear phosphoprotein (Soussi, T., *et al.*, 1990) which is 393 amino acid long (Figure 1). It has functional and structural domains that work together to contribute to its function regulating the cell cycle. Amino acids 1-42 and 43-63 in the amino terminus are involved in transactivation and region (amino acids 62-91) in the N-terminus has been shown to be associated with apoptosis (Oda, K., *et al.*, 2000). This acidic proline-rich (N-terminus) region of p53 has the ability to associate with such transcription factors as the TATA binding protein (TBP) and the TBP-associated factors (TAFs), which are components of the TFIID transcription complex (Thut, *et al.*, 1995; for review see Levine, A. J., *et al.*, 1997) (Figure 2).

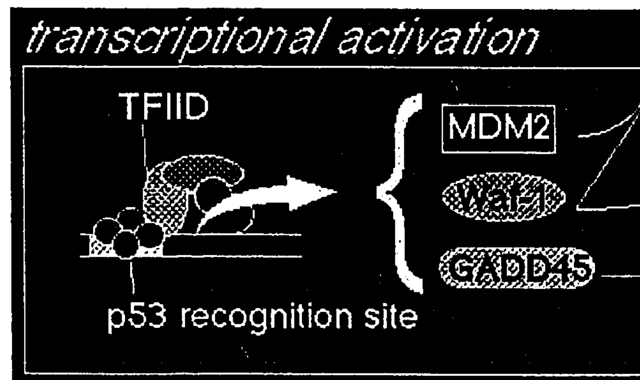

Figure 2: Schematic of the transcriptional activation of mdm2, p21^{WAF1} and gadd45 target genes by p53. Transcriptional activation of p53 involves direct interaction of the N-terminal transactivation domain of p53 with the multisubunit basal transcription factor TFIID (http://www.bogler.net/lab/p53expression.html).

These interactions illustrate a role for p53 in the regulating transcription. The N-terminus of p53 also interacts with the single stranded DNA-binding protein RP-A and

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the p62 subunit of the transcription repair factor TFIIH (Dutta *et al.*, 1993, Li and Botchan, *et al.*, 1993).

The central region (aa 100-300) contains a sequence-specific DNA binding domain (Bargonetti, J., *et al.*, 1993). We have learned by x-ray crystallography that this domain contains four highly conserved regions (II-V). These regions are involved in contacting the major and minor grooves of a p53 binding site (Cho, *et al.*, 1994). 80-90% of the mutations (mostly missense mutations) that occur within p53 lie within this region. There are a number of "hot spot" mutations that occur within the central region of p53, which are critical to suppressing the tumor suppressive effects of p53. X-ray crystallography studies show that the two most highly mutated residues were found within regions that directly contact DNA (residues 248 and 273). Four other "hot spot" residues (175, 245, 249, and 282) were found that are crucial in stabilizing the DNA binding region of p53 (Cho, *et al.*, 1994). Mutations in these residues can lead to alterations in p53 DNA binding activity and possible tumorigenesis (Hollstein, *et al.*, 1994).

The carboxyl-terminus (amino acids 300-392) is involved in oligomerization and non-specific DNA-binding with the last 30 amino acids of the carboxyl-terminus involved in the negative regulation of p53 DNA binding activity (Jayaraman and Prives, *et al.*, 1995, Reed, M. *et al.*, 1995). The C-terminal domain is subdivided into three regions: a flexible linker (300-360), oligomerization region and the last 30 amino acids of the C-terminus that is made up of mostly basic residues (360-393) (Jeffrey, *et al.*, 1995). The oligomerization region is made-up of beta-sheet-turn alpha helix motifs that are able to

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Consensus binding site for p53

- **2 copies of a 10 bp motif**
  5'-PuPuPuC(A/T)(T/A)GPyPyPy-3'
  separated by 0-13 bp

- **up to 300 binding sites for p53 protein**
  exists in the human genome

Figure 3: Consensus binding site (p53 binding site or p53 response element, p53RE) present in p53-target genes. p53 recognizes and binds to genes that contain this consensus sequence in a sequence-specific manner.

homodimerize (Pavletich, N. P., *et al.*, 1993). Two of these homodimers constitute the p53 tetramer (Pavletich, N. P., *et al.*, 1993).

p53 acts as a major molecular guardian for genomic stability or integrity. When DNA damage occurs, p53 is induced, stabilized or activated and arrests the cell until the damage can be repaired. If the damage is unrepairable, p53 may sensitize cells to or aid in programmed cell death (apoptosis) as observed in studies when wild-type p53 was

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added to certain types of tumor cells (Yonish-Rouach, E., *et al.*, 1991; Shaw, P., *et al.*, 1992).

The tetrameric p53 protein binds to two repeats of the DNA sequence 5'-Pu Pu Pu C (A/T) (T/A)G Py Py Py-3' which are separated by 0-13 bp (Figure 3). This is known as the p53 consensus sequence (El-Deiry, W. S., *et al.*, 1992). Several known p53-target genes containing p53-binding sites have been identified (Figure 4). Each of these genes contains DNA sequences with high homology to the p53 consensus sequence, varying only in a few base pairs. Bax has four potential p53-binding sites containing eight mismatches per 40 base pairs (Miyashita, T., et al., 1995). Bax contains one perfect p53 binding site (10 out of 10 base pairs, no mismatches) but, the other three contain eight mismatches/30 basepairs) and was thus labeled imperfect by Miyashita and coworkers. These imperfect p53 binding sites overlap with or are adjacent to the perfect one. Imperfect does not mean it is unable to bind to p53 because the other p53 binding sites within mdm2, gadd45 and waf1 also have mismatches and they have been shown to bind to p53. Mdm2 has two p53-binding sites containing five mismatches per 40 base pairs (Zauberman, A., et al., 1995). Waf 1 has two p53-binding sites containing four mismatches per 40 base pairs (El-Deiry, W. S., et al., 1995). Gadd45 has one p53-binding site containing one mismatch per 20 base pairs (Hollander, M.C., et al., 1993). Its p53RE has the highest homology to the p53 consensus sequence. Studies done by S. Kahu and co-workers showed that requirement for activation of p53 targeted genes $p21^{WAF1}$ and mdm2 differ based on the configuration and number of p53 binding sites within each target gene. There was a difference in the binding of p53 to the p53RE in the $p21^{WAF1}$ promoter which contains a single p53RE compared to the p53RE in the mdm2 promoter

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that contains two p53REs linked by a 17 bp spacer. PAb 421 antibody, which mimics C-terminal activation by phosphorylation or acetylation, was required in EMSAs when binding to the single p21^{WAF1} p53RE but this requirement was eliminated when the two p21^{WAF1} p53REs were linked with the 17bp spacer region from mdm2 consensus sequence. This suggested that target gene promoters with a single p53 RE requires C-terminal activation for p53 to bind unlike the two naturally occurring p53 binding sites in mdm2 (Kahu, S. et al., 2001). These two genes may be regulated by different mechanisms, one involving C-terminal activation. The MDM2 p53RE was reported to have 5 mismatches per 40bp and p21^{WAF1} p53RE to have 4 mismatches per 40bp but PAb 421 antibody was not required for recombinant p53 to bind to the p53RE in mdm2. This suggested that the degree of homology to the ideal p53 consensus sequence is not the only factor governing p53 binding ability to a specific target p53 element. This particular configuration of mdm2's p53RE could alleviate the requirement for p53 post-translational modification and activation and modulate transcriptional activation kinetics of different p53 genes to regulate p53 responses.

Another study by Xiao-Tang Kong and coworkers suggested that the p53 hinge domain, which is a short linker (300-327 aa) between the DNA binding domain and tetramerization domain within the C-terminus, differentially activate the promoters of p53 target genes p21^{WAF1} and bax. When a mutation or short deletions was generated in this region, p53's ability to transcribe each gene promoter was altered (kong, Xiao-Tang, et al, 2001). Not only modification of the last 30 aa such as phosphorylation by CKII antibody, but interactions such as binding of proteins or PAb 421 antibody to regions within the C-terminus can activate the binding of p53 to interact with particular target

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genes. Another study by Stros suggests that the binding of proteins to the p53 hinge region described in the previous study can activate that protein. They reported that the DNA-bending/looping chromosome proteins HMGB1 and HMGB2 can stimulate p73 which is a member of the p53 family that is involved in neurogenesis and natural immune response. p73 has been reported to show similar tumor suppressor and structural properties to p53 with differences in the N- and C-terminal domainsBenard, J., et al, 2003). They suggest that this stimulation requires the region between the DNA binding domain and the oligomerization domain of p73 possibly the p53 hinge region (Stros, M., et al, 2002). The nature and configuration of the hinge region within the C-terminal domain may allow p53 flexibility in binding to different p53 binding sites with each gene by protein triggered interaction that changes the configuration and thus affinity of p53 for specific target genes.

The protein has been implicated in the control of the cell cycle, DNA repair and in certain cell types, programmed cell death (Levine, A. J., *et al.*, 1997, Lane, D. P. *et al.*, 1992).

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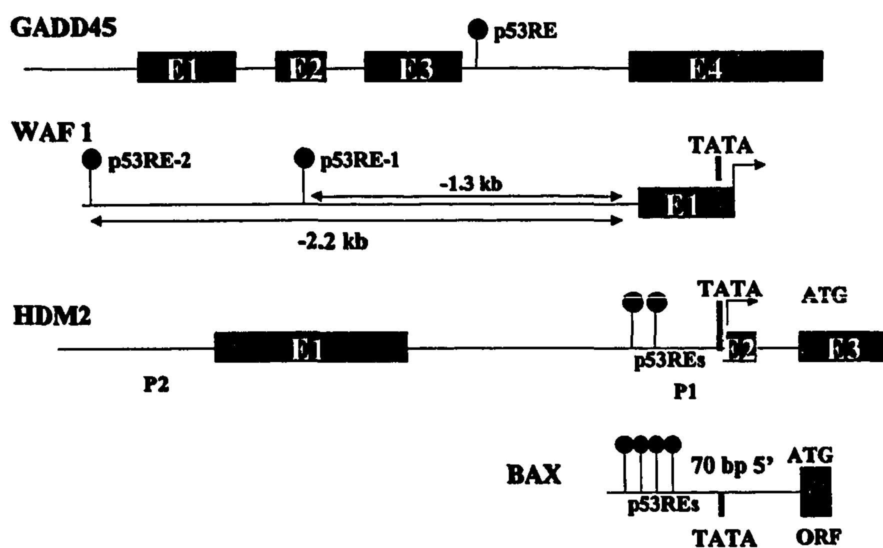
The p53 protein recognizes and binds in a sequence-specific manner to various target genes:



p53 consensus sequence:		PPPCWWGYYY	PPPCWWGYYY
GADD45	p53 RE:	GAACATGTCT	AAGCATGCT g
WAF1	p53RE1:	GAACATGTCC	c AACATGTT g
WAF1	p53RE2:	GAAgAAG aCT	GGGCAT GTCT
MDM2	p53RE1:	GG tCAAGT TC	AGACAcGTT C
MDM2	p53RE2:	AG t t AAGTCC	t GACTTGTC T
Bax	p53RE1:	AGACAAGCCT	
Bax	p53RE2:	t cACAAGTTa	
Bax	p53RE3:	GGGCg TGg gC	
Bax	p53RE4:	GGGCT At a TT	

Figure 4: Target genes that have been identified with p53REs or consensus sequences. Each contains a specific p53 binding sequence that is located at different sites from its transcription start site (TATA region). p53 has different binding affinities to the p53REs that have different versions of the p53 consensus binding sequence. Bottom panel shows the mismatches (lowercase letters) within the different p53 consensus sequences. Pu stands for purine (A or G), Py stands for pyrimidine (G or T) and W stands for A or T. Bax imperfect p53REs, 3-4, overlap with or are adjacent to the perfect one, p53RE1.

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These p53-induced cellular responses occur through transcriptional regulation of a number of genes such as p21 ^{WAF1}, gadd45, bax, and mdm2 (Ko and Prives *et al.*, 1996; El-Deiry, W. S., *et al*, 1998). We and others have seen that the ability of p53 to positively regulate different genes like p21^{WAF1}, gadd45, mdm2 (growth arrest and DNA repair-specific genes; Ko and Prives *et al.*, 1996), p53AIP1, puma, noxa and bax (apoptotic-specific genes; Oda, K., *et al.*, 2000; Nakano, K., *et al*, 2000) is not coordinately controlled. Rather different genes are activated by p53 under different circumstances (El-Deiry, W. S., *et al*, 1998). These differences might be explained by a number of different post-translational modifications such as cleavage (Molinari, M., *et al.*, 1996; Okorokov *et a*l., 1997), phosphorylation (Hupp, T. R., *et al.*, 1992 ; Milne *et al.*, 1992; McClure *et al.*, 1992; Ullrich *et al.*, 1992; Fiscella, *et al.*, 1993; Mayr *et al.*, 1995) and/or acetylation (Gu, W. and Roeder, R. G., *et al.*, 1997; Wang and Prives *et al.* 1995; Resnick-Silverman *et al.* 1998; Oda, K., *et al*, 2000). For instance, the severity of the damage determines whether a cell will undergo G1 arrest or apoptosis. Depending on the pattern of post-translational modification, p53 can be activated to bind to the promoters of G1 arrest genes (p21 ^{WAF1}), DNA repair genes, other genes such as mdm2 or apoptotic genes such as puma (Nakano, K., *et al*, 2001; Yu, J., *et al*, 2001), p53AIP1 (Oda, K., *et al*, 2000).

Other biochemical activities of p53 include its participation in the repair process. It has been shown to bind to and influence the DNA repair activity of such factors as XPB and XPD (Wang *et al.*, 1995). More recent data shows p53 interacting with the human Rad51 protein found to be a part of the mammalian recombination machinery (Buchlop *et al.*, 1997). This interaction with Rad51 can be inhibited with the PAb 421 antibody

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(epitope 372-381 aa) suggesting that a p53/Rad51 interaction may be involved in DNA recombination and repair (Buchlop *et al.*, 1997). p53 can also bind ss DNA ends non-specifically (Bakalkin *et al.*,1994) and has a strong DNA-DNA and RNA-RNA strand reannealing activity (Bakalkin *et al.*,1994; Oberosler *et al.*, 1993; Brain and Jeckins, *et al*, 1994). p53 may also regulate translation by directly affecting the translational machinery. Two studies provide evidence for interactions between p53 and ribosomes. In response to stress, murine p53 can be phosphorylated by casein kinase II at serine-398 (serine-392 in human p53) (Meek, D. W., and Eckhart, W., *et al*, 1990, 1988) and phosphorylation at these residues have been covalently linked to 5.8S rRNA (Fontoura, B. M. A., *et al*, 1992). This rRNA has an important role in the translation process (Walker, K., *et al*, 1990). It has been suggested that 5.8S rRNA may interact with the 28S rRNAs component of the 60S ribosomal unit. 5.8S rRNA has been shown to bind to proteins within both 60S and 40S ribosomal units. p53 through its possible interaction with 5.8S rRNA could influence or inhibit tRNA binding at the A site within the ribosomal unit and thus, translation of the protein (Walker, K., et al, 1990; Ulbrich, N., *et al.*, 1979). The 3' UTR sequence can repress translation supposedly through factors that bind RNA, which act at the 3' UTR (Fu, L.N., *et al*, 1996). The p53 protein itself has been shown to inhibit its own translation by binding to the 5' UTR of murine p53 mRNA (Mosner, J., *et al*, 1995). p53 also has a transrepressive activity on genes with p53 responsive sites. It requires an association between p53 and the TBP component of the TFIID complex (Seto *et al.*, 1992; Ragimov *et al.*, 1993; Truant *et al.*1993). It can also repress translation of proteins by through factors that bind RNA via its 3' untranslated region (UTR) (Fu, L. N., et al, 1996).

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Regulation of wild-type p53 stability, activation and DNA binding

Stability:

p53 is undetectable in healthy normal cells because of its rapid turnover rate (Kubbutant and Vousden, *et al,* 1998). Turnover is dependent on the ubiquitin-dependent 28S proteosome. Several copies of a small pepitide, ubiquitin (Ub), are added to the lysine residues of the protein to form chains (Varshavsky, *et al*, 1997). These chains act as flags to target p53 for degradation by the protein-degrading machinery (Varshavsky, *et al*, 1997). A family of enzymes called E1, E2, and E3 ubiquitin ligases participate in the addition of Ub to facilitate p53 degradation by the 28S proteosome (Varshavsky, *et al*, 1997).

The conformation of p53 may be an important determinant for its degradation (Medcalf and Milner., *et al*, 1993). The study provides evidence that two unrelated regions located within portions of p53 (aa residues 1-43 and aa residues 100-150) can serve as degradation signals (Haupt *et al*, 1997, Li and Coffino *et al*, 1996). Accessibility to the residues 100-150 may depend on the phosphorylation or acetylation status of the protein.

MDM2 plays a major role in inactivating p53 (Figure 5). MDM2 binds to the N-terminal trans-activation domain of p53 preventing its interaction with other components of the basal transcriptional machinery complex and downstream target genes (Momand *et al.*, 1992; Oliner *et al.*, 1993). MDM2 can also export p53 from the nucleus to the

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cytoplasm where its transcriptional activation activity is abolished (Kubbutat and Vousden, *et al.*, 1998; Kubbutat *et al.*, 1999). In tumor cells with mdm2-resistant p53 mutants, levels are very stable and abnormally high as compared to wild-type p53 (Kubbutat and Vousden, *et al.*, 1998). When MDM2 and wild-type p53 are present in these cells, these p53 mutants expressed in MCF-7 breast cells deleted of the mdm2 binding region (p53**del** I- II) or deleted of the C-terminus (p53 **del** II- 370) undergo rapid degradation like their wild-type counterparts (Midgley and Lane, *et al.*, 1997). This may be due to the formation of mixed tetramers. p53 mutants with deletions in their N-terminus and/or C-terminus (inactive mutant forms) which do not have the transcriptional activation ability do not elicit a tumor suppressor response; they are unable to activate expression of mdm2.

MDM2 also plays a role in the degradation of p53. It harbors the ability to function as an E3 ubiquitin ligase, thus promoting the ubiquitination of p53 (Honda *et al.*, 1997). MDM2 binds to the N-terminus of p53 through its RING finger region (p53-N/C-MDM2) located in its extreme C-terminus, which harbors it E3-ligase activity. Once bound, MDM2 can target p53 for degradation by ubiquitination (Kubbutat *et al.*, 1999).

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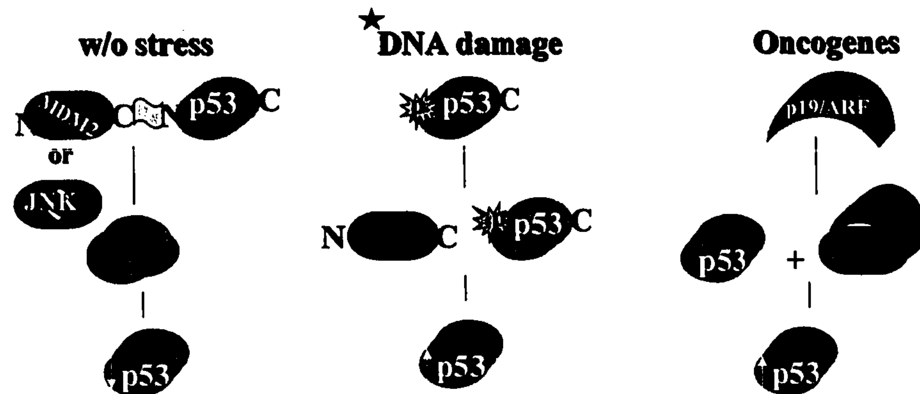

Figure 5: Regulation of p53 stability and activity. Wild-type p53 is degraded through the unbiquitin proteolysis pathway. Hdm2 or JNK proteins can bind to p53 and promote the ubiquitination of p53.

While MDM2 has been pegged the primary regulator of p53 stability, it may not be the only player in the ballgame. An alternative pathway involving the kinase JNK may play a role. JNK in its inactive form can regulate the stability of p53 independent of MDM2. p53 is able to interact with JNK when it is in its inactive form via JNK's p53 binding site to degrade p53. In response to UV damage, JNK becomes active and in this form it can no longer associate with the p53 protein to degrade it. Mice that are deficient in JNK have similar p53 dependent lethality characteristics as seen mice deficient in MDM2 (Fuchs, et al., 1998a).

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Changes in the phosphorylation sites within the N-terminus prevent p53 from forming complexes with MDM2 and/ or JNK resulting in it stabilization. Serine 20 (Unger *et al.*, 1999), serine 15 (Shieh *et al.*, 1997) and serine 33 (Fuchs *et al.*, 1998b) are resides that have been showed to reduce binding between p53 and MDM2 when phosphorylated. Different kinases phosphorylate these sites and others in the N-terminal region in response to different DNA damage signals (Figure 6). UV-induced DNA damage can induce p53 modifications that involve ATR and JNK (Jimenez, G. S., *et al*, 1999) (Figure 6). ATM (mutated in the ataxia telangiectasia gene product) may play a role in stabilizing p53 after IR-induced DNA damage (Kastan, M. B., *et al.*, 1992; Xu, Y. and Baltimore, D., *et al.*, 1996).

Studies have shown that DNA strand breaks induced by IR occur through an ATM/Nbs-1/Chk2 connection (Buscemi, G., et al 2001;Girard, P. M., et al., 2002). Nbs-1, a gene product of Nijmegan breakage syndrome, has been shown to play an important role in the activation of chk-2. In NBS defective cells, the phosphorylation and induction of chk-2 is defective. It has also been proposed by Girand and co-workers that Nbs-1 facilitates ATM-dependent phosphorylation of downstream substrates that are involved in G1 arrest such as the phosphorylation of p53 at serine 20 by chk-2 to generate a p53-mediated G1 arrest.

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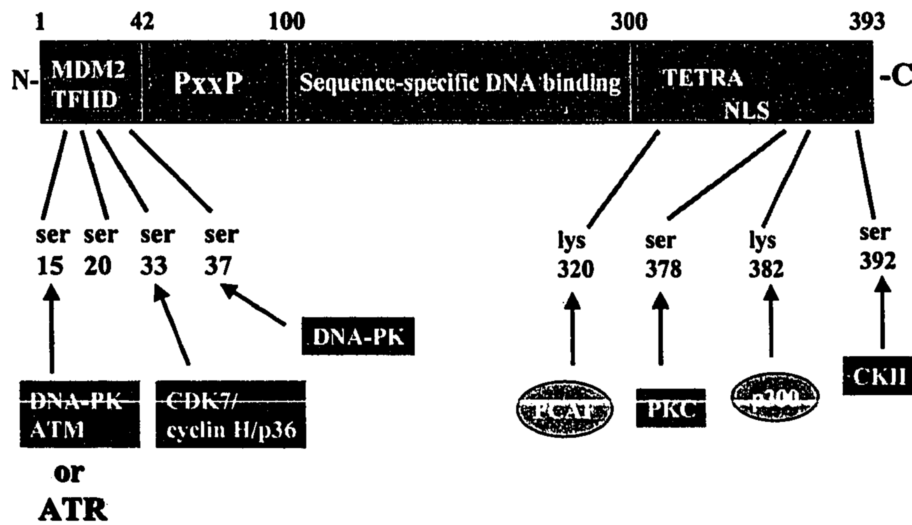

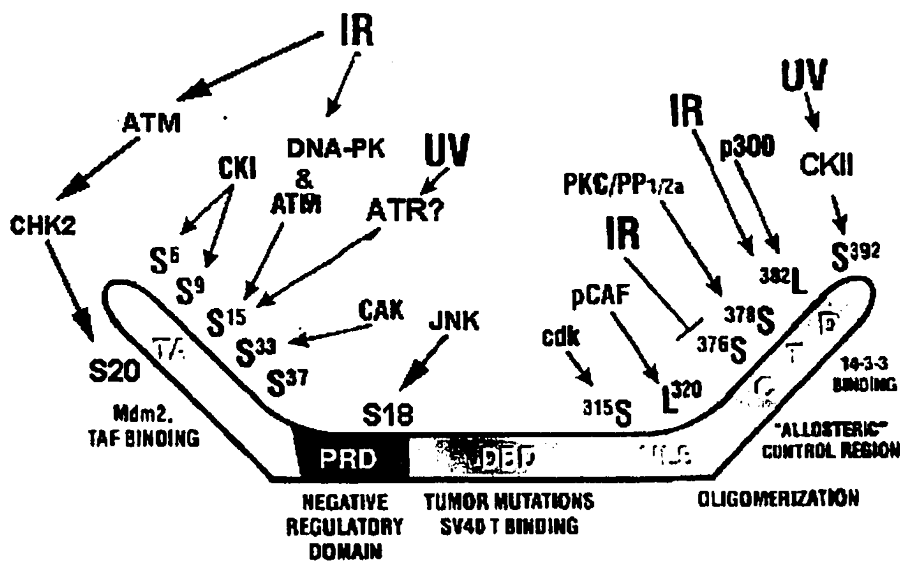

Figure 6. Schematics of the domains of p53. These schematics include many of the

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modifications (phosphorylation and acetylation) sites that occur within the N and C-terminus of 53 that are involved in its stabilization, activation or repression. Giaccia, A.J. and Kastan, M. B. Genes and Development 12: 2973-2983 (1998).

DNA double strand breaks (dsbs) have been shown to activate a kinase called DNA-dependent protein kinase (DNA-PK). This enzyme phosphorylates p53 at serine 15 and 37. However, it is not clear whether DNA-PK plays a role in stabilizing p53 from studies performed with cells derived from DNA-PK-deficient SCID (severe combined immunodeficient) mice. These studies show no detectable defects in their p53 response after DNA damage (Huang, L., *et al.*, 1996b; Rathmell, W, K *et al,* 1997). PKC has two sites, serine 376 and 378 that form a binding for 14-3-3 sigma that plays a role in G2/M arrest. This regulatory protein is dephosphorylated at serine 376 by a phosphatase that is activated by an ATM-dependent mechanism (Waterman, M. J., *et al.,* 1998). In response to stress p53 associated proteins can also be modified, MDM2 is phosphorylated by ATM (Maya, R. *et al,* 2001; Khosravi, R., *et al,* 1999) to impede its ability to bind p53 preventing its ubiquitin ligase activity and the export of p53 from the nucleus into the cytoplasm where it degraded (Mayo *et al.*, 1997).

Phosphorylation-dependent changes are but one mechanism which affects stability, p53 can also be covalently modified by acetylation at its C-terminus at lysine residues 320, 373 and 382 in response to several types of DNA damage (Figure 6). Not much is known about cellular responses initiated by acetylation, but based on several studies we can speculate lysine residues that are acetylated on p53 are no longer recognized by ubiquitin and degraded (Nakamura, S., *et al,* 2000).

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Oncogenes can play a role in regulating p53 stability and activity (Figure 5). A small tumor suppressor protein called p14^{ARF} (alternative reading frame) in humans and p19^{ARF} in the mouse that is encoded by the CDKN2/INKa gene can bind to MDM2 that is already bound to p53 and blocks MDM2's ubiquitin ligase activity (Honda and Yasuda, *et al*, 1999) and p53 export from the nucleus for degradation (Weber *et al*., 1999). The stabilization of p53 can also occur through oncogenes Ras, Myc and E1A through their activation of p14^{ARF} expression (de Stanchania, *et al*, 1998; Palmero, *et al*, 1998; Zindy, *et al.*, 1998). A p14^{ARF} /MDM2/p53 complex is formed as an attempt to prevent against abnormal growth.

Incubation with either double-strand or single-strand DNA can activate autoproteolytic activities of p53 that play a role in stabilizing p53. Cleavage of p53 generates p53 fragments lacking either the N or C-terminus. The smallest fragment of p53 generated, its DNA-binding core only, has been shown to have proteolytic activity with similarities of an aminopeptidase. p53 fragments lacking either its N or C-terminus could play a role its stabilization (Molinari, M., *et al*, 1996; Okorokov, A. L., *et al*, 1997). Deletion of the N-terminus could mimic the effects obtained when p53 becomes phosphorylated at its N-terminus. If the N-terminus is deleted, MDM2 can not bind to p53 and degrade it.

p53 has an element in its UTR (Fu, L., *et al*, 1996) that can inhibit the translation of its own mRNA and this inhibition is reversed in response to DNA damage. It has been reported that p53 levels increase rapidly in response to DNA damage without any clear changes in its mRNA levels (Fu, L. and Benchimol, S., *et al*, 1997). We explain this elevation of the protein level may be due to enhanced translation of the protein.

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DNA binding activity:

Following stabilization of the protein, p53 must be bound to DNA to affect the activation of transcription of its downstream target genes. Evidence suggests that the C-terminus regulates the sequence specific binding characteristics of the core region. First, the DNA binding activity of p53 is stimulated when the carboxyl-terminal 30 amino acids are removed from the C-terminal domain (Hupp *et al.*, 1992). Second, the monoclonal antibody PAb 421 binds to the C-terminal domain of p53 (aa 371-382) and can activate p53 DNA binding. Third, the DNA binding activity of p53 can also be stimulated by phosphorylation at serine 378 by protein kinase C (Hupp *et al.*, 1992; Takenaka *et al.*, *et al,* 1995), at serine 392 by casein kinase II (Meek, D. W., and Eckhardt, W., et al., 1990, 1988; Kapoor, M. and Lozano, G., *et al,* 1998). It has been shown by Prives and Wang that cyclin-dependent kinase phosphorylates p53 at the C-terminus at serine 315. This influences the targeting of p53 to specific p53 binding sites (Wang and Prives *et al.*, 1992).

There is evidence that modifications of the N-terminus can affect DNA binding. Cdk7 is a part of CAK (Cdk-activating kinase) complex which also is composed of cyclin H and Mat-1 proteins (Ko, L. J., *et al,* 1997; Lu, H., *et al,* 1997). The phosphorylation of p53 at serine 33 by CAK requires Mat-1, and increases p53 DNA binding activity (Ko, L. J., *et al,* 1997). p53 can also regulate CAK activity by binding with cycin H, this suggest a regulatory feedback loop relationship between p53 and CAK as in the case of

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MDM2 and p19^{ARF}. The post-translational modifications mentioned above show that some regulation of DNA binding activity is governed at the C-terminus. But, it is not clear as yet how regulation of p53 specific DNA-binding by the C-terminus occurs. One model proposed by Hupp stated that p53 is converted from an inactive DNA binding form to an active form by interaction with the C-terminus. Studies done by Jayarman and Prives strengthen the possibility of regulation via the C-terminus. Peptides constructed from a region of the C-terminus were found to have strong stimulatory effects on DNA binding activity of full-length p53. Longer double strands of DNA can inhibit the DNA binding activity of p53 through the C-terminal region (Jayarman and Prives *et al.*, 1995). They also found that small deletions in the C-terminus result in constitutive DNA-binding activity (Jayaraman, L. and Prives, C., *et al,* 1997). Lutzker and Levine did another study that supports the existence of inactive forms of p53. They proposed that stabilization of p53 may not be essentially accompanied by activation of the protein, suggesting that a latent form of p53 exist in cell. They found that Teratocarcinomas have high synthesized levels of transcriptionally inactive p53 (Lutzker, S. G. and Levine, A. J., *et al,* 1996). The domains of p53 may be communicating in some way with each other through possible removal or alterations via intermolecular cleaving of p53 itself (Molinari *et al.*, 1996) or other p53 molecules (Okorokov *et al.*, 1997), some type of post-translational modifications or a combination of all. The DNA binding activity cannot only be affected by phosphorylation but by other factors that activate p53 such as the high mobility group protein-1 (HMG-1) and Redox/repair protein (Ref-1) through protein-protein interactions (Jayarman and Prives *et al.*, 1997, 1998). It has been shown that p53 DNA binding can be activated through its interaction with the protein p300 that is involved in chromatin

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remodeling. The transcriptional co-activators CBP (CREB-binding protein) and PCAF have histone acetyl transferase (HAT) activity. They are able to bind to and acetylate the lysine residues in the extreme C-terminus of p53. CBP acetylates lysine residues 382 and PCAF acetylates lysine residues 320. Acetylation of these residues cause a conformational change releasing the negative inhibition exerted by the extreme C terminus of p53. This results in enhancement of sequence-specific DNA binding of p53 (Gu, W., *et al,* 1997a; Gu, W., *et al,* 1997b; Liu, L., *et al,* 1999).

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Cellular consequences of p53 induction

The loss of p53 function is a common event in many human cancers, suggesting it plays a major role in preventing the development of tumors. The transformation of a normal cell into a cancerous one requires the acquistion of genetic instability and loss of the apoptotic response that prevents abnormal growth (Figure 7) (Donehower, L. A., et al., 1992). P53 has been implicated as the "guardian of the genome" participating both in the maintance of genomic integrity and apoptosis. Thus, if the loss of p53's function contributes to cancer, this suggests that p53 has an important role in preventing tumor formation (Mercer, W. E., et al., 1990a, 1990b; Chen, P. L., et al., 1990).

Loss of p53 activity

Mechanisms of Inactivating p53	Effect of inactivation
- Amino-acid-changing mutation in the DNA-binding domain	- Prevents p53 DNA binding
-amplification of the MDM2 gene in the genome	- stimulates the degradation of p53
- Viral infection	- inactivate p53. in some cases stimulate p53 degradation
- Deletion of the p14 ^{ARF} gene	- Failure to inhibit MDM2
-Mislocalization of p53 to the cytoplasm	- Lack of p53 function

Figure 7: Mechanisms that inactivate p53 and their effects on p53 activity.

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In response to different forms of stress, there is an accumulation of p53 due to stabilization and conversion of protein to an active form, as well as sequestration within the nucleus (Hupp, T. R., 1999). Signals that induce p53 include DNA damaging agents (drugs, IR, UV) that create DNA strand breaks or that produce other cellular lesions (bulky adducts) (Kastan, M. B., 1996), hypoxia (Graeber, T. G., *et al.*, 1994), ribonucleotide reduction (Linke, S. P., *et al*, 1996), oxidative stress (Tishler, R. B., et al., 1993) and activated oncogenes (Lowe, S. W. et al., 1993a) (Figure 8). The extent, duration and biological consequences of p53 induction vary according to the nature and intensity of the stimuli, as well as the tissue or cell type considered.

For my work I will concentrate on the p53 pathways that are activated in response to DNA damage (Figure 9). Ionizing (IR) (Kastan, M. B., *et al,* 1991) and UV radiation (UV) (Nelson, W. G., 1994; Maltzman, W. and Czyzyk, L., *et al,* 1984) rapidly elevate the p53 levels to respond to DNA damage. An accumulation of p53 has also been shown after introduction of DNA restriction enzymes or nucleases into the nuclei of cultured cell. The process of DNA transfection can also increase p53 levels (Linke, S. P., *et al,* 1996). In response to DNA damage, the half-life of p53 increases and this leads to the transactivation of target genes such as gadd45, $p21^{WAF1}$, mdm2, bax that results in one of the following: 1) cell cycle arrest or suppression of cell proliferation (Mercer, W. E., *et al.*, 1990a; Chen, P. L., 1990; Mercer, W. E., *et al.*, 1990b; or 2) apoptosis (programmed cell death) (Yonish-Rouach, E., 1991; Shaw, P., *et al.*, 1992) (Figure 9 & 10).

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The p53 Pathway

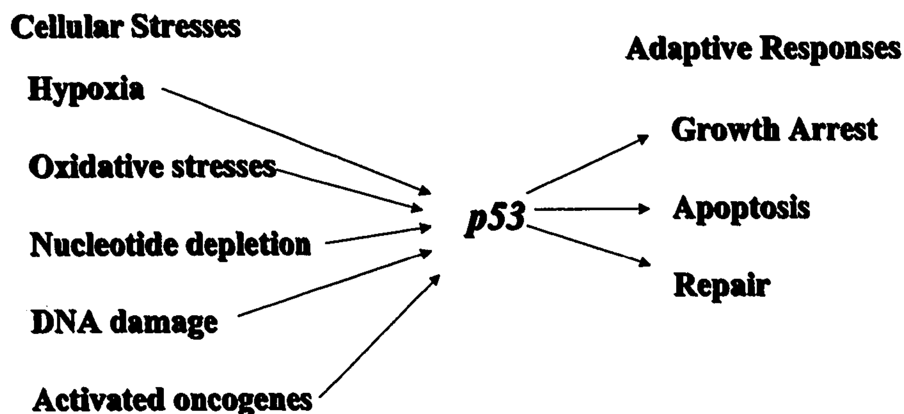

Figure 8: p53 pathways activated in response to cellular stresses.

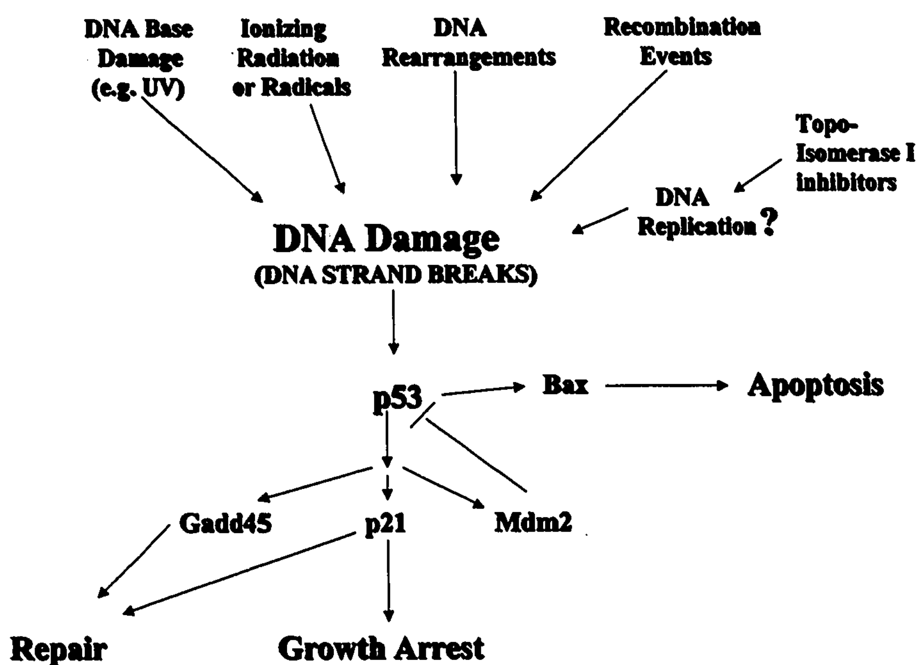

Figure 9: p53 pathways activated in response to DNA damage.

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There is evidence that suggests that p53 also plays a role in repair (Zho, J., et al., 2001; Wang, X., et al., 1995) and replication (Brain, R., and Jeckins, J. R., 1994), and that non DNA-damaging agents can also induce p53.

G1 Arrest

Wild-type p53 is required for the induction of a G1 arrest in response to irradiation (Keurbitz *et al.*, 1992; Kessis *et al.*, 1993). Embryonic fibroblasts obtained from mice that are p53-deficient (both alleles) do not induce a G1 arrest in response to ionizing radiation (IR) (Kastan *et al.*, 1991) and normal diploid fibroblasts generate a long term p53-dependent arrest after IR treatment. This arrest is accompanied by a prolonged induction of $p21^{WAF1}$ protein (DiLeonardo *et al.*, 1994). $p21^{WAF1}$ is a critical component of the p53-mediated G1 arrest response. Expression of the $p21^{WAF1}$ Cdk inhibitor is controlled by p53 through two specific p53 DNA binding response elements which are located on the $p21^{WAF1}$ promoter (El-Deiry, *et al.*, 1993; El-Deiry, et al., 1995). Activated $p21^{WAF1}$ binds to cyclin-CDK complexes and impede their kinase activity and prevent cells from entering S (El-Deiry, *et al.*, 1993). $p21^{WAF1}$ inactivates high levels of Cdk/cyclin E complexes in irradiated G1 phase populations. Blocking the activity of G1 phase specific Cdks traps pRb in its hypo-phosphorylated state. pRb inhibits E2F-related transcription genes that are required for cells to enter S phase. This inhibition of E2F-transcription genes prevents cell cycle progression (G1 into S) (Harper, J. W., *et al.*, 1993; Xiong, Y., *et al.*, 1993).

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G2/M Arrest

Genes targeted by p53 such as 14-3-3 σ (Hermeking, H., *et al.*, 1997) may act as potential mediators of a p53-mediated G2 arrest. 14-3-3 σ generates a G2 arrest by binding to the phosphatase Cdc25c in its cytoplasmic form. It masks the phosphatase activity of Cdc25c preventing it from activating cyclin B/cdc2 which is involved in progressing cells pass the G2 checkpoint into mitosis (Hermeking, H., *et al.*, 1997). Cdc2 becomes active when it is dephosphorylated by cdc25c. Cdc2 is active in its phosphorylated state and causes G2/M arrest.

The role of p53 in G2/M arrest may occur also indirectly through its relationship with $p21^{WAF1}$. $p21^{WAF1}$ can interact with cyclin A and cyclin B complexes that are present during the later stages of the cell cycle. This association with cyclin A and B suggest that $p21^{WAF1}$ may also interact with their respective kinases such as cdc2 and influence them in some way in a similar manner as 14.3.3 by preventing the dephosphorylation of cdc2. $p21^{WAF1}$ mRNA levels have also been observed in human fibroblasts during the later stages of the cell cycle (G2/M). This also suggests a role for $p21^{WAF1}$ at the beginning of mitosis (Li Y., *et al*, 1994). During late G2, approximately half of Cdk/cyclin A is complexed with $p21^{WAF1}$. $p21^{WAF1}$ may inhibit the kinase activity (Poon R. Y. C., *et al.*, 1996) or block interaction between Cdk/cyclin A and potential substrates (Adams P. D., *et al*, 1996). When $p21^{WAF1}$ is complexed with Cdc2 in *Xenopus* extracts, the ability of Cdc2 to activate Cdkl/cyclin B complexes is inhibited (Guadagno T. M., *et al*, 1996). The importance of nuclear accumulation of $p21^{WAF1}$ or

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its presence in late G2 may be a part of a p21^{WAF1}-dependent mitotic attenuation mechanism causing inactivation of Cdk/cyclin complexes at the beginning of mitosis (Dulic V., *et al,* 1998). p21^{WAF1} plays an important function in DNA damage-induced G1 arrest, but it is not necessary for an immediate G2/M arrest (Burgarolas, J., *et al,* 1995; Levedakou, E. N., 1995). Several studies do show that this G2/M arrest is p53-mediated (Stewart, N., *et al,* 1995; Schwart, D., *et al,* 1997; Agarwal, M. L., *et al,* 1995).

Apoptosis

The p53 tumor suppressor protein plays a key role in programmed cell death, otherwise known as cellular suicide or apoptosis. This word was first used by Kerr & Wyllie et al, 1972 to describe the characteristic morphology of programmed cell death i.e. nuclear condensation, membrane blebbing, loss of cellular volume and membrane integrity and DNA fragmentation. Since then, a vast body of evidence has highlighted the role of the proteolytic cascade of cysteine proteases, called caspases in disrupting the functions of important regulatory proteins driving the cell to commit suicide (Martin, S., *et al.*, 1995). Regulation of apoptosis is necessary for development and homeostasis. When defects occur in the cell that give rise to an insufficient apoptotic response, this can result in cancer.

Recent data showed that conformational changes in p53 after phosphorylation at serine 46 can promote p53 to bind to apoptosis-related genes such as p53AIP1 (Oda, K., *et al*, 2000), noxa (Oda, E., *et al*, 2000), and puma (Nakano, K., *et al*, 2001). Oda and

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coworkers observed that p53 mutants with single amino acid substitutions in the DNA binding domain decreased the induction of p21^{WAF1} and MDM2 without affecting the induction level of p53AIP1 (Oda, K., *et al*, 2000). Resnick-Silverman and co-workers found that conformational change in p53 could cause a change in selectivity among different p53 target promoters. Work by Friedlander showed that tumor-derived p53 mutants that had a single amino acid substitution in the DNA binding domain, could induce G1 arrest genes (p21^{WAF1}) but could not induce apoptosis at high temperatures (Friedlander *et al*, 1996; Ryan and Vousden, 1998).

Eventhough the activation of p53 has been shown to be involved in apoptosis in some cell types, it is still unclear if p53 is a direct mediator or just sensitizes cells to apoptosis induced by DNA damage. Overexpression of p53 in rat vascular smooth muscle cells is not sufficient to induce apoptosis alone and other signals such as E1A or c-myc appear to be required (Bennett, M.R., 1995). A study done by Tsuji and coworkers provide evidence that overexpression of p19^{ARF} in mouse embryonic fibroblast devoid of p53/ARF induced apoptosis irrespective of MDM2 status (Tsuji, k., et al, 2002). This suggests that there is a p19^{ARF} -induced apoptotic pathway present that is not dependent on p53. Another study reported that an adenosine derivative C1-1B-MECA can induce apoptosis via the up-regulation of FAS (a death receptor) in the absence of p53 (Kim, S. G. et al., 2002). But, the most compelling evidence that p53 sensitizes cells to apoptosis comes from a study done by Aladjem and coworkers. They observed that embryonic stems that were subjected to DNA damage underwent p53-independent apoptosis. This suggests that the apoptosis induced during embryogenesis was not dependent upon p53

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(Aladjem, M. I., et al, 1998). p53 may be only one of many signals that the cell integrates before deciding whether to undergo apoptosis.

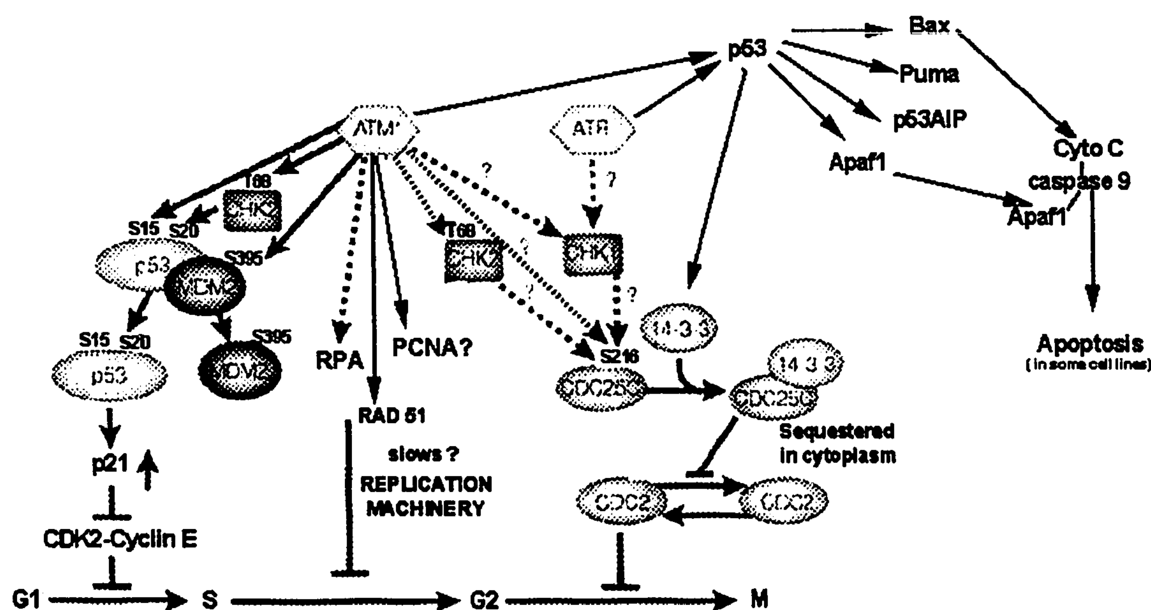

Figure 10. ATM and other signals controlling cell-cycle checkpoints. The specific activity of ATM is increased after introduction of DNA double-strand breaks (DSBs) in DNA through ionizing radiation or other means. Nature Reviews Molecular Cell Biology 1; 179-186 (2000)

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Replication and Repair

In addition to its effects on transcription, recent studies have attributed a role for p53 in the downregulation of S phase progression by its ability to bind to single stranded nucleic acids and promote their re-association. This activity may slow down the progression of DNA replication (Brain, R., and Jeckins, J. R., *et al,* 1994). P53 can also affect repair and recombination through its interact with proteins Proliferating Cell Nuclear Antigen Promoter (PCNA) which is a subunit of DNA polymerase delta and epsilon and replication protein A (RPA). Both proteins play roles in regulating DNA replication and nucleotide-excision repair. P53 can interact with the promoter of Proliferating Cell Nuclear Antigen Promoter (PCNA) protein which contains a p53 binding site. At low levels of p53, it transactivates PCNA promoter to perhaps allow for stimulation of its DNA repair activity. At high levels of wild-type p53, the PCNA promoter is inhibited after binding to p53 C-terminus. This may slow down or impede replication (Shivakumar, C. V., *et al.*, 1995). Mutant p53, which does not require a p53 binding site, does the opposite at high levels. It can also affect PCNA indirectly via $p21^{WAF1}$. $p21^{WAF1}$ can bind to PCNA to inhibit its effects on replication and cell-cycle progression. It has no control over its DNA repair activity (Smith, M L., et al, 1994).

p53 can inhibit the onset of replication through its interaction with the replication protein through a 20 amino acid region in its N-terminus. p53 can inhibit the activity of RPA in the absence of DNA damage (radiation). In the presence of UV, p53 can no longer interact with RPA. Each protein is able to carry out its own functions for instance p53 is able to carry out transcriptional activation and RPA can carry out nucleotide-

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excision repair (Dutta, A., et al, 1993; Abramova, N. A., et al, 1997). The interactions of p53 with RPA and PCNA may illustrate a link between p53 and DNA repair.

p53 can also bind to components of the basal transcription-repair complex TFIIH to regulate the activity of TFIIH-associated helicases ERCCB/XPB and ERCC2/XPD (Oberosler, P., *et al.*, 1993). p53 binds through its C-terminal domain to the helicase motif III of XPB and inhibits the DNA helical activities of the complex (Wang, X. W., *et al,* 1995). After DNA damage, p53 binds to TFIID to regulate DNA-repair efficiency. This study also suggests a role for p53 in DNA repair.

In response to IR, PARP is rapidly activated (Agarwal, M. L., *et al,* 1997). It is also involved in recruitment of DNA repair enzymes to the site of damage (Fernet, M., *et al,* 2000). It has been shown in PARP-1 gene deficient mice that in response to IR, low levels of p53 accumulate. PARP could be the first steps in the signaling process by DNA damage by controlling the levels of p53 induction.

Huang and co-workers found that as little as one double strand break could induce p53 levels and thus cell arrest. p53 has the ability to interact directly with DNA in several different ways and thus signals emanating from the DNA itself is a plausible mechanism for up-regulating p53 (Huang *et al.*, 1996a).

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Figure 11. Possible role of p53 in DNA repair. p53 in its tetrameric form binds ss-DNA and DNA ends via its C-terminal domain (Reed, M., *et al,* 1995).

p53 binds to double-stranded and single-stranded DNA (Figure 11) in a non-sequence specific manner (Steinmeyer, K and Deppert, W., *et al.*, 1988), to ends of double-strand breaks (Bakalkin, G., *et al.*, 1995), to Holliday junctions (Lee, S. M. *et al.*, 1997), and to DNA mismatches leading to DNA "bulges" or adducts (Lee, S., *et al.*, 1995). This suggests that p53 can recognize (sense) and bind strongly (specifically) to damaged DNA. This is seen with DNA damage induced by IR (Reed. M. *et al.*, 1995) and p53 ability to bind to or form complexes with mutated DNA regions e.g. insertion/deletion mismatches (Lee, S., *et al.*, 1995). p53 binding is mediated through its C-terminal domain, which has been shown to play an important part in regulating the activity and function of p53. This suggests that p53 may sense DNA damage via its C-terminus. Then this may lead to the activation of sequence-specific DNA binding of p53 and transactivation of downstream target genes (Jayaraman, L. and Prives, C., 1995) or its interactions with such proteins as RPA, PCNA or TFIID that may participate in and enhance DNA repair.

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Modulation of p53 expression and stability during the cell cycle

The information presented in this section of the introduction is the basis for the majority of work presented in my thesis. The observation that p53 is a critical regulator of p21 function in G1 and G2 cells but not S-phase cells in response to DNA damage (Maeda, T., et al., 2002) suggested that changes during the cell cycle may modulate the inducibility and activity of the p53 protein. The protein is considered to be regulated mostly at the post-translational level by covalent and non-covalent modifications. These modifications, including phosphorylation, modulate protein stability and accumulation. The degree of p53 phosphorylation at different sites during the cell cycle (Meek, D. W., *et al*, 1998) and the role of those phosphorylation patterns remains a subject of ongoing investigation. For instance, site-directed mutagenesis of all phosphorylation sites was not sufficient to inactivate the protein or prevent its accumulations (Blattner, C., *et al*, 1999b).

This suggest that there are other ways p53 can be activated when there is a lack of phosphorylation for e.g. acetylation (Gu, W., and Roeder, R. G., *et al*, 1997) or other post-translational modifications such as cleavage (Molinari *et al*, 1996; Okorokov *et al*, 1997).

p53 is subjected to kinases that regulate the cell cycle as well as ones that are activated by stress. Cyclin B or A can complex with cdc2 to phosphorylate p53 on serine 315 and phosphorylation of the serine 315 seems to be up-regulated during S and G2/M stages of the cell cycle (Linke, S. P. *et al*, 1996; Wang, Y., *et al*, 1995; Sturzbecher, H. W., *et al*, 1990). As a result of phosphorylation at serine 315 by cdc2, p53 undergoes a

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conformational change. This affects its sequence-specific DNA binding activity to different p53 target genes. This phosphorylation enhances binding activity to particular p53 binding sites within the $p21^{WAF1}$ and gadd45 genes, but not to the one in the bax-1 gene (Wang, Y., *et al,* 1995). Chk-1 is a protein kinase that works downstream of ATM/DNA-PK/ATR network that is expressed specifically at S to M phases. Chk-1 may interacts with 14-3-3 proteins to regulate the activity of cdc25c phosphatase at G2/M (Chen, L., *et al,* 1999; Furnari, B., *et al,* 1999).

Another post-translational modification of p53 that may be involved in its regulation during the cell cycle may be acetylation. Many proteins such as CBP (CREB-binding protein) and p300 have histone acetyl transferase (HAT) activity. This HAT activity is regulated at the G1/S border by phosphorylation (Ait, S. A., *et al,* 1998) to increase the HAT activity at this border (Gu, W., *et al,* 1997a; Gu, W., *et al,* 1997b).

Proteins that play a role in regulating the cell cycle may directly or indirectly modulate the stability of p53 during the cell cycle. MDM2 has a role in regulating the stability of transcription factor called E2F1. This transcription factor is critical for cells to enter S from G1 phase. The Rb gene plays a role in transcriptional regulation of genes such as E2F1 (Blattner, C., *et al,* 1999; Reinke, V., *et al,* 1999). This suggest that MDM2 is present at the G1/S border and when MDM2 is present, it not only regulates E2F but it may also down-regulate p53 levels at the G1/S border where p53 has been shown to affect the Rb gene and G1-S progression via transactivation of the $p21^{WAF1}$ gene. It has been shown that the HAT activity of CBP is increased at the G1/S border (Ait, S. A., et al, 1998). MDM2 can interact with the N-terminus of CBP to block its interaction with p53. MDM2 can prevent p53 transactivation indirectly by masking p53 transactivation

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domain from potential co-activators (Avantaggiati, M. L., *et al,* 1997; Lambert, P.F., *et al,* 1998; Wadgaonkar, R., *et al,* 1999). This may prevent p53 DNA binding sequence-specific activity to decrease p53 affinity for different gene at the G1/S border to influence the regulation of the progression of cells from G1 into S. The CDKN2/INK4a gene encodes a protein called $p19^{ARF}$. This protein plays a role in regulating the cell cycle. $p19^{ARF}$ is able to bind to MDM2 that is already bound to p53 and decrease its inhibitory effect on p53 transcriptional activity (Larsen, C. J., *et al,* 1998) and allow for increased p53 stability

Studies show that MDM2 can complex with p53 in S and G2/M and that p53 can complex with JNK during G0/G1 phases of the phases of the cell cycle (Fuchs, S. Y., *et al,* 1998a). The two proteins, MDM2 and JNK (Jun N-terminal Kinase), play a role in targeting p53 for degradation. This suggests that two forms of p53 exist. One form of p53 binds to and is regulated by JNK expression and the other binds to and is regulated by MDM2 expression. This also suggests that the stability of p53 is regulated by its association with either protein, depending on the phase of the cell cycle.

The effects of MDM2 on p53 stability and transactivation functions during these stages of the cell cycle could be off set by the phosphorylation of p53 at S 315 by cell cycle regulated kinases cyclin B and A to influence p53's DNA binding activity during S and G2/M phases to target different genes during the course of the cell cycle. In response to stress such as IR, MDM2 can become phosphorylated by ATM (stress-activated kinase), this phosphorylation of MDM2. ATM expression has been observed to be induced independent of the cell cycle (Pandita, T. K., et al., 2000). This suggests that DNA damage sensors are present throughout the cell cycle. Upon phosphorylation

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MDM2 is no longer able to inhibit p53 transactivation activity at the G1/S border or S and G2/M phases. JNK can become active in response to UV damage preventing its inhibitory effects on p53 within G0/G1 phases of the cell cycle.

Overall these data suggest: 1) that the stabilization of p53 is under a very sensitive regulatory mechanism that includes at least MDM2, $p19^{ARF}$, E2F1, Myc and JNK during the cell cycle to ensure a rapid and precise G1-arrest response or G1 to S progression, 2) that p53 is present throughout the cell cycle and it is able to be regulated at the translational level as well as through protein-protein interactions and 3) that DNA damage pathways (at least the one involving ATM) are present during the different stages of the cell cycle by the ability of ATM to be expressed independent of the cell cycle. This lends support to the fact that there is a need for p53 to be present during all phases of the cell cycle to response rapidly to protect the genome.

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Summary of Thesis Work

Studies mentioned in the previous section suggested that different types of post-translational modifications can occur within different stages of the cell cycle and that these modifications may regulate p53 activity during the course of the cell cycle.

Some studies have investigated the effect of the cell cycle on p53 induction. The levels of p53 in the nucleus may vary during the course of the cell cycle. In human T-lymphoblastoid cell lines, p53 protein was low in those fractions containing mostly G1 populations and peaked when cells crossed the G1/S boundary and into S phase. In the later fractions (G2/M), the protein level decreased (Bischoff *et al*, 1990). In normal mammary epithelial cells, the p53 levels were low throughout G1 and increased 3 to 4 fold as the cells entered S phase (Gudas *et al*, 1994). These observations correlate with the results that high levels of wild-type p53 induced by DNA damage or introduced as a result of gene cloning techniques block the G1/S transition, arresting cell cycle progression (Kastan *et al*, 1991; Dileonardo *et al*, 1994) and G2/M transition (Agarwal *et al*, 1995) to allow for DNA repair by controlling the expression of regulatory gene products (Lane *et al*, 1992).

Some studies have been done to investigate the effect of the cell cycle on p53 function (Haapajarvi, T., *et al*, 1997; Cotter, T. G., *et al*, 1992; Pitkanen, K., *et al*, 1998). Pitkanen and co-workers found that the activation of p53 by UV radiation is dependent on the cell cycle (Pitkanen, K., *et al*, 1998). However these studies made use of drugs to synchronize cells. These drugs may have interfered with specific p53 functions.

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To investigate if p53 levels are able to be stabilized throughout the course of the cell cycle or if this is unique to specific phases of the cell cycle, we used a centrifugal elutriation technique. This technique separates cells based on their size and weight, avoiding the artifacts common with cell cycle blocking agents. Nine fractions of cells were isolated representing early-G1, mid G1, late G1/early-S, early-S, mid S, late S, late S/G2 and G2/M cells. A portion of each fraction was fixed in 30% ethanol and the DNA content was determine by flow cytometry (FACS analysis). Each cell cycle fraction was also monitored by Coulter counter for size to ensure the elutriation procedure was working. We conducted this study with the myeloid leukemia cell line (ML-1) that contains wild-type p53. To activate p53, we used the drugs Camptothecin (Cpt) and Zeocin (Figure 12). Cpt is a topoisomerase I inhibitor which has been reported to cause replication-dependent DNA double strand breaks (Ryan, A. J., *et al*, 1994). Cpt has also been shown to produce cytotoxicity within cells independent of replication (Morris, E. J., *et al*, 1996). Zeocin, a member of the bleomycin family of antibiotics, is an ionizing radiation mimetic that causes strand breaks in the DNA. These DNA strand breaks are the result of free radical attacks on the deoxyribose moieties of each DNA strand (Villeponteau, B. and Martinson, H. G., *et al*, 1987, Lopez-Larraza, D., *et al*, 1990). For this proposal, the ML-1 cells in each fraction were treated with 20 uM Cpt for 6 hours, 0.5 uM Cpt for 3 hours or 400 ug/ml of Zeocin for 6 hours. Nuclear extracts from each cell cycle fraction were subjected to Western blot analysis with specific p53 antibodies PAb 240 specific to

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Camptothecin

- Topoisomerase I inhibitor
- Forms a ternary CPT-topoI-DNA complex that upon collision with DNA polymerase causes double-strand breaks

Zeocin

- Copper chelating glycoprotein.
- Member of the bleomycin family of antibiotics
- Upon entry into the cell, the copper becomes reduced, allowing zeocin to become active. It then binds to DNA in the presence of O_2 and fe (BLM- O_2- Fe II) and cleaves DNA directly.
- With the formation of this complex there is a removal of a H from the deoxyribose to form a free radical, resulting in the release of the sugar ring, thus breaking the DNA backbone

Figure 12: Mechanisms by which Cpt and Zeocin induce DNA damage.

the central DNA binding domain, PAb 1801 specific to the N-terminus, and/or PAb 421 specific to the C-terminus of p53. By Western blot analysis, we observed that Cpt was able to increase p53 levels in all cell cycle phases but not as well as it did in non-elutriated cells. Although the amount of p53 induced by Cpt in non-elutriated cells was reproducibly greater than that seen in the elutriated fractions, this was not seen for Zeocin drug treatments. Zeocin was able to induce p53 levels in all the stages of the cell cycle equal to the extent of non-elutriated cells. Even when a further examination was carried out with a shorter treatment time and lower dose, p53 was still able to be induced in ML-1 cells by Cpt during all stages of the cell cycle.

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By using EMSAs, we observed that there were increases in p53-dependent DNA binding activity in every cell cycle fraction after Cpt or Zeocin treatment for the super consensus sequence (SCS) and gadd45 oligonucleotides, illustrating that the p53 protein induced in every cell cycle fraction was able to bind DNA. This shows that the machinery necessary to stabilize and activate p53 DNA binding was present in all cell cycle stages. There was no direct correlation seen between increased DNA binding activity and the induction of p53 levels for both Zeocin and Cpt treatments. An EMSA with competitor DNA was performed to determine the affinity of p53 induced in each cell cycle fraction for different p53REs. The nuclear extracts used were from the Cpt drug treatment where a lower dose and time point was used. Using the oligonucleotide super consensus sequence, competition was carried out with 100-fold excess of superconsensus sequence (SCS), gadd45, mdm2, $p21^{WAF1}$ (p53 binding site upstream of $p21^{WAF1}$ promoter), $p21^{WAF2}$ (p53 binding site downstream of $p21^{WAF1}$ promoter), bax, mutant RGC and mutant $p21^{WAF}$ oligonucleotides. Two DNA binding forms of p53 were observed to bind to the oligonucleotide SCS. These forms have different affinities for different p53REs. These forms of p53 seem not only to have different affinities for different p53REs, but they are present within different cell cycle phases. One form was found mainly in the G1 phase, non-stressed cells and possibly G2/M phase. The other form was present in all phases of the cell cycle only after cells were stressed. p53 induced in cells stressed (allowed to over grow) prior to drug treatment with Cpt (20 uM for 6 h) showed a difference in DNA binding for the mdm2 oligonucleotide as compared

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to cells not stressed prior to drug treatment. The p53 induced in stressed (allowed to over grow) cells prior to treatment with Cpt showed binding to the mdm2 oligonucleotide.

Three signaling cascades in response to genotoxic stress have been observed (Figure 14) (Liu, Z. G., *et al*, 1996) and they involve the activation of DNA-PK, ATM (a c-Abl pathway), ATR, CKII, c-Jun kinase (JNK), and p53. One or the other of these signaling pathways could be activated in response to specific gentotoxic stress such as UV or IR. ATM, c-Abl, DNA-PK and p53 are activated in response to ionizing radiation (gamma), but JNK is not. ATR, p53, JNK, and CKII are activated in response to UV radiation, but c-Abl (ATM -mediated pathway) is not. In both pathways, p53 is activated but each induces a specific phosphorylated product of p53 governing its binding and activation of downstream targets and thus, its cellular response. UV damage induces a form of p53 phosphorylated at serine 392 which is not seen after ionizing radiation exposure (gamma) (Kapoor, M., and Lozano, G., *et al*, 1998).

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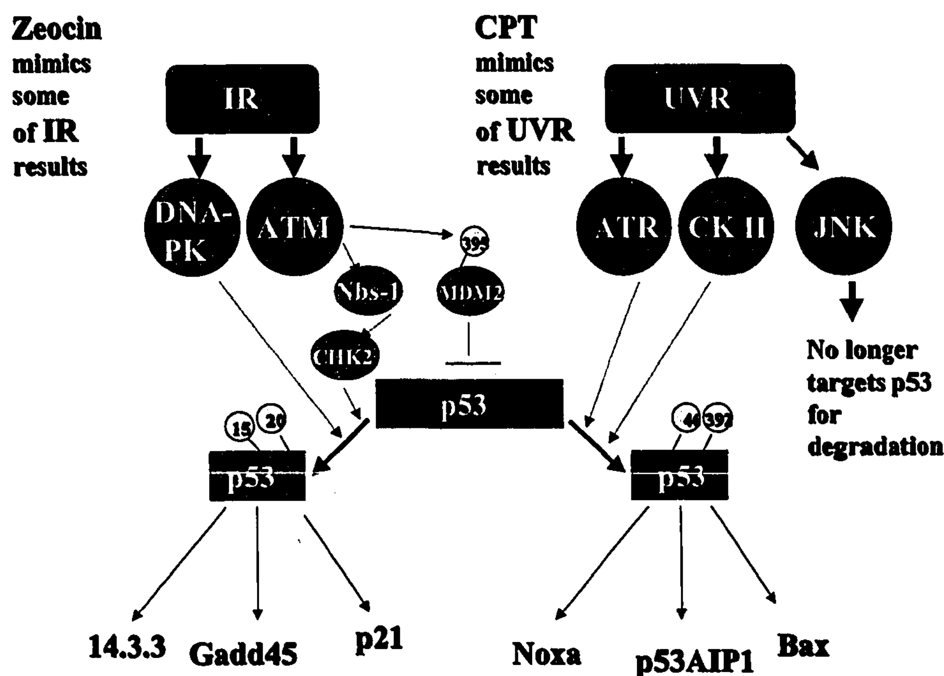

Figure 14: Signaling cascades in response to IR and UV genotoxic stress. IR stands for ionizing radiation.

We observed similar results with Cpt (which mimics UV results) and Zeocin (which mimics gamma IR results) treatments (Houser, S., *et al,* 2001). We examined the cell cycle regulation of p53 phosphorylation at serine 392 using an antibody directed against phosphoserine 392 (a gift from Mini Kapoor and Gigi Lozano). Phosphorylation at serine 392 is not induced under all DNA damage conditions. Phosphorylation at this serine has been shown to associated with the DNA binding activity of p53 (Kapoor, M. and Lozano, G., *et al*, 1998). Cpt was able to induce serine 392 phosphorylation in every cell cycle phase and this phosphorylation did not correlate with the DNA binding activity of p53 in each cell cycle fraction. Western blots containing nuclear extracts from Zeocin-treated cell cycle fractions were not probed with the antibody against p53 phosphorylated at serine 392 because phosphorylation at this site was not observed in pilot experiments

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in which asynchronous ML-1 cells were treated with Zeocin even though the p53 level was induced to a level similar to that of Cpt-treated cells. Western blots of nuclear extracts were probed with $p21^{WAF1}$ antibody because in asynchronous cells only cells treated with Zeocin induced robust $p21^{WAF1}$ protein expression while Cpt produce barely detectable or no $p21^{WAF1}$ at all. A clear increase in nuclear $p21^{WAF1}$ protein levels was observed in all Zeocin-treated cell cycle phases while the cell cycle populations enriched for G2/M cells showed scarce amounts.

We investigated if different forms of p53 are generated in ML-1 cells treated with Zeocin and Cpt in different cell cycle fractions and found different forms of p53 in these fractions. The generation of different forms of p53 was further examined in asynchronous cells treated with Zeocin or Cpt at different doses and time points. Interestingly, Zeocin induced a form of p53 not reactive with PAb 421 antibody (specific to the extreme C-terminus of p53) that was phosphorylated at serine 15 whereas Cpt induced a form of p53 not reactive with PAb DO-1 antibody (specific to the extreme N-terminus of p53) that was phosphorylated at serine 392. When the protease inhibitor leupeptin was added to samples treated with Cpt, the phosphorylated form of p53 at serine15 as seen in Zeocin treated cells was able to be retained.

Even though some studies showed that Cpt induced apoptosis does not require the replication process (Morris, E. J., and Geller, H, M., *et al*, 1996), many have reported the p53 effect does (Ryan, A. J., *et al*, 1994; Nelson, W. G., and Kastan, M. B., 1994). To further investigate if Cpt requires cells to be in a replicating state to induce p53, we treated asynchronous cells with aphidicolin to stall DNA synthesis prior to treatment with Cpt and Zeocin. Aphidicolin binds to dNTPs to form dNTPs/aphidicolin complexes.

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Cells within the S phase stop replicating immediately while cells in G1 and G2 cycle until they accumulate at the G1/S border. It does not affect protein or mRNA synthesis (Spadari et al, 1984). We observed that Cpt and Zeocin (even at low levels) did induce p53 levels in non-replicating cells. We also observed by using anti-bromodeoxyuridine (BrdU) labeling that non-replicating cells were able to regain their ability to take up BrdU in the presence of higher doses of either Cpt or Zeocin. The uptake of BrdU could be the result of cells undergoing DNA repair and taking up dNTPs during the repair process. The protein ribonucleotide reductase (p53R2) is required for production of dNTPs and it is important in DNA repair and replication (Nakano, K., *et al*, 2000; Tanaka, H., *et al.*, 2000). This gene is a direct target of p53. p53 (wild-type) is able to stimulate base pair excision repair and stabilize the interaction between DNA polymerase and DNA (Zhou, J., *et al*, 2001). This suggest that replication coupled repair may be efficient to activate p53. Our data showed that Cpt and Zeocin can induce different modified forms of p53. This shows that Cpt and Zeocin can signal through different pathways, but both pathways are cell cycle independent.

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Chapter 2

Materials and Methods

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Reagents. CPT, nocodazole, propidium iodide, APH, bromodeoxyuridine and anti-actin were purchased from Sigma Chemical. Anti-BrdU and FITC-conjugated F(ab')$_2$ Goat Anti-Mouse IgG (H + L chains) from CALTAG Laboratories. Zeocin was purchased from Invitrogen. RPMI 1640 and fetal calf serum were purchased from Gibco/BRL. The apoAlert annexin V-FITC kit was purchased from Clontech.

Cell Culture. The ML-1 cells were a generous gift from Micheal Kastan. This myeloid leukemia cell line was grown in RPMI 1640 with 10% fetal bovine serum and 5% CO_2. The cells were seeded at a density of 2.5 X 10^5/ml and exponentially growing cells were used in all experiments. Cells grown beyond 5 X 10^5/ml began to show signs of stress as indicated by increase in the p53 level before drug treatment. HL-60 cells were from ATCC and were grown under the identical conditions.

Flow Cytometry. FACS analysis was carried out on a Becton Dickinson FACS Scan at the Sloan Kettering Cancer Center and the Bio-imaging facility at Hunter college. Values were derived by Becton Dickinson FACS Scan and Phenox Flow System Multi-Cycle software. 5 X 10^6 cells were fixed for each analysis. Cells were spun down at 2300 rpm for 7 min., washed 2 times with PBS and resuspended in 20 ml of PBS containing 2% BSA and 0.1% NaN_3. Ethanol (9 ml) was then added dropwise while overvortexing. Propidium iodide staining and RNase treatment were carried out at 37^0C for 30 min. 24 h prior to flow cytometry. Cells were also labeled with anti-BrdU (Bromodeoxyuridine) and propidium iodide for flow analysis (protocol provided by Becton-Dickinson.com, Book/Source). Cells in culture were incubated with 1uM BrdU for 30 minutes in a CO_2 incubator at 37°C prior to harvesting. 1x 10^6 cells were fixed for

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each analysis. Cells were spun down at 2300 rpm for 7 min, washed 2X with PBS containing 1% BSA, spun at 500 *x g* for 15 minutes at room temperature and resuspended in 100ul of normal saline on ice. Cells were then added dropwise in 70% ethanol while vortexing and incubated for 30 minutes on ice. Cells were then centrifuged at 500 *x g* for 10 min. at 10°C, supernatant was aspirated carefully and the pellet was loosened by vortexing. 1 ml of 2N HCL/Trition X-100 were added to fixed cells, incubated for 30 min, then centrifuged at 500 *x g* for 10 min. The supernatant was aspirated and the pellet was resuspended in 1 ml of 0.1 M $Na_2B_4O_7$, pH 8.5 to neutralize the acid. 10^6 cells were placed in micro centrifuged tubes, centrifuged, and resuspended in 50ul of PBS containing 0.5% Tween and 1% BSA. 20ul of Anti-BrdU to 10^6 cells, incubated for 30 min. at room temperature, centrifuge 500 *x g* for 5min., resuspended in 50ul of PBS containing 0.5% Tween ,1% BSA and 1ug of FITC-conjugated $F(ab')_2$ Goat anti-mouse IgG and incubated for 30 min. at room temperature. Cells were centrifuged at 500 *x g* for 5 min. resuspended in 1ml of PBS containing 0.5% Tween , 0.5% BSA and 5ug/ml of propidium iodide and analyzed on a FACS flow cytometry.

Centrifugal Elutriation. Four liters of ML-1 cells grown to $5x10^5$ ml were separated aseptically using a Beckman JE 5.0 elutriation system. Cells were spun down at 1800 rpm for 10 minutes and pooled into 50-55ml of 1X RPMI with 1% FBS. Reservoir buffer used for counter flow was 0.25 X RPMI with 1% FBS. Cells were then run through an 18g needle 2X and 1X through a 20g needle to reduce clumps. With the rotor speed set at 1700 rpm and a flow rate of 5.25ml/min., the 50 -55 ml suspension was injected into the rotor containing a large 40 ml A chamber at a pump speed of 115 (Master Flex pump 7520-25, Cole Palmer Instruments INC.). Once the cells had entered

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the large A chamber, the rotor speed was maintained at 1700 rpm with a flow rate of 5.25ml/min. for 5 minutes. Then the rotor speed was changed to 1670 rpm with a flow rate at 5.12 ml/min. for 5 minutes. Then the rotor speed was changed to 1650 rpm with a flow rate at 5.0 ml/min. for 5 minutes and the pump speed was changed to 120. Then the rotor speed was changed to 1630 rpm with a flow rate at 4.8 ml/min. for 2 minutes. Then the rotor speed was changed to 1610 rpm with a flow rate at 4.7 ml/min. for 2 minutes and collections of cell volumes of 500 ml were begun as follows (coulter counter readings were also carried out to verify that the gradient was established):

Bottle #	Exact Volume collected	Exact Rotor speed	Approximate Flow rate	Exact Pump speed	T_L & T_U settings (%) 7-10	7-11	7-12	7-14
1	500 ml	1610 rpm	4.7 ml/min.	125	46	-	-	-
2	"	"	5.5	131	-	20	11	-
3	"	"	6	137	-	20	14	-
4	"	"	7	147	-	27	14	-
5	"	"	7.5	157	-	55	19	-
6	"	"	8	172	-	50	41	-
7	"	"	8.5	187	-	50	61	-
8	"	"	10.5	212	-	-	84	31
9	"	"	11.5	237	-	-	94	35
10	"	"	12.5	262	-	-	97	43
11	"	"	13.75	287	-	-	99	54
12	"	"	14	312	-	-	-	56
13	"	"	16.5	337	-	-	-	59
14	"	"	17	362	-	-	-	23
15	"	"	18.5	387	-	-	-	23
16	"	"	20	412	-	-	68	22
17	"	"	21	437	-	-	60	-
18	"	"	22.5	462	-	-	-	-

First 5 bottles were pooled into one fraction, fraction 1.

Bottle number 6 = Fraction 2 corresponded to G1 if the elutriation procedure was successful.

Bottle number 7 = Fraction 3 corresponded to G1.

Bottle number 8 = Fraction 4 corresponded to G1-S.

Bottle number 9 = Fraction 5 corresponded to G1-S.

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Bottle number 10 = Fraction 6 corresponded to S.

Bottle number 11 = Fraction 7 corresponded to S-G2/M.

Bottle number 12 = Fraction 8 corresponded to G2/M.

Bottle number 13 = Fraction 9

Bottle number 14 & 15 = Fraction 10

Bottle number 15, 16 & 17 = Fraction 11

Note: Cell cycle populations were determined from FACS analysis.

Note: Small sized cells came off first and as we increased the flow rate, cell populations of increasing size exited the centrifuge. We used the Coulter Counter to analyze the size of the cell population within each bottle to show that the elutriation procedure was working. Cells of the same sizes were also pooled based on the results from the Coulter Counter. Coulter Counter analyzed a small volume of cells from each bottle numbered 1-18. The selected numerical value of size (um) for the T_{lower} (T_L) threshold and T_{upper} (T_u) threshold were entered into the machine. Next, the count mode was selected:

a) Between = will count the % of cells between T_L and T_u.

b) Above T_u = will count the % of cells above T_u.

Each volume of cells was analyzed at each count mode to give a % of cells between T_L& T_u and above T_u, giving an approximate size of the cell population within that bottle. The size of the cell population should be increasing with each bottle number. We started out with T_L =7 um and T_u = 10 um, then we change the T_u to 11 um, then T_u was changed to 12 um, then T_u was changed to 13, 14 and 15 um. A coulter counter was used during the collection of fractions to determine the upper and lower limits of cell size in order to

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determine when cell cycle fraction pools should be made. Each fraction was split in two and one half was treated with drugs as indicated while the other served as control.

Nuclear Extract Preparation and Western Blot Analysis. Nuclear extracts were prepared using a variation on the Dignam protocol (Dignam, J. D., *et al.*, 1983). Cells were spun down and resuspend in 5 packed cell volumes of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCL, 0.5 m M DTT). They were then put on ice for 10 min. prior to centrifugation for 10 min. at 2000 rpm. The pellet was resuspended in 2 packed cell pellet volumes of buffer A (volume prior to initial wash). The cells were passsed twice through a 25-gauge needle and the nuclei were then spun down at 2000 rpm for 10 min. followed by an additional 20 min. spin at 14,000 *x g*. The pellet was resuspended at 10^9 cells per 3 ml of buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) and by passing it twice through a 25-gauge needle. The suspension was rocked gently for 30 min. at 4^0C. The extract was centrifuged for 30 min. at 16,000 rpm and the supernatant aliquots were stored at -80^0C. Samples were electrophoresed on a 10% SDS-PAGE and electrotransferred to nitrocellulose. Blots were probed with the monoclonal antibodies specific to p53 (PAbs 1801, 240 and 421). Polyclonal antibodies against phosphoserine-392, phosphoserine-15 or anti-actin were as indicated. The signal was visualized after incubation with goat anti-mouse or goat anti-rabbit secondary antibody using ECL solutions (Amersham).

Electrophoretic Mobility Shift Assays. Synthetic oligonucleotides were purchased for this study from Operon. The SCS site contained three adjacent p53 half sites. The sequence of this nucleotide was the following: Top, 5'- TCG AGC CGG GCA TGT CCG CCG

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GGC ATG TCC GGG CAT GTC - 3' and Bottom, 5'-GAC ATG CCC GGA CAT GCC CGG ACA TGC CCG GCT GGA-3' (Halazonetis et al, 1993). A sequence of natural p53 binding sites obtained from Operon, gadd45 nucleotide, was the following: Top, 5'-AAT TCT CGA GGA ACA TGT CTA AGC ATG CTG CTC GAG - 3' and Bottom, 5'-AAT TCT CGA TGC TTA GAC ATG TTC CTC GAG-3' (Kastan et al, 1992); $p21^{WAF1}$ nucleotide, was the following: Top, 5'-AAT TCT CGA GGA ACA TGT CCC AAC ATG TTG CTC GAG -3'and Bottom, 5'-AAT TCT CGA ACA TGT TGG GAC ATG TTC CTC GAG- 3' (El-Deiry et al, 1993); MDM2 nucleotide, 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'and Bottom, 5'-GAT CCG GAG ACA TGT CAG GAC CCT CCT CCG ACA GCC GAC GCC GGA CGT CCA ACT TGA CCA GG-3'; bax nucleotide, was the following: Top, 5'- GAT CTC ACA AGT TAG AGA CAA GCC TG- 3' and Bottom, 5'-TCG ACA ATA TAG CCC ACG CCC AGG CTT GTC TC-3'(Miyashita and Reed, 1995); $p21^{WAF2}$ nucleotide, was the following: Top, 5'- AAT TCT CGA GGA AGA CTG GGC ATG TCT CTC GAG- 3' and Bottom, 5'-AAT TCT CGA GAG ACA TGC CCA GTC TTC CTC CTC GAG-3' (Macleod et al, 1995); RGC nucleotide, was the following: Top, 5'- TCG AGT TGC CTG GAC TTG CCT GGC CTT GCC TTT TC - 3' and Bottom, 5'- GAA AAG GCT TGG CCA GGC AAG TCC AGG CAA CTC GA-3'; mutant $p21^{WAF}$ nucleotide, was the following: Top, 5'-AAT TCT CGA GGA AGA ATT CAA GAT ATA TTC CTC GAG-3'and Bottom, CTC GAG GAA TAT ATC TTG AAT TCT TCC TCG AGA GGA CTT GCC TGG CCA AGC CTT TTC-3' (El-Deiry, et al., 1993); and mutant RGC nucleotide, was the following: Top, 5'- TCG AGT TTA ATG GAC TTT AAT GGC CTT TAA TTT TC -

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3'and Bottom, 5'-GAA AAT TAA AGG CCA TTA AAG TCC ATT AAA CTC GA-3'. Labeling of the oligonucleotide was *performed* with large fragment of DNA polymerase and gamma ^{32}P dCTP. EMSA experiments (30 μl) were carried out in reaction mixtures with 150 fmol of ^{32}P oligonucleotide. 10ug of nuclear extract protein was added and the binding reaction was incubated for 20 min. at room temperature in a reaction buffer containing 20 mM Hepes, pH 7.8, 100 mM KCL, 1 mM EDTA pH 8.0, 1 mM DTT; 1ug sheered salmon sperm DNA and 10% glycerol. Reactions were carried out in the presence and absence of PAb 421 as indicated. Samples were separated by 4% polyacrylamide gel electrophoresis (gels were perun at 100 V for 15 min. at 4^0C) at 200 V for 3-3.5 h. Gels were dried for 1 h at 55^0C and auto radiography was performed.

RNA Isolation and RT-PCR with Molecular Beacon Analysis. 5 $X10^6$ cells were added to 1ml of TRIzol Reagent (Sigma), lysed by repetitive pipetting and incubated for 5 min. at room temperature. 0.2 ml of chloroform was added per 1ml of TRIzol, shaken vigorously for 15 seconds and incubated for 2 to 3 min. at room temperature. Cells were centrifuged at no more than 12,000 *x g* for 15 min. at 4°C to separate mixtures into a lower red, phenol-chloroform phase, an interphase and a colorless upper phase. RNA in the aqueous phase was carefully removed, transferred to a fresh tube, 0.5 ml of isopropranol was added to this RNA solution and incubated at room temperature for 10 min. Cells were centrifuged for no more than 12,000 *x g* for 10 min. at 4°C. Supernatant was removed, RNA precipitate was washed 1X with 75% ethanol, mixed by vortexing, and centrifuged for no more than 7,500 *x g* for 5 min. at 4°C. The RNA pellet was vacuum-dried briefly or 5- 10 min., dissolved in RNase-free water.

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Reverse transcription (RT). For each sample, 0.5ug of total cytoplasmic RNA was incubated at 65°C for 10 min with 250 umol oligo(dT)$_{15}$ primer (Bohringer) in total volume of 10 ul. After cooling on ice, 10 ul of RT mix was added: 2X AMV buffer, 15U AMV (Amersham), 2.5 uM 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 2min. Samples were stored at – 80°C. *Molecular beacons with RT-PCR.* Molecular beacons were designed with a DNA folding program (http:www.ibc.wustl.edu/~zuher/dna/form1.cgi) to have hairpin-shaped stem that dissociate at a temperature 10°C higher than the detection temperature. The molecular beacons were synthesized as described by Tyagi and Kramer et al, 1996.

2 ul of RT products were used in the PCR reaction carried out under the following conditions: 1X TagMan Buffer (Perkin-Elmer), 2.5 uM MgC12, 250 uM dNTP, 15 pmol of each primers, 2.5U AmpliTag 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. Values for waf1, gadd45 and bax were normalized with GAPDH. Results are expressed as % of control (untreated cells).

The primer pairs used for PCR reaction were following:

Gadd45, Forward primer: 5' – CCATGCAGGAAGGAAAACTATG – 3'; Antisense primer: 5' – CCCAAACTATGGCTGCACACT – 3'.

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Waf1, Forward primer: 5' – ACCTTCCAGCTCCTGTAACATACT – 3'3'; Antisense primer: 5' – GTCTAGGTGGAGAAACGGGAA – 3'.

GAPDH, Forward primer: 5' –AGAGCACAAGAGGAAGAGAGAGACC– 3'; Antisense primer: 5' –AACTGTGAGGAGGGGAGATTCAG– 3'. PCR primers for each target gene selected were designed using Mac vector NTI computer software.

The sequences of the molecular beacon were following:

Gadd45, 5'-CGCTGCAGAATGGTTGAGTTACATTAAAATAAACCGCAGCG –3';

p21^{WAF1}, 5' – CGCTGCAGGACACATGGGGAGCCGAGCAGCG – 3';

GAPDH, 5' – GGACGCGGTGGGGGACTGAGTGTGGCGTCC – 3'.

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Chapter 3

CPT and Zeocin can increase p53 levels during all cell cycle stages

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Introduction

It has not been determined if during all stages of the cell cycle similar signals can increase the levels and activate the DNA binding activity of p53. The work presented here begins to address this idea using cells separated by centrifugal elutriation and subsequent treatment with the topoisomerase-I inhibitor, Cpt or Zeocin which is a member of the bleomycin family of antibiotics. We used the human myeloid leukemia cell line, ML-1, because this was the initial cell line used to demonstrate p53 stabilization after exposure to Cpt and bleomycin (Nelson, W. G., and Kastan, M. B., *et al,* 1994). Additionally, ML-1 cells grow in suspension and this makes the cell line excellent to be separated by centrifugal elutriation. The topoisomerase I-targeted drug, Cpt exhibits cell cycle-independent toxicity under certain conditions (Cotter, G. *et al.*, 1992; Morris, E. J. and Geller, H. M., *et al,* 1996). However, it has been suggested that in order for Cpt to stabilize wild-type p53, S phase DNA replication is required. In the presence of replication inhibitors, p53 levels had previously been shown not to increase. We found that p53 can be induced by Cpt and Zeocin in ML-1 cells treated with APH (which stalls DNA synthesis) (chapter 5). Nelson and Kastan reported that Cpt can stabilize topoisomerase I-DNA complexes throughout the cell cycle, but the stabilized complexes are non-toxic unless DNA replication is allowed (Nelson, W. G., and Kastan, M. B., *et al,* 1994; Haapajarvi, T., *et al.*, 1997; Pitkane, K., *et al.*, 1998), suggesting that the replication fork colliding with the cross-linked Cpt/DNA complex causes a double-strand

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break which is toxic. The evidence indicating that DNA replication is required for both Cpt cytotoxicity and the induction of p53 has been interpreted as an S phase requirement. However, the experiments that generated the evidence made use of chemical replication inhibitors and thus the interpretations of these experiments have failed to acknowledge that these inhibitors also inhibit the coupling of DNA replication to DNA repair. DNA replication coupled to DNA repair can occur in cell cycle stages other than S and may activate pathways needed for the stabilization of p53.

This thesis describes the first study performed to investigate the activation of p53 by Cpt and Zeocin over the cell cycle in the absence of cell cycle inhibitors. Zeocin is a bleomycin analogue marketed by Invitrogen as a means to obtain clonal selection of cells. Vectors can be designed that carry a drug resistance marker for Zeocin for selection in mammalian cells, plants, yeast and prokaryotes. E.coli strains carrying the complete Tn5 (i.e. DH5αF'IQ, SURE, SURE2) transposable element that encodes the Shble (bleomycin) resistance gene have resistance to Zeocin. Zeocin^R cells will continue to divide at regular intervals after plated on medium containing Zeocin to form distinct colonies. Concentration for selection range from 50 to 1000 ug/ml. Zeocin does not require DNA replication to damage the DNA as it binds to DNA directly and cleaves it. By using ML-1 cells synchronized by centrifugal elutriation, we have monitored p53 levels, the DNA binding activity of p53 and p53 phosphorylation at serine-392 before and after both Cpt and Zeocin drug treatments.

It has been predicted that between 200 and 300 p53 response elements exist in the human genome (Tokino, T., *et al.*, 1994), suggesting that p53 may be coordinately regulating the transcription of a number of genes. The ability of wild-type p53 to arrest

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the cell cycle at the G1/S border is associated with the ability of p53 to activate the $p21^{WAF1/Cip1}$ gene, which produces a potent cyclin-dependent kinase inhibitor (El-Deiry, W. S., *et al.*, 1992; Harper, J. W., *et al.*, 1993). The p53-mediated growth arrest at the G2/M border appears to be through activation of the 14-3-3 σ gene whose product inhibits the activity of cdc25 (Hermenking, H., *et al.*, 1997) and activates the DNA binding activity of p53 (Waterman, M. J., *et al.*, 1998). Additionally, activation of gadd45 (which is a part of the DNA damage response pathway) may inhibit DNA replication until DNA repair has taken place and is also part of a G2/M checkpoint (Kearsey, J. M. *et al.*, 1995; Smith, M. L., *et al.*, 1994; Smith, M. L., *et al.*, 1996). There are some genes that are directly involved in apoptotic signaling pathways, for example the ba*x* gene (Miyashita, T., *et al.*, 1994; Miyashita, T. and Reed, J. C., 1995), the KILLER/DR5 gene (Sheikh, M. S. *et al.*, 1998), IGF-BP3 (Buckbinder, L., *et al.*, 1998), p53AIP1, Noxa, Puma (Oda, K., *et al.*, 2000; Nakano, K., *et al*, 2000). The protein products of p53 target genes have diverse and opposing functions and may therefore need to be activated at very different times under variable conditions. Our results showing differential cell cycle activation of p53 by Zeocin and Cpt suggests that post-translational modifications of p53 and increases in p53 levels may contribute to different cellular outcomes within the cell. Diverse combinations of post-translational modifications of p53 may be involved in controlling differential-mediated gene expression. Recent data showed that phosphorylation at serine 46 initiated as a result of severe damage can change the conformation of p53 increasing its affinity for apoptosis-related genes such p53AIP1 (Oda, K., *et al.*, *et al*, 2000). This also suggests the possibility that during moderate damage p53 is phosphorylated at serine 15 and 20.

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These phosphorylations would increase p53 affinity to G1 arrest or DNA repair-related genes.

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Results

p53 Levels Increase When ML-1 Cells Are Treated with CPT

A variety of chemotherapeutic DNA damaging agents can elevate wild-type p53 levels in the human myeloid leukemia cell line ML-1. We began by analyzing nuclear extracts derived from ML-1 cells after drug treatment with 20 uM CPT at various time points by Western blotting with a mixture of p53-specific monoclonal antibodies (Fig. 3.1A).

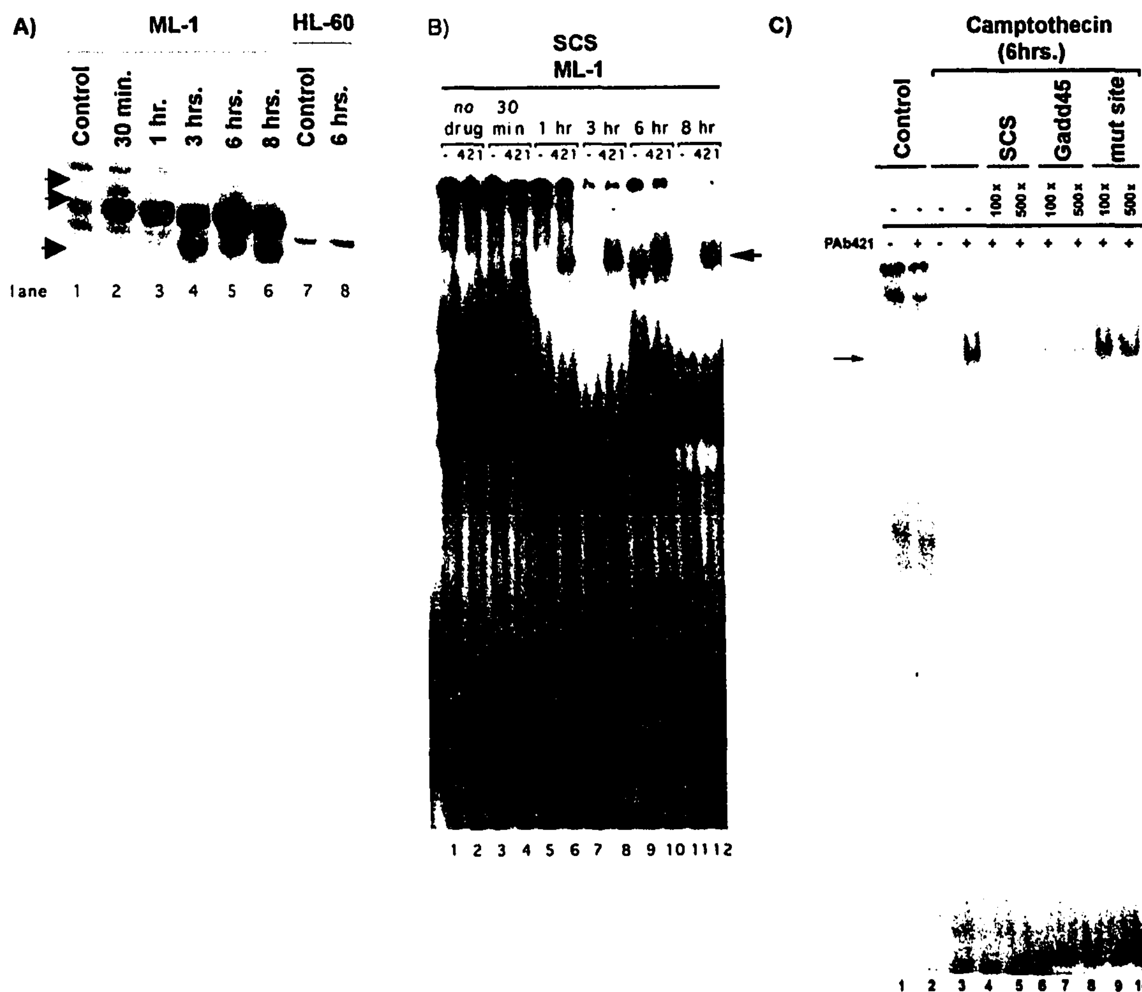

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Figure 3.1: Time curve for CPT treatment: A peak in p53 protein level and electrophoretic mobility shift at 6 h. ML-1 cells were incubated with 20 uM CPT for 30 min., 1 h, 3 h, 6 h, and 8 h, then placed on ice for 10 min. Nuclear protein extracts were prepared according to conditions described by Dignam, J., *et al*, 1983. (A) 100 ug of nuclear extracts was resolved by 12% SDS-PAGE and visualized by Western blot analysis with a mixture of the p53 antibodies 421, 1801, and 240 followed by detection with ECL reagent. Lanes are as indicated. (B) Electrophoretic mobility shift analysis was carried out with the same extracts and a ^{32}P-labeled super consensus sequence (SCS) oligonucleotide. All reactions were carried out in the presence and absence of PAb 421 as indicated. (C) Competition of the PAb 421 induced super-shift of SCS with ML-1 nuclear extract derived from cells treated for 6 h with 20 uM CPT. Competition was as indicated with non-labeled SCS, Gadd45 and mutant sequence oligonucleotide. **Top** arrow (band **a**) indicates an antibody reactive p53 species with a mobility of 53kDa, **Middle** arrow (band **b**) indicates an antibody reactive p53 species with a mobility of 50kDa (represents two bands in close proximity to each other) and **lower** arrow (band **c**) indicates an antibody reactive p53 species with a mobility of 40-42 kDa.

The Cpt concentration used to begin the studies was 20 uM which was in the range of that used by Nelson and Kastan (Nelson, W. G. and Kastan, M. B., *et al,* 1994). At this concentration, Cpt binds both with topoisomerase I (topo I) and the DNA. In this way a topoisomerase/Cpt/ DNA ternary complex is formed and this complex inhibits the progression of both transcription and DNA replication, while irreversibly trapping the topo I-induced cleavage site (Pommier, Y., *et al.*, 1998). Three p53 reactive species with mobilities of 53kDa (band **a**), 50kDa (band **b**) and 40-42 kDa (band **c**) were observed after drug treatment. The maximum elevation of the p53 antibody reactive species with a mobility of approximately 50 kDa (band **b**) was seen at 6 h and no induction of a similar band or band **a** or **c** was observed in the p53 negative cell line HL-60 (Fig. 3.1A, compare lanes 5 and 8). The p53 reactive species with the mobility of approximately 40-42kDa was shown to react with p53 antibody 240 and was phosphorylated at serine 392 in the presence of Cpt (fig. 3.3 A & B). Nuclear extracts derived from the same time course were then tested for p53 DNA binding ability. The binding of p53 to DNA was monitored by electrophoretic mobility shift assays (EMSAs) of a ^{32}P-labeled (or radiolabeled) oligonucleotide containing the p53 super consensus sequence (SCS) in the presence or absence of the p53-specific monoclonal antibody PAb 421. PAb 421 is

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routinely used to activate the DNA binding activity of p53. The density of this band **b** which has been shown to react with 1801, 421 and not DO-1 p53 antibodies (fig.5.1) correlates with the DNA binding actvitiy of p53 to SCS oligoNT (fig. 3.1B). Both a peak in p53-dependent DNA binding activity (PAb 421 activated supershift; fig 3.1B compared lanes 10 and 12) and protein levels (fig 3.1 A) was observed at 6 h. Therefore we chose 6 h of 20 uM CPT treatment as the initial set of conditions for drug treatment (other drug treatment will be shown in later figures). The specificity of the PAb 421 induced gel shift species was further tested by competition with different DNA oligonucleotides. The shift induced by 6 h of 20 uM Cpt treatment was able to be efficiently competed by 100-fold excess of cold SCS competitor and was also competed to some extent by the p53 binding site gadd45, while the mutant site competitor was unable to elicit competition (Fig. 3.1C).

CPT Induced p53 stabilization during All Cell Cycle Stages

We have used centrifugal elutriation to separate cells into discrete cell cycle stages in order to examine if the pathways necessary for stabilization of p53 are present throughout the cell cycle. Centrifugal elutriation of tissue culture cells allows cell cycle fractions to be separated on the basis of their migration in a gradient. For examining p53 protein level, this method has an advantage over using drugs to block the cell cycle at specific

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stages because drug treatment often induces damage that can affect the activity and level of p53. During Cpt treatment, signaling pathways are activated that not only increase p53 but also culminate in programmed cell death (Kaufmann, S. H., *et al.*, 1998). Lymphoid cells often undergo p53-dependent apoptosis (Lowe, S. W., *et al.*, 1993a and 1993b). In some cell types apoptosis can occur in all cell cycle stages (Cotter, G. *et al.*, 1992; Morris, E. J. and Geller, H. M., *et al.*, 1996; Allday, M. J., *et al.*, 1995; Wagner, A. J., *et al.*, 1994).

We analyzed if Cpt treatment of ML-1 cell cycle fractions separated by elutriation could accumulate high levels of p53 regardless of their cell cycle stage. FACS analysis was used to examine the cell cycle stage of elutriated fractions prior to drug treatment and after drug treatment (Fig. 3.2). By Western blot analysis we saw that Cpt was able to induce an increase in p53 protein levels and the DNA binding activity in all the cell cycle stages examined but not as well as it did in exponentially cycling non-elutriated control cells (Figs. 3.3A and 3.3 E, respectively). Three bands **a**, **b** and **c** similar to bands observed in figure 3.1 were generated in Cpt treated exponentially growing cells (Fig. 3.3A, lane 9) in comparison to two bands **b** and **c** in the elutriated fractions of Cpt treated cells (Fig. 3.3A, lanes 10-16). This suggests that the loss of the upper band in fractions may be a result of an additional stress in the elutriation of the cells treated with Cpt. Pertained molecular weight markers were used as indicators of size for Western blot analysis (they are indicated by arrows through out fig 3.3).

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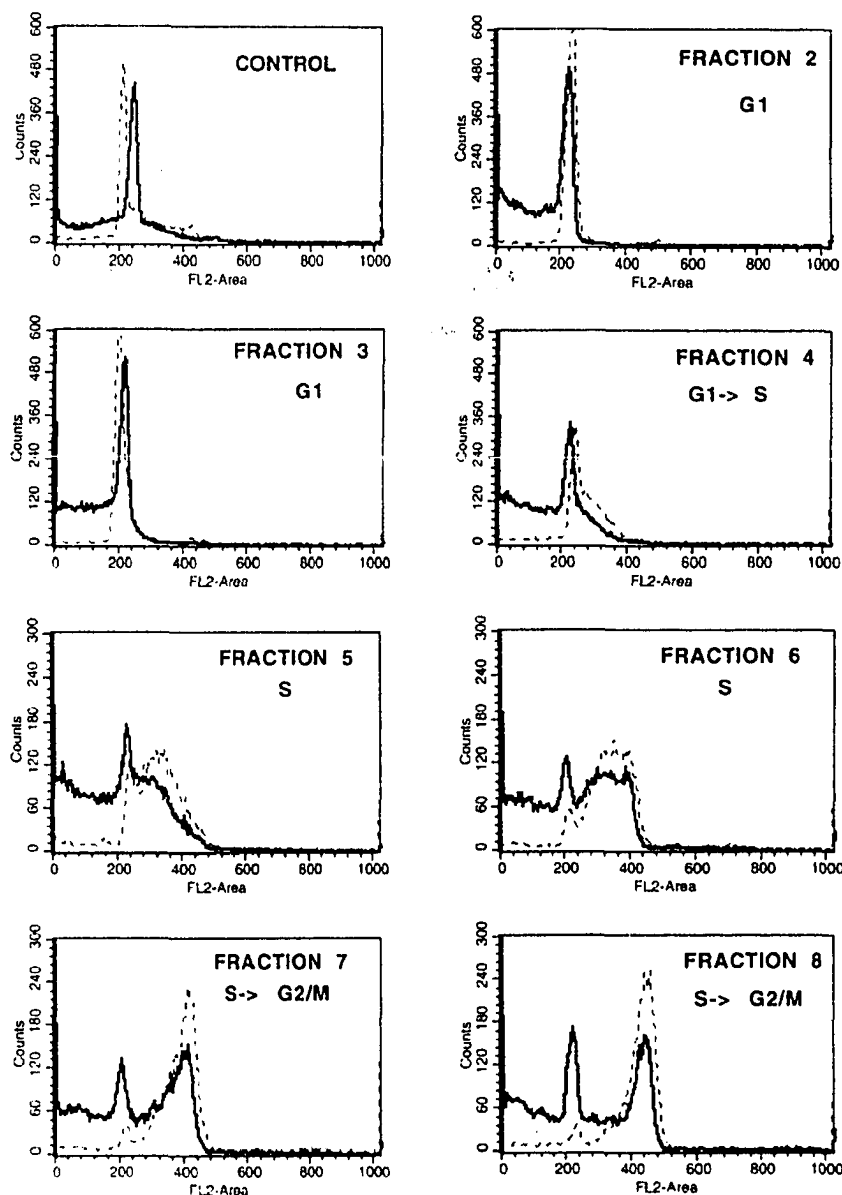

Figure 3.2: FACS Analysis of Elutriated Cell Cycle Fractions with and without CPT treatment. ML-1 cells were separated into enriched cell cycle populations by centrifugal elutriation. Each fraction was then treated with 20 uM CPT for 6 h. The cell cycle distribution was determined by flow cytometry profiles of fixed and propidium iodide-stained cells. Values were derived by Becton Dickinson FACS Scan and Phenox Flow System Multi-Cycle software. The dashed line represents the ML-1 cells before treatment with drug. The bold black line represents the ML-1 cells after treatment with 20 uM CPT for 6 h. The cell cycle distributions were as follows: The control sample contained cells that were not separated by centrifugal elutriation. The viable cell distribution was 45% G1, 50% S and 5% G2/M; the sub-G1 DNA content increased by 24% after drug treatment. The viable cell distribution of fraction 2 before drug treatment was 98% Gland 2% S; after drug treatment the sub-G1 DNA content increased by 44%. The viable cell distribution of fraction 3 before drug treatment was 97% Gland 3% S; after drug treatment the sub-G1 DNA content increased by 42%. The viable cell distribution of fraction 4 before drug treatment was 36% Gland 64% S; after drug treatment the sub-G1 DNA content increased by 43% (here the viable

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cell distribution began changing and was 62% G1, 37% S, and 1% G2/M. The viable cell distribution of fraction 5 before drug treatment was 13% G1and 85% S and 2% G2/M; after drug treatment the sub-G1 DNA content increased by 41% (here the viable cell distribution began changing and was 22% G1, 76% S, and 2% G2/M. The viable cell distribution of fraction 6 before drug treatment was 4% G1and 92% S and 4% G2/M; after drug treatment the sub-G1 DNA content increased by 31% (here the viable cell distribution began changing and was 11% G1, 63% S, and 26% G2/M. The viable cell distribution of fraction 7 before drug treatment was 4% G1and 89% S and 7% G2/M; after drug treatment the sub-G1 DNA content increased by 30% (here the viable cell distribution began changing and was 15% G1, 36% S, and 49% G2/M. The viable cell distribution of fraction 8 before drug treatment was 4% G1and 70% S and 26% G2/M; after drug treatment the sub-G1 DNA content increased by 31% (here the viable cell distribution began changing and was 23% G1, 38% S, and 39% G2/M. Performed 3 times.

The activation of p53 by DNA damage involves signal transduction pathways that affect the phosphorylation of p53 (Waterman, M. J., *et al.*, 1998; Siliciano, J. D., *et al.*, 1997). Therefore, we examined the cell cycle regulation of p53 phosphorylation at serine-392 because it is not induced under all DNA damage conditions and it has been reported that this phosphorylation can activate the DNA binding activity of p53 (Kapoor, M. and Lozano, G., *et al.*, 1998).

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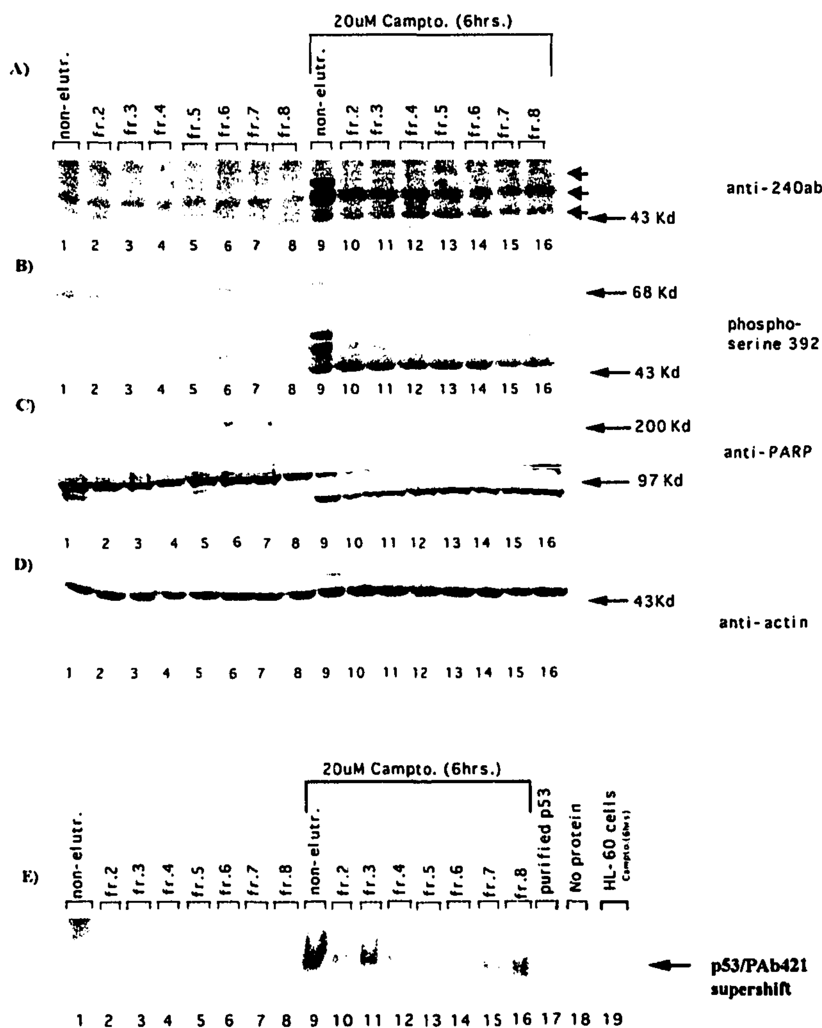

Figure 3.3: An increased level of p53 and serine-392 phosphorylation occurred after CPT-treatment independent of the cell cycle stage. 100 ug of nuclear lysates from elutriated cell cycle fractions with and without CPT treatment were resolved by 10% SDS-PAGE and transferred to nitrocellulose. (A) The levels of p53 were examined by immunoblotting with the monoclonal anti-p53 antibody PAb 240 that recognizes the central region of p53. (B) The specific phosphorylation at serine-392 was visualized with a polyclonal anti-p53 antibody for phosphoserine-392, corresponds to band **c**. (C) PARP cleavage was detected using a monoclonal anti-PARP antibody from PharMingen. PARP cleavage is considered to be a marker of apoptosis. (D) The blot was probed with anti-actin (Sigma) to normalize for gel loading. Arrows indicate the position of molecular weight markers. (E) Mobility shift assay with the p53 SCS oligonucleotide and nuclear extracts from CPT-treated ML-1 cell cycle fractions. EMSA reactions were carried out with nuclear extracts from non-drug-treated ML-1 cells (lanes 1-8) and 6 h CPT-treated ML-1 cells (lanes 9- 16). The extracts from each elutriated cell cycle fraction were as indicated. All reactions contained PAb 421 and p53 super-consensus site (SCS). Nuclear extract from HL-60 cells (containing no p53) treated with CPT for 6 h (lane 19, no protein (lane 18) and immuno-purified p53 from baculovirus infected insect (lane 17) were used as negative and positive controls. **Top** arrow (band **a**) indicates an antibody reactive p53 species with a mobility of 53kDa, **Middle** arrow (band **b**) indicates an antibody reactive p53 species with a mobility of 50kDa (represents two bands in close proximity to each other) and **lower** arrow (band **c**) indicates an antibody reactive p53 species with a mobility of 40-42 kDa.

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Cpt not only induced stabilization of p53 in every cell cycle fraction examined but also induced serine-392 phosphorylation in every cell cycle stage (Fig.3.3B). Interestingly, the p53 phosphorylated at serine-392 was the fastest migrating form of the protein which was also reactive with anti-p53 antibody 240 (Fig. 3.3A). Two p53 reactive species with mobilities of approximately 53 and 50 kDa were detected by 3 anti-p53 antibodies: PAb 421 (the C-terminal specific antibody), PAb1801 (more internal N-terminal specific antibody; data not shown) and PAb240 (the DNA binding domain specific antibody; fig 3.3A). The p53 reactive species with mobility of approximately 53kDa (band **a**) was not detected with the most N-terminal antibody DO-1 after Cpt treatment (Fig. 3.4B, see lane 7) as constrasted in figure 5.1b when the some protein degradation in the nuclear extract was prevented by the addition of additional proteases.

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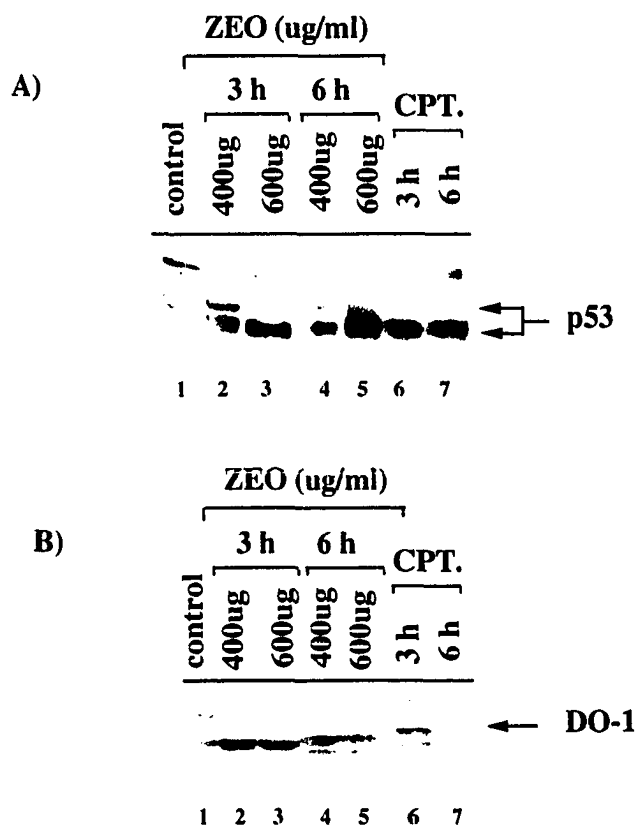

Figure 3.4: CPT induces a form of p53 that is not reactive to the DO-1 antibody. Nuclear extracts from ML-1 Cells grown in 1X RPMI with 10% FBS with and without drug treatments as indicated. Exponentially growing cell were treated with 400and 600ug/ml of Zeocin and 20 uM CPT for 3 and 6 h at 37°C. 95ug of nuclear extract was electrophoresed on 10% polyacrylaimde gel, transferred to nitrocellulose membrane and analyzed with (A) anti-p53 antibody PAb 1801 (specific to the N-terminus, aa 46-55), **Top** arrow (band **a**) and **lower** arrow (band **b**), Experiment performed 3 times, and (B) DO-1 (specific to the extreme N-terminus, aa 18-30, corresponds to band **a**). Experiment performed once.

Interestingly, the amount of p53 induced by Cpt in non-elutriated cells was reproducibly greater than that seen in the elutriation fractions although this was not the case for Zeocin drug treatments (compare Figs. 3.3 and 3.8). We do not have a clear explanation for this but we have noted that after elutriation the cells are no longer healthy (cells undergoing p53 independent apoptosis) and this may affect the signaling pathway

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utilized by Cpt (see Fig. 3.7). The same Western blot was also analyzed for activation of poly (ADP-ribose) polymerase (PARP), an abundant protein that recognizes certain types of DNA damage including that induced by Cpt (Kaufmann, S. H., *et al*, 1998). During all forms of apoptosis PARP is cleaved by caspases after activation. PARP cleavage was detected in every cell cycle stage (Fig. 3.3C).

Nuclear extracts from elutriated synchronized cells were also examined for p53-dependent DNA binding activity by EMSA to assess the functional activation of p53. The increases in p53-dependent DNA binding activity in every cell cycle fraction for the super consensus sequence and gadd45 oligonucleotide also demonstrated that the p53 in every cell cycle fraction was able to bind to DNA (Fig.3.3 E). Therefore the machinery necessary to stabilize and activate p53 DNA binding was present in every cell cycle stage. The observed p53 DNA binding activity was highest in fractions 2, 3, and 8 (Fig.3.3E). A direct correlation was not observed between increased DNA binding activity, the induction of p53 levels (p53 reactive species with a mobility of approximately 50 kDa as seen in figure 3.1a) and phosphorylation of p53 at serine-392. It is possible that different forms of p53 in the nucleus function together as active oligomers to bind to DNA. We reproducibly saw the same level of apoptosis in all cell cycle stages (Fig 3.2). Therefore, in this experimental system, none of the p53 activities assayed correlated with the induction of apoptosis. In fact, apoptosis could be induced in the absence of p53 (Fig.3.9 and Fig. 4.2A and B, as indicated by PARP cleavage in the absence of p53 induction).

To rule out if PAb 421 antibody affects p53 DNA binding activity, another EMSA using the oligonucleotide derived from the gadd45 p53 binding site was performed with

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nuclear extracts (1 ug/ul) from non-elutriated and elutriated ML-1 cells with and purified p53 protein from baculovirus insect cells (SF9) (no Pab 421 was added). The DNA binding activity profile in each fraction (Fig. 3.5C) correlates with the DNA binding activity from the previous EMSA where PAb 421 was present in each fraction (fig. 3.5A). This eliminates the concern that PAb 421, in some way, influences the DNA binding activity of p53.

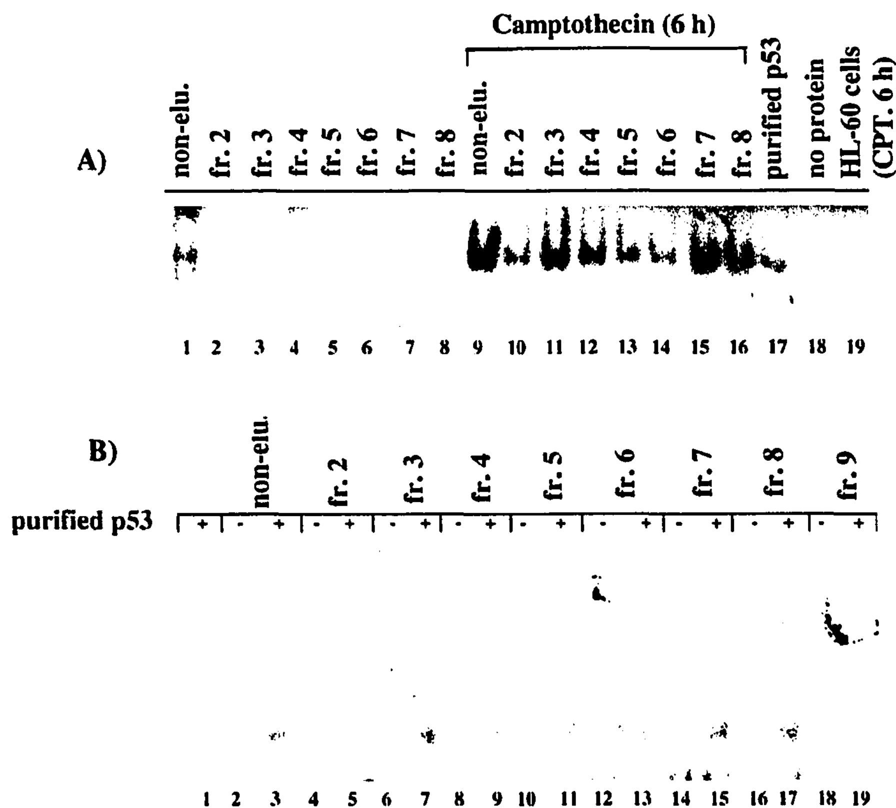

Figure 3.5: Binding of purified p53 from baculovirus insect cells to Gadd45 oligonucleotide in the absence of 421 antibody. EMSA reactions were carried out with nuclear extracts from non-elutriated and

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elutriated ML-1 cells isolated after 6 h. Nuclear extract derived from ML-1 cells treated with 20 uM CPT were incubated with [P32]-labeled DNA corresponding to Gadd45 p53 binding site and then electrophoresed on a 4% gel. All lanes contained PAb421 antibody. HL-60 cells treated with CPT for 6hr and samples containing no protein were used as negative controls. Performed 3 times. (B) In a separate experiment, a similar analysis was carried out using the same extracts from experiment (A) in the absence of PAb421 antibody. Nuclear extracts (1ug/ml) were incubated with purified p53 (isolated from baculovirus infected insect cells) in DNA binding buffer for 20 min. at 37°C. Performed once.

Zeocin Treatment of ML-1 cells Results in the stabilization of p53 without Phosphorylation at serine 392

Many different DNA damaging drugs in addition to Cpt can stabilize wild-type p53 in ML-1 cells. Zeocin has been marketed as an agent for antibiotic selection when selecting stable cell lines. We tested this agent for its ability to increase the stability of p53 in ML-1 cells and found that it was a potent agent for the stabilization of p53 (Fig. 3.6A).

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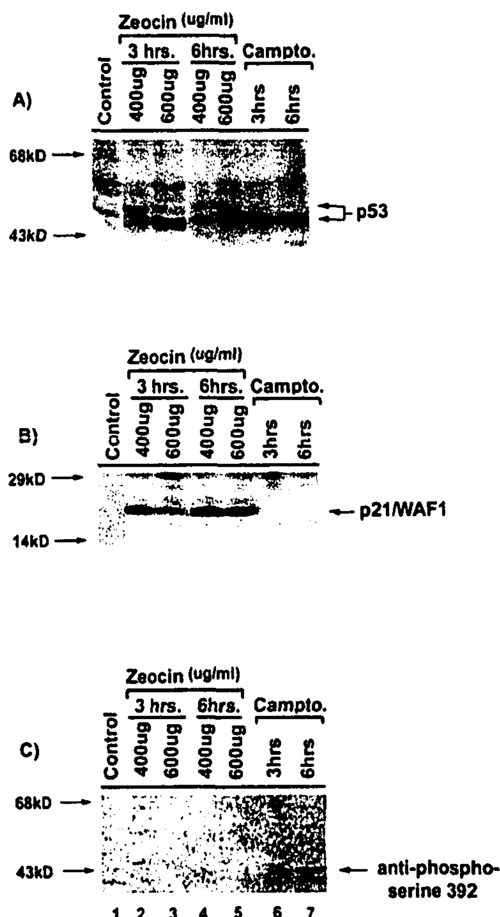

Figure 3.6: Zeocin and CPT induce the same level of p53 but the pathways differ. Nuclear extracts were prepared from ML-1 cells grown in 1X RPMI with 10% FBS with and without drug treatments as indicated. Exponentially growing cells were treated with 400 and 600 ug/ml of Zeocin and 20 uM CPT for 3 and 6 h at 37°C. 95 ug nuclear extracts was electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose membrane and analyzed (A) anti-p53 antibody PAb 1801, **Top** arrow (band **a**) and **lower** arrow (band **b**) (B) anti-p21^{WAF1}antibody from Oncogene Science and (C) with anti-phosphoserine 392 antibody, corresponds to p53 reactive species with a mobility of approximately 40-42 kDa ,band **c** (a gift from G. Lozano). Performed 3 times.

We found that with a Zeocin concentration of 600 ug/ml, we were able to induce a similar level of p53 to that seen when the cells were treated with 20 uM Cpt. At lower Zeocin concentrations, p53 was also induced but not to a level that was comparable to previous CPT treatment that was obtained with 20 uM (Fig. 3.4). Interestingly, when we

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compared nuclear extracts for p21^{WAF1} protein levels, the cells treated with Zeocin showed increased p21^{WAF1} protein levels while those cell treated with Cpt reproducibly gave barely detectable levels of p21^{WAF1} (Fig. 3.6B).

We then examined the phosphorylation of p53 at serine-392 by Western blot analysis with a p53 phospho-specific antibody directed against this residue. We found that the p53 in nuclear extracts derived from the Zeocin-treated samples was not detected with the phospho-specific antibody for serine 392, while the p53 reactive species with a mobility of approximately 40-42 kDa from the samples treated with Cpt showed a detectable band with the same phospho-specific antibody (Fig. 3.6C). The ML-1-derived p53 stabilized by Zeocin clearly was not exactly the same species as the p53 stabilized by Cpt. Therefore, we reasoned that the pathways initiated for p53 stabilization by the two different drugs were different. This concept will be expanded on in chapter 5. We then examined if Zeocin, like Cpt, was able to stabilize p53 regardless of the cell cycle stage of ML-1 cells.

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Zeocin Treatment of ML-1 Cells Can Stabilize p53 in All Cell Cycle Stages

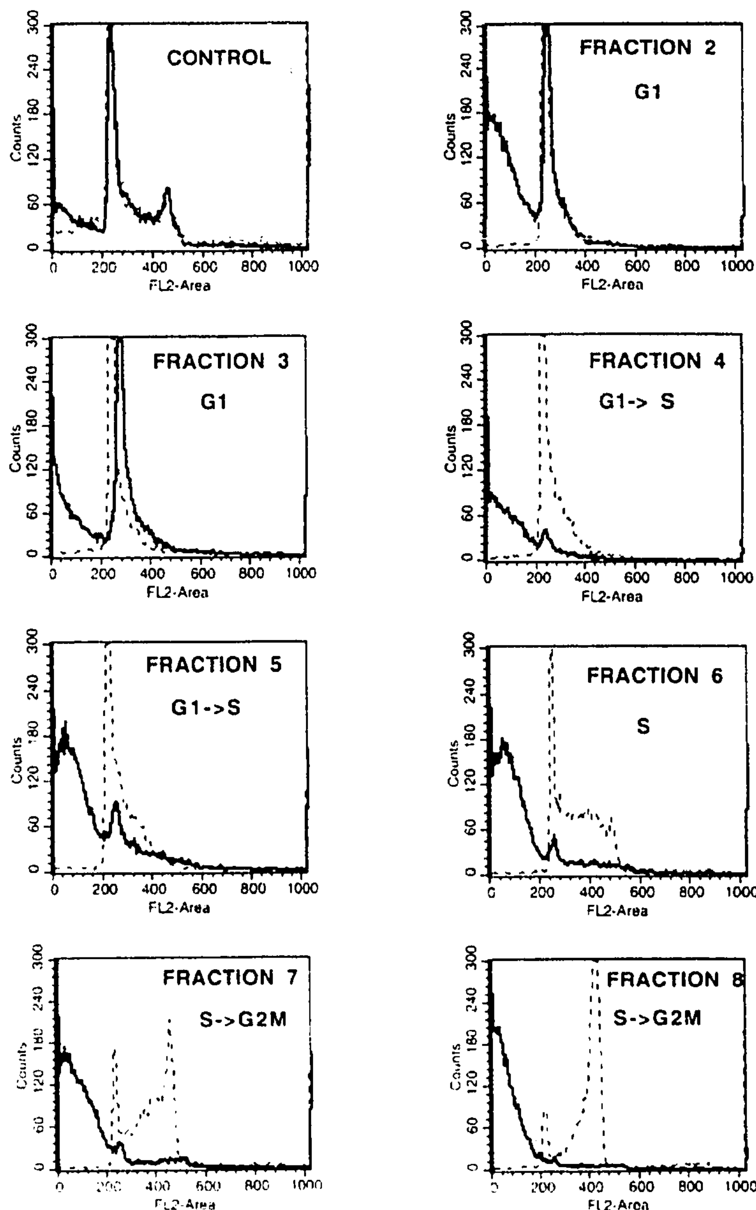

Figure 3.7: FACS Analysis of Elutriated Cell Cycle Fractions with and without Zeocin treatment. ML-1 cells were separated into enriched cell cycle populations by centrifugal elutriation. Each fraction was then treated with 400 ug/ml Zeocin for 6 h. The cell cycle distribution was determined by flow cytometry profiles of fixed and propidium iodide-stained cells. Values were derived by Becton Dickinson FACS Scan and Phenox Flow System Multi-Cycle software. The dashed line represents the ML-1 cells before treatment with drug. The bold black line represents the ML-1 cells after treatment with 400 ug/ml

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Zeocin for 6 h. The cell cycle distributions were as follows: The control sample contained cells that were not separated by centrifugal elutriation. The viable cell distribution was 50.5% G1, 44% S and 4.8% G2/M; the sub-G1 DNA content increased by 24% after drug treatment. Fraction 2 prior to drug treatment contained 90.1% G1and 9.9% S; after drug treatment the sub-G1 DNA content increased by 43.82%. Fraction 3 prior to drug treatment contained 89% G1and 10.9% S; after drug treatment the sub-G1 DNA content increased by 22.72%. Fraction 4 prior to drug treatment contained 70% G1and 29% S; after drug treatment the sub-G1 DNA content increased by 62.58%. %. Fraction 5 prior to drug treatment contained 48% G1and 52%; after drug treatment the sub-G1 DNA content increased by 68.51. Fraction 6 prior to drug treatment contained 18.7% G1and 79% S and 1.9% G2/M; after drug treatment the sub-G1 DNA content increased by 72.28%. 7 prior to drug treatment contained 10% G1and 72.4% S and 17.6% G2/M; after drug treatment the sub-G1 DNA content increased by 72.15. Fraction 8 prior to drug treatment contained 5.7% G1and 50.2% S and 44.2% G2/M; after drug treatment the sub-G1 DNA content increased by 56.78%.

An analogous experiment to that of the Cpt treatment of the cell cycle fractions separated by centrifugal elutriation (Figs. 3.2 and 3.3) was carried out for ML-1 cells treated with Zeocin (Figs. 3.7 and 3.8). Once again we observed that the p53 was stabilized in all cell cycle fractions examined (Fig. 3.8A) and that the DNA binding activity of p53 was also induced in every cell cycle fractions examined (Fig.3.8D). In fact the induction of both p53 level and DNA binding activity appeared to be more uniform in the Zeocin treated cell cycle treated fractions. All the cell cycle fractions showed a clear increase in the level of p53 reactive species with a mobility of 50 kDa, and the level was similar in the non-elutriated sample. This increase in S phase could not be due to G1 or G2 contamination because fraction 6 (figure 3.7) has the same % of G1, S and G2 cells as in fraction 5 (figure 3.2) and the levels of p53 were still low. Also there was a change in p53 levels between fractions 2 and 3 (figure 3.7) and there was no difference in G1, S and G2 cell populations. This suggests that the levels of p53 induction do not depend on the cell cycle. Because Zeocin-treated samples showed a clear increase in nuclear $p21^{WAF1}$ (as seen in Fig. 3.6B), the Western blot of the Zeocin elutriated fractions was probed with anti-$p21^{WAF1}$ antibody. An increase in nuclear $p21^{WAF1}$ was detected in every cell cycle fraction (Fig. 3.8E). It was noted that the cell cycle fractions enriched for G2/M cells

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demonstrated the lowest level of Zeocin-induced p53 as well as the lowest level of nuclear p21[WAF1] and p53 DNA binding activity.

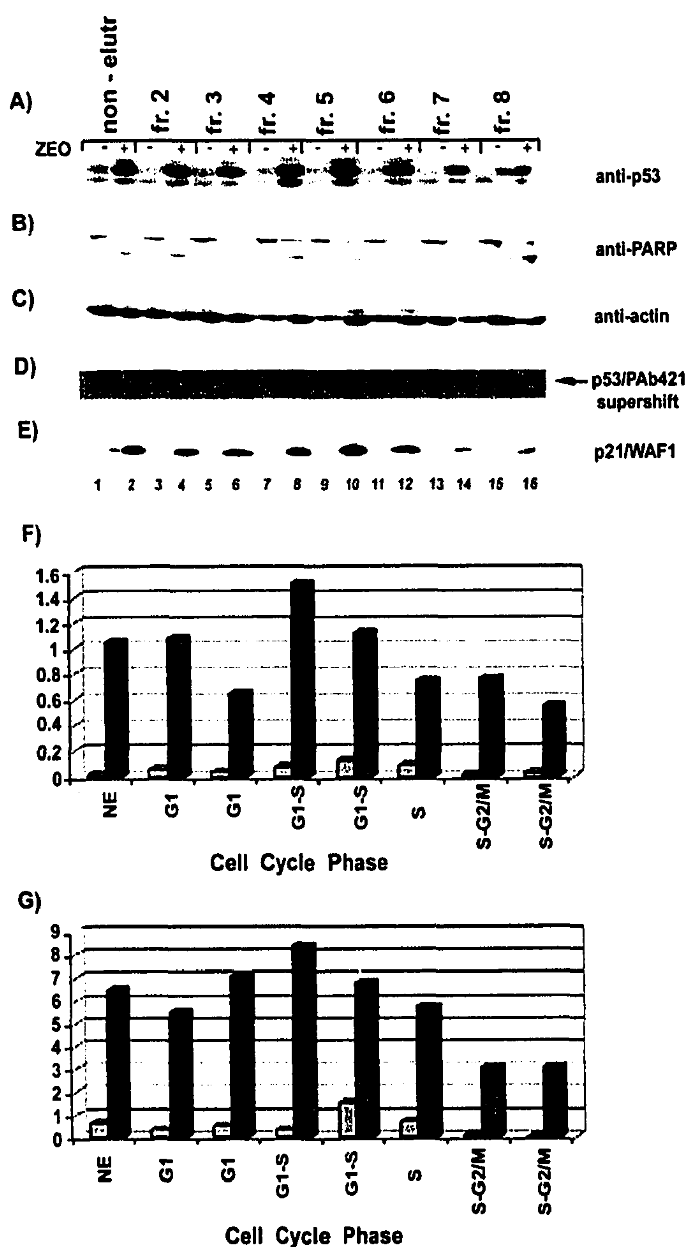

Figure 3.8: An increased level of p53 occurred after Zeocin-treatment independent of the cell cycle. 95 ug of nuclear lysates from elutriated cell cycle fractions with and without 400 ug/ml of Zeocin for 6h at 37oC. Nuclear extracts were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membrane and analyzed for p53 level (A) with a mixture of p53 antibodies PAb 421,1801, and 240 followed by detection with ECL reagent. Lanes are as indicated. (B) Poly (ADP-ribose) polymerase which is cleaved in cells undergoing apoptosis with anti-PARP, (C) anti-actin demonstrates equal loading and (D)

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EMSA with p53 super consensus site and nuclear extracts from Zeocin treated ML-1 cells analyzed by western blot were carried out as described under Materials and Methods. (E) The nitrocellulose membrane was probed with an anti-p21^{WAF1} antibody (Oncogene Science Ab-1) (F) Histogram of the expression of p53 and (G) and p21^{WAF1} after normalization for actin levels using a densitometer and ImageQuant software.

A further examination of the ability of p53 to be induced in ML-1 cells during all stages of the cell cycle was carried out with a shorter time treatment (3 h) and a lower dosage of Cpt (0.5 uM). The negative control consisting of untreated elutriated cells which were then allowed to grow for 3 h, showed no increase in the p53 levels or DNA binding activity (Figs. 3.9A and 3.9D). Cpt treatment at this concentration showed that the cell cycle fractions enriched for S-phase cells had the highest level of p53 induction. Again, induction of p53 in the elutriated fractions was far less than in the non-elutriated cells. It should be noted that the stress of taking the cell through the elutriation was enough to cause the cells to undergo apoptosis as seen by examination with anti-PARP (Fig. 3.9B). All the cell cycle fractions demonstrated apoptosis whether or not they had been drug treated. Although centrifugal elutriation of cells did not induce the stabilization of p53, it did stress the cell enough to induce apoptosis when the cells were actively growing again. This observation does not decrease the validity of the p53 activation data but does show cells undergo p53-independent apoptosis. There appears to be both a p53-independent and p53-dependent apoptotic pathway. Thymocytes from p53$^{-/-}$ mice are resistant to IR-induced apoptosis but not to apoptosis induced by other stresses (Clarke, *et al,* 1993; Lotem and Sach, *et al,* 1993; Lowe *et al,* 1993a & b; Symonds *et al,* 1995).

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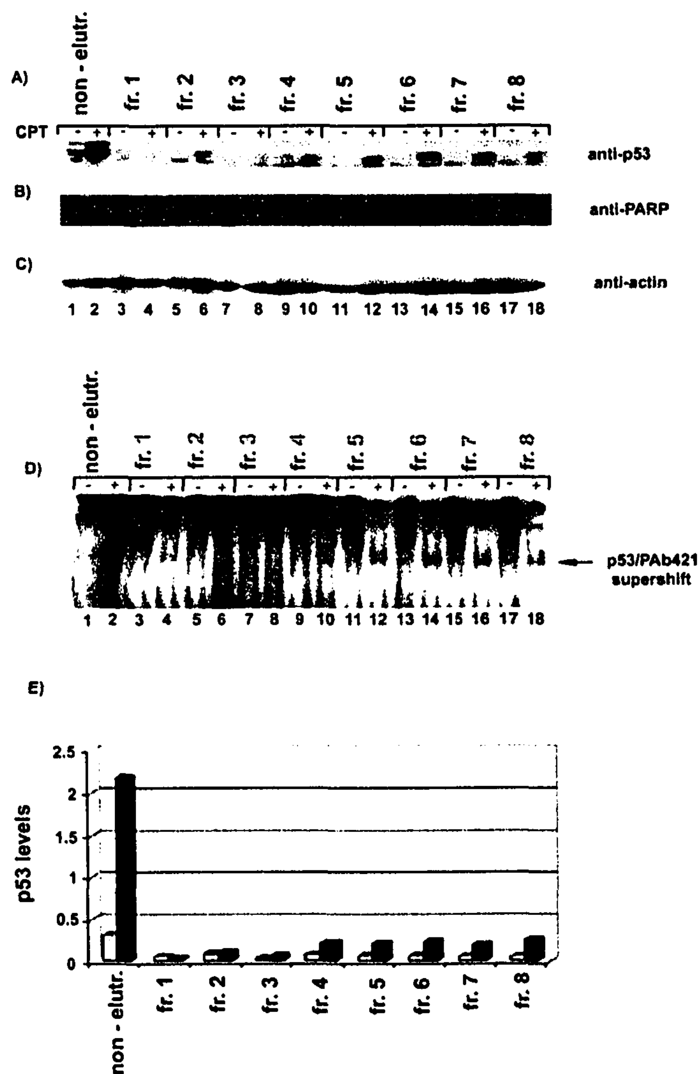

Figure 3.9: An increased level of p53 occurred after 0.5 uM CPT-treatment independent of the cell cycle. 95 ug of nuclear lysates from elutriated cell cycle fractions with and without 0.5 uM of CPT for 3h at 37°C. Nuclear extracts were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose membrane and analyzed for p53 level (A) with a mixture of p53 antibodies PAb 421,1801, and 240 followed by detection with ECL reagent. Lanes are as indicated. (B) Poly (ADP-ribose) polymerase which is cleaved in cells undergoing apoptosis with anti-PARP and (C) anti-actin was used to monitor loading. (D) EMSA with p53 super consensus site and nuclear extracts from CPT-treated ML-1 cell cycle fractions. (E) Histogram of the expression of p53 after normalization for actin levels using a densitometer and ImageQuant software.

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Discussion

We have shown that in ML-1 cells synchronized by centrifugal elutriation both Cpt and Zeocin induce an increase in p53 protein during every stage of the cell cycle, however, through different pathways. This is the first study to examine the effects of cell cycle position on p53 activation in normally cycling cells by two different DNA damage pathways. Although centrifugal elutriation allowed us to examine the effects of drug treatment on p53 activity in different cell cycle fractions, it did not allow us to examine apoptosis. The process of spinning the cells down and injecting them into the gradient was enough to make the ML-1 cells enter p53-independent apoptosis. Elutriation did not cause p53 levels to be increased in the absence of drug treatment and therefore all the analyses of the cell cycle dependent activation of p53 is valid (Fig.3.9). Therefore the results presented address, for the first time, the ability of p53 levels to be increased by different drug treatments in cell cycle fractions isolated by elutriation. DNA-damaging agents that activate cell cycle checkpoints typically cause a high level of wild-type p53 accumulation (Kastan, M. B., *et al.*, 1991). High levels of p53 can induce both apoptosis (Lowe, S. W., *et al.*, 1993a and 1993b) and growth arrest (Zhan, Q., *et al,* 1993; Agarwal, M. L., *et al.*, 1995; Canman, C. E., *et al.*, 1995).

Activation of p53 occurs by at least two independent pathways. One pathway responds to DNA double-strand breaks (major DNA damage caused by IR), the other is activated by bulky DNA adducts e.g. crosslinks (such as those caused by exposure to UV light) (Agarwal, M. L., *et al.*, 1995; Sakaguchi, K., *et al.*, 1998). The pathway activated

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by bulky adducts responds in part by signaling for phosphorylation of p53 at serine-392 (Kapoor, M., and Lozano, G., *et al,* 1998) and this pathway was seen to be activated by Cpt. In addition to Cpt causing a covalent trapped topo I-DNA intermediate, it has been suggested that double strand breaks result during DNA replication and that these activate p53 (Nelson, W. G., and Kastan, M. B., *et al,* 1994; Pommier, Y., *et al.*, 1998; Kaufmann, S. H., *et al,* 1998; Ryan, A. J., *et al.*, 1991). Cpt has been shown to induce apoptosis during all cell cycle stages (Morris, E. J., and Geller, H. M., *et al.*, 1996). However, the Cpt induction of DNA double strand breaks have been proposed to only occur during S-phase (reviewed in Kaufmann, S. H., *et al.*, 1998). It is these DNA double strand breaks that have been proposed as the major cause for induction of p53 by Cpt. The results presented here suggest that active S-phase DNA replication assists p53 induction but it is not essential for Cpt to signal for some activation of p53. The ability to observe p53 induction in all the cell cycle fractions after 6 hours of treatment may be due to the fact that during the 6 hours treatment the cells cycled far enough to cause every fraction to contain some cells in S phase. Additionally, cells blocked in G2/M by nocodazole also showed an increase in p53 levels when treated with Cpt (data not shown). It should be considered that DNA replication coupled to DNA repair occur in cell cycle stages other than just S-phase (Budd, M. E., and Campbell, J. L., *et al.*, 1995; Sancar, A., *et al.* 1995), and this may have been sufficient to allow for low levels of activation in non-S phase cells.

Interestingly, we have shown that although Cpt and Zeocin signal through different pathways, both pathways are cell cycle independent. Different types of DNA repair may be important for the DNA damage signaling pathways that communicate to p53.

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Therefore, it is not surprising that we observed cell cycle-independent induction of p53 in normally cycling cells, presumably because the DNA repair signals are elicited in all cell cycle stages.

Phosphorylation of p53 at serine-392 in response to Cpt occurred in all the cell cycle fractions examined and the p53 in every fraction was able to bind to DNA. Phosphorylation of p53 at serine-392 has also been shown to enhance tetramer formation of p53 and may cross-talk with the DNA-damage induced increases of the p53 protein to help provide a switch that activates a specific p53 pathway (Sakaguchi, K., *et al.*, 1997). When ML-1 cells were treated with Zeocin, the protein $p21^{WAF1}$ was detected in nuclear extracts and this was visible in all the cell cycle fractions (although practically no detectable p21 was observed in Cpt treated cells). Although the level of p53 can be induced in all the cell cycle fractions, Cpt clearly stabilized p53 more effectively in healthy growing cells. However, when we used APH to block DNA replication, p53 was induced equally efficiently by both Cpt and Zeocin (this will be expanded in chapter 4 see fig.4.2B). The fact that Zeocin induced more p53 in elutriated cells suggests that the cells do not need to be as healthy to generate an effective Zeocin induced p53 response. Clearly the drugs Cpt and Zeocin signaled through differing pathways as evident by the differential phosphorylation of p53 at serine-392 and the differential ability of both drugs to cause an increase in the nuclear $p21^{WAF1}$ level. We are in the process of investigating the reasons for these differences. We observed a difference in p21 mRNA levels within the different cell cycle phases of ML-1 cells treated with 400ug/ml of Zeocin (figure A.1). p21 mRNA levels from figure 3.8 were highest in G2/M cell cycle populations. In summary, we have shown that two different p53 stabilization pathways are intact during

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all stages of cell division cycle. Therefore, a cell cycle specific pathway is not required for the activation of p53. Recently ionizing radiation has been shown to activate the ATM kinase throughout the cell cycle (Pandita, T. K., *et al.*, 2000). We would like to suggest that activated DNA repair is required in order to activate ubiquitous factors that signals to p53 during all stages of the cell cycle. The different damage signaling pathways may cross talk with p53 differently.

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Chapter 4

CPT induces p53 stabilization in non-replicating ML-1 cells

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Introduction

The induction of p53 by Cpt was previously thought to have an S-phase requirement (Cotter, T. G., *et al.*, 1992). It has been reported that camptothecin creates TopoI/DNA cleavable complexes that are converted to DNA damage as a result of the replication fork colliding with these complexes during replication (Cotter, T. G., *et al.*, 1992). This damage then triggers p53 induction. This concern arose when acquiring data in Chapter 3. It was suggested that the fact that we saw p53 in all cell cycle fractions (Fig. 3.3A) after 6 hours of Cpt drug treatment might have been due to the fact that during the 6 hour treatment, cells cycled far enough to cause some fractions to contain some cells in S phase (replicating cells). Even though the answer to these questions were answered in Chapter 3 by using a lower dose of Cpt at a shorter treatment (Fig. 3.9), as well as studies with cells blocked in G2/M by nocodazole showing an increase in p53 levels when treated with Cpt (data not shown) and by studies performed by Morris and Geller that showed that aphidicolin (stalls DNA synthesis) did not prevent Cpt-induced toxicity in neurons (Morris, E. J., and Geller, H. M., *et al.*, 1996), we further investigated p53 stabilization in ML-1 cells when DNA synthesis was blocked.

To rule out the possibility that DNA replication is required for Cpt-induced induction of p53 or that cells only in S phase (replicating cells) can induce p53, we examined the stabilization and response of p53 when DNA synthesis was blocked by aphidicoiln (APH) for a short period of time alone and in the presence of Cpt or Zeocin. Aphidicolin blocks eukaryotic polymerases-alpha, delta and epilison or viral induced polymerases by competing with dNTPs for binding to pol-DNA complexes to form a pol-DNA-

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aphidicolin ternary complex. It does not affect RNA, protein or nucleotide biosynthesis (Spadari, et al, 1984). ML-1 cells were also treated with Zeocin because it has not been reported to induce damage dependent on replication (Lopez-Larraza, d et al, 1990). Zeocin as mentioned binds to DNA directly and nicks it. We observed as a result of anti-BrdU and/or PI labeling/FACS cytometry, Western blot anaylsis of p53, $p21^{WAF1}$ and MDM2 and RT-PCR (using molecular beacons) analysis of p21 and Gadd45 mRNA that ML-1 cells pretreated with APH for a short period of time induced transcriptionally active p53 in the presence of Cpt and Zeocin. Cpt, which has been reported to require replication to induce damage, induces p53 levels to a similar extent as Zeocin, which does not require replication to induce damage. This suggests that Cpt's ability to induce p53 in ML-1 cells is not solely dependent on replication. ML-1 cells pretreated with aphidicolin and subsequent treatment with Cpt or Zeocin were able to incorporate BrdU (dUTPs) after increased DNA damage by Cpt and Zeocin which could be the result of cells undergoing increased DNA repair synthesis. We also observed that apoptosis could occur in a p53-independent manner in these cells.

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Results

Increased uptake of BrdU in non-replicating ML-1 cells following Drug treatment

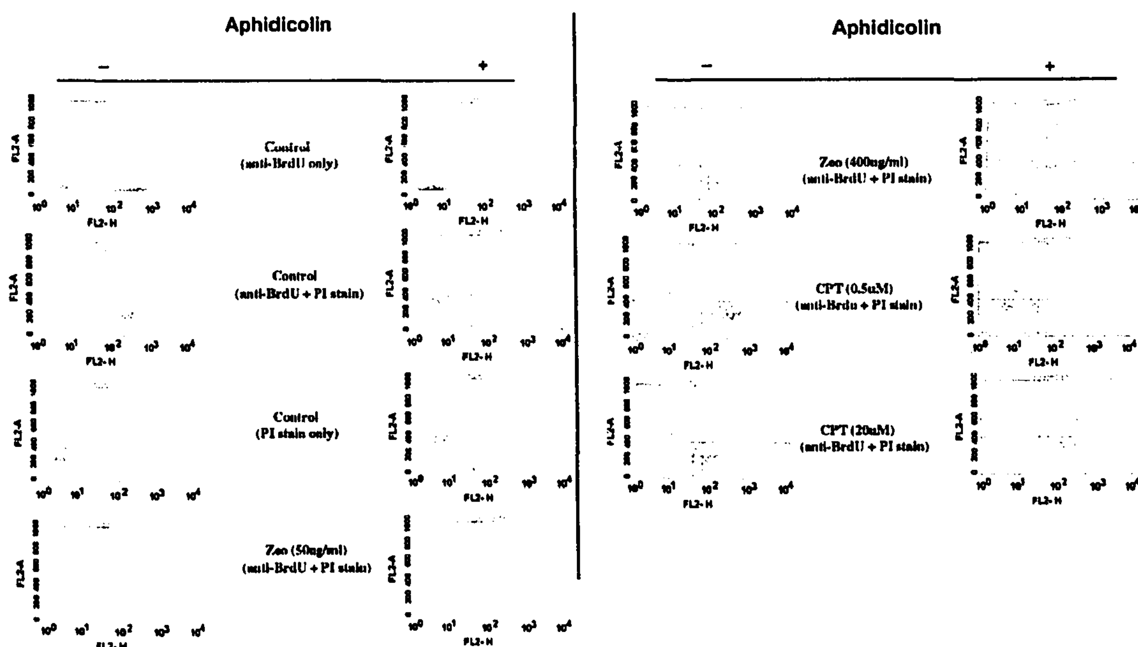

Figure 4.1: Increase in BrdU incorporation in drug-treated ML-1 cells. FACS analysis of non-drug and drug treated ML-1 cells with and without 10 uM APH for 4 hours. ML-1 cells were treated as follows: 50 ug/ml Zeocin, 400 ug/ml Zeocin, 0.5 uM CPT, or 20 uM CPT for 3 hours. Samples were fixed and stained with either propidium iodide, 5-bromodeoxyuridine or both. Stain cells were then analyzed by flow cytometry with Becton Dickinson Scan. Experiment repeated twice. These results are representative of two independent cell treatments. The **Y-axis** represents ML-1 cells stained with PI (FL2-A/linear scale: **0, 200, 400, 600, 800 and 1000**) and **X-axis** represents ML-1 cells stained with anti-BrdU (FL3-H/Log scale:**10^0, 10^1, 10^2, 10^3 and 10^4**).

Exponentially growing ML-1 cells grown in 1 X RPMI with 10% FBS were treated with 10 uM APH for 4 hrs. and then subsequent treatment with 0.5 uM Cpt, 20 uM Cpt, 50 ug/ml Zeocin or 400 ug/ml Zeocin for 3 hrs. (fig. 4.1). The addition of APH to the growing culture resulted in an inhibition of nuclear DNA synthesis within 4 hrs. as assessed by FACS analysis. In the presence of aphidicolin, cells that entered the S phase

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no longer synthesize the DNA (incorporate BrdU), whereas cells in other phases of the cycle continue their growth cycle and accumulate at the G1/S border (Pedrali-Noy *et al.*, 1981; Dinter-Gottlieb and Kaufmann, *et al*, 1983; Spadari *et al.*, 1984). As a consequence, mitosis and cell division decreased gradually and are fully suppressed within a few hours. BrdU (bromodeoxyuridine)-labeled ML-1 cells treated with lower doses of both Zeocin (50 ug/ml) and Cpt (0.5 uM) did not seem to incorporate BrdU (Fig.4.1). But cells treated with higher doses of Zeocin (400 ug/ml) and Cpt (20 uM) incorporated BrdU after pretreatment with APH. This increase in BrdU incorporation was not due to the ability of the cells to start cycling again after treatment because the percent of cells analyze by PI FACS in each cell population G1, S and G2/M did not change after the addition of Zeocin or Cpt (data not shown). The incorporation of BrdU was most likely due to increased DNA repair synthesis.

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Accumulation of p53 occurs in response to DNA damage when DNA synthesis is blocked : associated changes in target activation

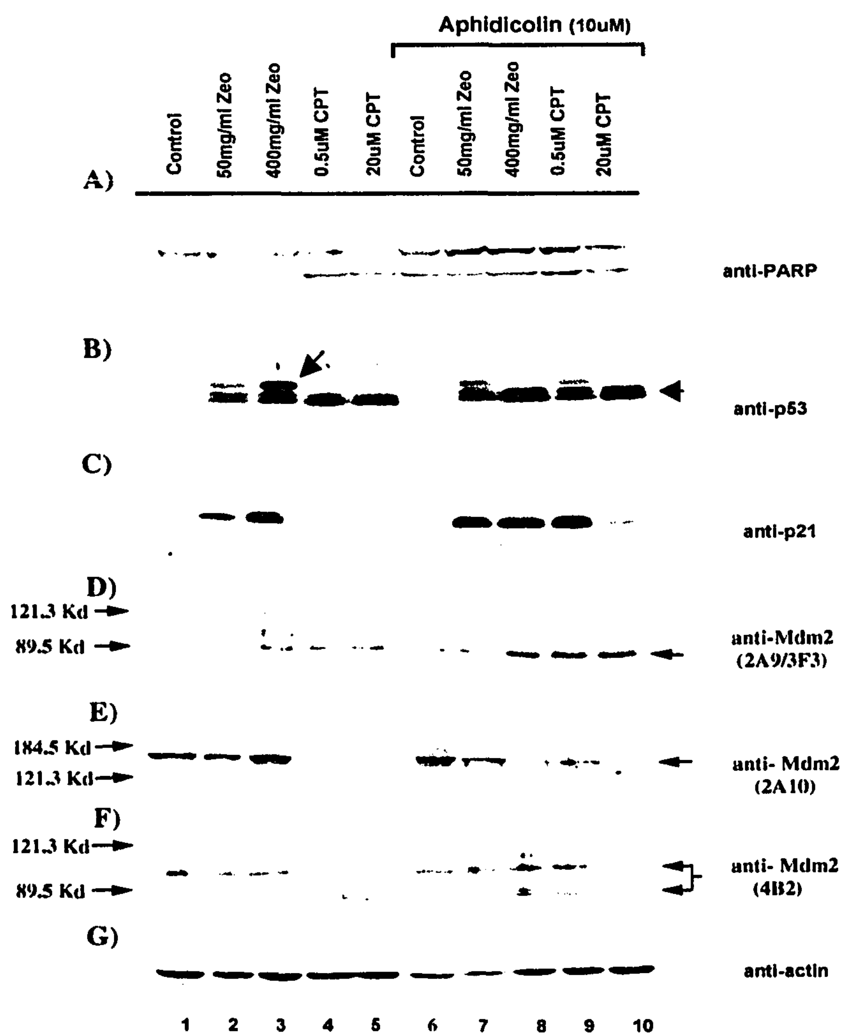

Figure 4.2: Increase in p53 levels in ML-1 cells when DNA synthesis is blocked following drug treatments. Nuclear extracts were prepared from ML-1 cells grown in 1 X RPMI with 10 % FBS with and with drug treatments. Exponentially growing cells were treated with 50 ug/ml of Zeocin, 400 ug/ml of Zeocin, 0.5 uM CPT or 20 uM CPT for 3 h at 37°C with and without pretreatment with 10 uM APH for 4 h. 95ug of nuclear extract was electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose membrane and analyzed with (A) anti-PARP antibody, (B) anti-p53 antibodies 240/1801, **Top** arrow (band **a**) and **lower** arrow (band **b**) (C) anti-p21^{WAF1}antibody, (D) anti-MDM2 antibodies, 2A9 (contains NLS signal) /3F3 (contains p53 binding site), (E) 2A10 (contains DYS motif and Zinc-finger), and (F) 4B2 (contains p53 binding site). (G) anti-actin was used to show equal loading

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Control non-drug treated replicating ML-1 cells did not induce p53 or p21 protein. There was reactivity for MDM2 with 2A10 (which interacts with unphosphorylated MDM2) and 4B2 (which contains the epitope of the MDM2 p53 binding site) suggesting an unphosphorylated form of MDM2 with an intact p53 binding site was present. No PARP cleavage was observed which is indicative of cells undergoing apoptosis. Only the intact PARP protein of 116 kDa was observed. Replicating ML-1 cells treated with 50 ug/ml of Zeocin induced nuclear p53 (bands **a**, full-length p53 & **b**, truncated form of p53, p50) and p21 protein levels. There was reactivity for MDM2 with 2A10 but there was decrease MDM2 reactivity to 4B2 suggesting that an unphosphorylated form of MDM2 with and without an intact p53 binding site was synthesized. There was no PARP cleavage observed. Replicating ML-1 cells treated with 400 ug/ml of Zeocin induced twice the nuclear p53 (bands **a** and **b**) and p21 protein levels as compared to cells treated with 50 ug/ml of Zeocin. There was MDM2 reactivity with 2A10, 4B2 and with 2A9 (contains NLS signal epitope for MDM2) suggesting an unphosphorylated form of MDM2 containing the NLS was present. We observed a faint cleavage product of PARP which may indicate the onset of apoptosis and/or the recruitment of DNA repair enzymes to the sites of damage. Replicating ML-1 cells treated with 0.5 uM Cpt induced nuclear p53 (band **b**) protein but not p21 protein levels. The decrease in p21 protein levels was not due to decreased p21 mRNA levels because we did observe p21 mRNA levels similar to ML-1 cells treated with 400 ug/ml of Zeocin (figure 4.3). There was no MDM2 reactivity to 2A10 (2A10 does not react with phosphorylated MDM2). However there

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was reactivity with 2A9 and faster migrating form reactive to 4B2 suggesting a phosphorylated form of MDM2 containing NLS with and without an intact p53 binding site was present. The form without a p53 binding site was possibly cleaved. The cleavage product of PARP (86 kDa) was 2-fold higher than observed in ML-1 cells treated with 400 ug/ml of Zeocin. Replicating ML-1 cells treated with 20 uM Cpt induced nuclear p53 (bands **b**) protein but not p21 protein levels. The decrease in p21 protein levels could be due to decreased p21 mRNA levels observed in figure 4.3. There was no MDM2 reactivity to 2A10 but there was MDM2 reactivity to 2A9 and a faster migrating form of MDM2 reactive to 4B2 suggesting a phosphorylated form of MDM2 containing NLS was present. We observed total cleavage of the intact PARP protein which may indicate cells actively within apoptosis. APH causes ML-1 cells to undergo apoptosis as indicated by PARP cleavage (Fig. 4.2A, lane 6). However there was no induction of p53 (or the $p21^{WAF1}$) protein, suggesting that p53 may aid in the apoptotic response but may not be necessary for it to occur. This was also shown in Chapter 3. This suggests that apoptosis can occur in both a p53 independent and dependent manner in ML-1 cells (Yonish-Ronach, *et al,* 1991).

Non-replicating/non-drug treated ML- cells pretreated with 10 ug/ml of aphidicolin for 4 hours mimics replicating /non-drug treated cells except for the induction of a faint p21 protein band and a decreased reactivity to 4B2. We observed the same levels of intact and cleaved PARP in all non-replicating samples (lanes 6-10). Non-replicating ML-1 cells treated with 50 ug/ml of Zeocin mimics replicating cells treated with the same dose of Zeocin except for PARP cleavage and a higher level of nuclear p21 protein. Non-replicating ML-1 cells treated with 400 ug/ml of Zeocin mimics replicating cells treated

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with the same dose of Zeocin except for the induction of a different form of p53 (band **b** only), decreased MDM2 reactivity to 2A10 and increased MDM2 reactivity to 4B2. This suggests that a form of phosphorylated MDM2 was present. Non-replicating ML-1 cells treated with 0.5 uM Cpt mimics replicating cells treated with the same dose of Cpt except for the induction of a different form of p53 (bands **a** & **b**), induction of p21 protein, increased MDM2 reactivity to 2A10 and increased MDM2 reactivity to 4B2. This also suggests a form of phosphorylated MDM2 was present. Non-replicating ML-1 cells treated with 20 uM Cpt mimics replicating cells treated with the same dose of Cpt.

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p53 accumulates but becomes functionally impaired in non-replicating and replicating cells in response to excessive DNA damage

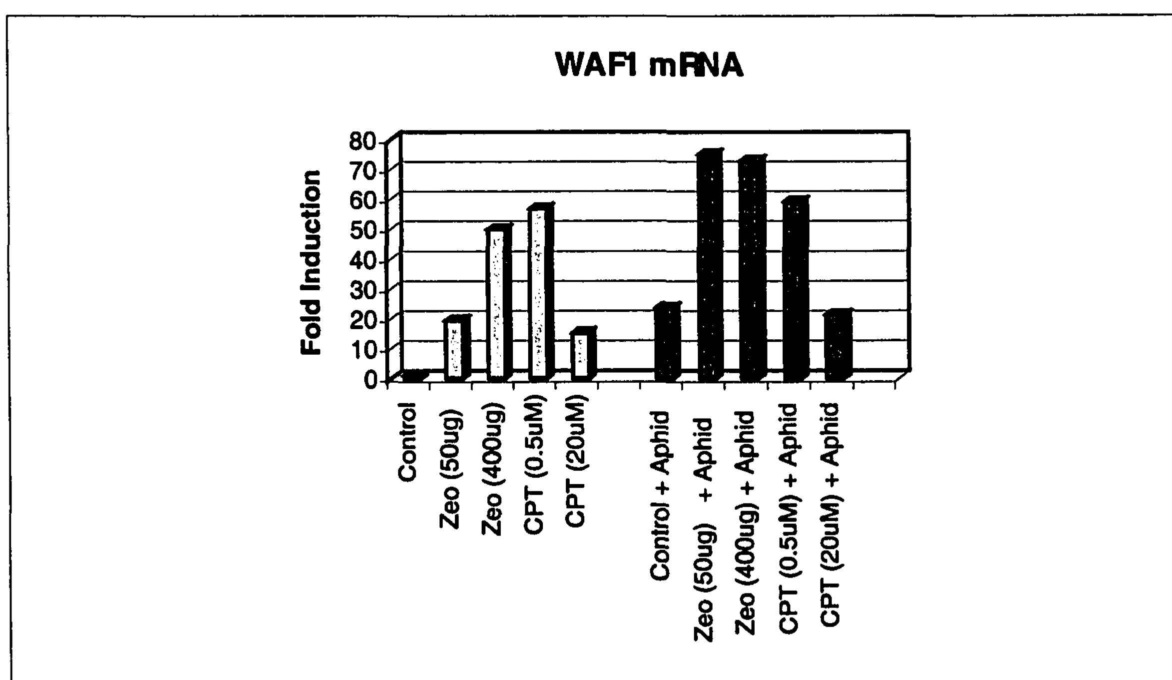

Figure 4.3: Increased expression of p21^{WAF1} mRNA levels in non-replicating ML-1 cells.
Cytoplasmic RNA was extracted from ML-1 cells treated for 3 h with either 50 ug/ml of Zeocin, 400 ug/ml of Zeocin 0.5 uM of CPT and 20 uM of CPT at 37°C with and without pretreatment with APH for 4 h. 0.5ug of total RNA was used for each RT-PCR in the presence of Waf1-specific molecular beacons. Values for Waf1 were normalized with GAPDH. Results are expressed as percent of control (untreated cells). Experiment performed once

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Quantitative RT-PCR with molecular beacons was used to investigate the transactivation of the $p21^{WAF1}$ gene in ML-1 cells after drug treatment (fig. 4.2C). This allowed us to analyze if the lack of $p21^{WAF1}$ protein seen in ML-1 cells after Cpt (20 uM) treatment was due to an inhibition of $p21^{WAF1}$ mRNA at the transcriptional level and if blockage of DNA replication causes inhibition of $p21^{WAF1}$ mRNA synthesis. $p21^{WAF1}$ mRNA was induced by treatment of replicating ML-1 cells with Zeocin (50 and 400 ug/ml) and a lower dose of Cpt (0.5 uM), however, treatment with a higher dose of Cpt (20 uM) did induce $p21^{WAF1}$ levels but not to levels comparable to the levels seen with 400 ug/ml of Zeocin and 0.5 uM Cpt. The $p21^{WAF1}$ levels were lower than that seen with 50 ug/ml of Zeocin (Fig. 4.3). The induction of $p21^{WAF1}$ mRNA seem to be inhibited at the 20 uM Cpt concentration. This may be because this high level of Cpt globally inhibits transcription due to crosslinks. In the case of the p53 induced by 0.5 uM Cpt, $p21^{WAF1}$ mRNA levels were increased but the protein was not observed (Fig 4.3 and 4.2C). At a higher dose of Cpt (20 uM), there seems to be an inhibition of $p21^{WAF1}$ mRNA levels as compared to Zeocin (50 and 400 ug/ml) and Cpt (0.5 uM) treated samples. A similar experiment was conducted with ML-1 cells following an initial treatment of ML-1 cells with a DNA synthesis blocking agent (APH) (Fig. 4.3). $p21^{WAF1}$ mRNA was markedly increased in Zeocin (50 and 400 ug/ml) treated cells. Cpt treatment did not show a difference at either 0.5 or 20 uM concentrations. The $p21^{WAF1}$ mRNA profile of induction in replicating cells mimics the $p21^{WAF1}$ mRNA profile seen in non-replicating cells. The pattern of increase for each drug dose in samples with and without APH was similar. We also observed that in non-replicating cells treated with Cpt (0.5 uM), both $p21^{WAF1}$ mRNA and protein levels were elevated, unlike the $p21^{WAF1}$ protein levels seen

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in replicating ML-1 cells treated with 0.5 uM Cpt. In replicating ML-1 cells treated with 0.5 uM Cpt, only an elevation in $p21^{WAF1}$ mRNA levels was seen (Fig. 4.2C and 4.3). The inhibition of the $p21^{WAF1}$ protein that is exhibited in replicating (without APH) ML-1 cells seems to be blocked in non-replicating cells (with APH) at this dose of Cpt (0.5 uM), allowing for the induction of the protein. We conclude that treatment of cells with a compound that blocks DNA synthesis does not alter the ability of p53 to induce nuclear $p21^{WAF1}$ mRNA but it does rescue the ability of $p21^{WAF1}$ protein to be either stabilized or translated in the presence low levels of damage caused Cpt (0.5 uM).

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p53 is sequestrated in the cytoplasm in replicating but not non-replicating ML-1 cells unlike PARP

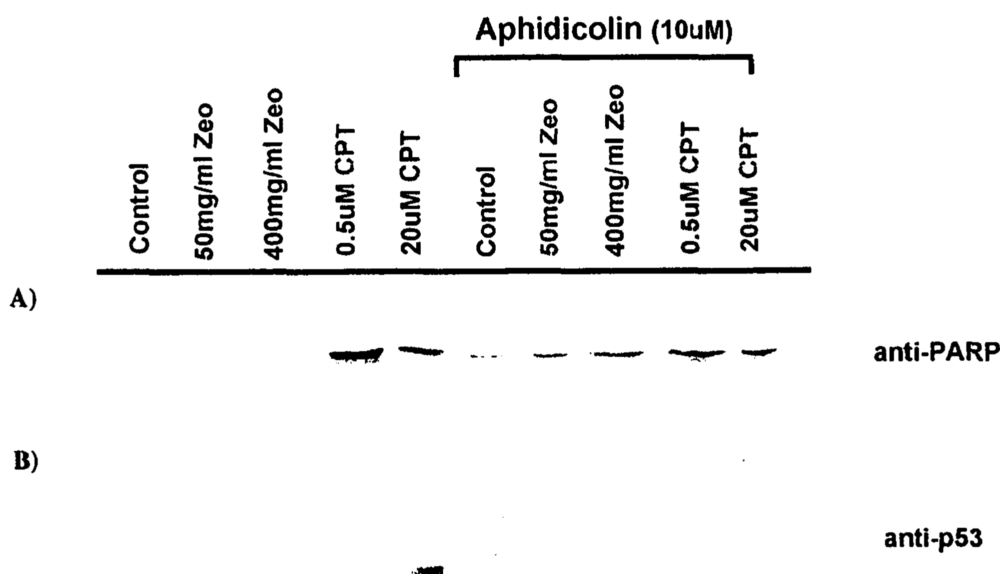

Figure 4.4: Increase in cytoplasmic p53 levels in replicating ML-1 cells following drug treatment. Cytoplasmic extracts were prepared from ML-1 cells grown in 1X RPMI with 10% FBS with and with drug treatments. Exponentially growing cells were treated with either 50 ug/ml of Zeocin, 400 ug/ml of Zeocin, 0.5 uM of CPT or 20 uM of CPT for 3 h at 37°C with and with pretreatment with 10 uM APH for 4 h. 95ug of cyotplasmic extract was electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose membrane and analyzed with (A) anti-PARP antibody, and (B) anti-p53 antibodies 240/1801.

When p53 is shuttled, exported or localized to the cytoplasm by MDM2, its transactivation and tumor suppressive activity are severely impaired or abolished

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altogether as a result of this cytoplasmic sequestration (Tao, W., *et al*, 1999). There was no detectable p53 observed in the cytoplasmic samples treated with Zeocin (50 and 400 ug/ml) and a lower dose of Cpt (0.5 uM) but, there was p53 presence when a higher dose of Cpt (20 uM) (severe damage) was used in replicating ML-1 cells that were not blocked for DNA synthesis (Fig.4.4A). At a higher dose of Cpt (20 uM), detectable p53 was observed in the cytoplasm. There was no $p21^{WAF1}$ protein detected in either Cpt-treated samples in replicating cells, suggesting that production of $p21^{WAF1}$ protein was somehow changed at the translational or post-translational level because transcript was made as shown in figure 4.3). At the lower dose of Cpt, there was no sequestration of p53 protein in the cytoplasmic samples. It has been shown that p53 might also regulate translation by directly affecting translation. Phosphorylation at phosphoserine 398 (in murine) and 392 (in humans) (Meek, D. W., and Eckhart, W., 1988, *et al*, 1990) have been covalently linked to 5.8S rRNA (Fontoura, B. M. A., *et al*, 1992) which has an essential role in translation (Walker, K., *et al*, 1990). A possible covalent linkage between p53 (phosphorylated at serine 392) and 5.8S rRNA may allow p53 access to the ribosomal machinery (A site) allowing it to affect translation of certain target proteins, perhaps inhibiting $p21^{WAF1}$. But, at a higher dose of Cpt (20 uM), p53 begins to be sequestrated to the cytoplasm via the MDM2 protein where its function becomes impaired as seen in figure 4.2 (this p53 form was not able to transcriptional transactivate the $p21^{WAF1}$ gene as efficiently as the p53 form induced at 0.5 uM Cpt treatment, as seen in fig. 4.3) disabling it from further transcribing the $p21^{WAF1}$ gene. We observed that phosphorylation of p53 at serine 392 occurs within ML-1 cells at this higher dose of Cpt treatment as shown in figure 3.4. Phosphorylation at serine 392 is able to stabilize p53 tetramer formation while

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serine 315 phosphorylation reverses this effect (Sakaguchi, K., *et al*, 1997). Because the nuclear localization signal (NLS) of p53 is next to the oligomerization domain and the nuclear export signal (NES) is contained within the oligomerization domain (Stommel, J. M., *et al*, 1999), this may suggest that the oligomerization of p53 may affect its nucleocytoplasmic transport. Phosphorylation at serine 392 could change the conformation of p53 to affect these regions ability to bind to their receptors. This change in conformation may mask the NLS while making the NES more accessible to its receptor, thus enhancing export of p53 (Stommel, J. M., *et al*, 1999). However, there were detectable levels of $p21^{WAF1}$ protein in the cytoplasmic samples from both Zeocin treated samples (data not shown), suggesting that during Zeocin damage the p53 enters the nucleus where it becomes fully active in transcribing target genes such as $p21^{WAF1}$.

In the case of non-replicating cells, there was no detectable p53 in the cytoplasmic extracts from both Zeocin (50 and 400 ug/ml) and Cpt (0.5 and 20 uM) treated ML-1 cells as was observed in studies done by Gottifredi and coworkers. They also found that p53 was localized exclusively to the nucleus when DNA replication was blocked (Gottifredi, V., *et al*, 2001). But, there was detectable $p21^{WAF1}$ protein in Zeocin (50 and 400 ug/ml) and at a lower dose of Cpt (0.5 uM) treatments (data not shown).

PARP (86 kDa) was in the cytoplasmic extracts treated with Cpt (0.5 uM and 20 uM) in replicating cells and in all samples from non-replicating cells whereas two forms of PARP (intact and cleaved form) were seen in nuclear extracts (Figs. 4.2A and 4.4A). PARP has been shown to act as a beacon for enzymes involved in DNA repair by recognizing and binding to sites of DNA lesions (Fernet, M., *et al.*, 2000). PARP may be cleaved as a result of its interaction with damaged DNA and this may not only act as a

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signal to recruit DNA repair enzymes to the site but PARP cleavage may be involved in the very first signals initiated by DNA damage that govern the degree of p53 induction.

Discussion

We found that when ML-1 cells were treated with the DNA replication inhibitor APH for a short period of time, p53 levels could be induced by Cpt and Zeocin and its transactivation of target molecules such as $p21^{WAF1}$ was not impaired. But, in the presence of excessive damage such as that induced by higher doses of Cpt, p53 was unable to transcriptionally activate the $p21^{WAF1}$ gene. There was a difference in the presence of protein products generated from genes such as mdm2 and $p21^{WAF1}$ as detected by Western blot analysis comparing replicating to non-replicating cells. p53 induction by Cpt and Zeocin was not inhibited by pretreatment with APH suggesting that the induction of p53 is not solely dependent on DNA replication. Our data suggests that ML-1 cells with stalled DNA replication synthesis for short period of time alone does not trigger p53 induction. Breaks or structures caused by Cpt or Zeocin treatment of a shorter treatment time than with APH can initiate the signal pathway of p53 if damage is not that extensive. The ability of Cpt and Zeocin to induce p53 strongly suggest that stalled DNA synthesis for a short period of time does not repress p53 or it tranactivation function. Also we observed an increase in cellular $p21^{WAF1}$ mRNA synthesis by both

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drugs but there was a reduction in the presence of extensive damage. Synthesis was actually up-regulated by both Zeocin treatments and Cpt treatment (at the lower dose). The $p21^{WAF1}$ mRNA profile in non-replicating cells mimics the $p21^{WAF1}$ mRNA profile in replicating cells.

Somehow the induction of the $p21^{WAF1}$ protein at the lower dose of Cpt (0.5 uM) in replicating cells is prevented from occurring perhaps due to the fact that Cpt at this lower dose does not inflict considerable damage to harm cells because the DNA synthesis has already been halted. Cpt, which is a topoisomerase inhibitor, works by nicking and unwinding DNA to eventually allow DNA replication to occur. So, if DNA synthesis is stalled, Cpt at this lower dose does not inflict much damage as evident as when 20 uM Cpt was added. Within stalled DNA ML-1 cells, 0.5 uM Cpt seems to mimic the damage caused within Zeocin treated samples. Its ability to bind and nick DNA is still occurring in these cells. This also adds support to the suggestion that when DNA is stalled, damage can still be detected and responded to. At a higher dose of Cpt (severe damage), the p53 seems to be incapable of functioning which is not due to sequestration of the protein. p53 protein was not observed in the cytoplasmic samples when DNA synthesis was stalled as observed in replicating cells. Perhaps MDM2 is able to enter the nucleus and bind to p53 to inhibit its transcriptional activity. But it is prevented from shuttling p53 to the cytoplasm by some factor (that requires replication) where it can be degraded. The $p21^{WAF1}$ mRNA level seen in 20 uM Cpt treatment could result from p53 that has not as yet been bound and inactivated by MDM2 in the nucleus.

Forms of the MDM2 protein were also observed when DNA synthesis was stalled. By using Western blots probed with antibodies specific for epitopes of the MDM2 protein as

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2A10 (contains the core motif DYS and zinc-finger region), 2A9 (contains nuclear localization signal region), 3F3 or 4B2 (both contain the p53 binding site) (Chen, J., *et al,* 1993), we observe 3 forms of MDM2 based on the sizes of the bands observed in the western blot reactive with the different MDM2 monoclonal antibodies. They were approximately 168.5, 90 and 85.9 kd. It is unclear at this time if these results are due to different post-translational modifications or splicing variants. The 2A10 epitope of the MDM2 protein contains the core motif DYS, this motif which is present at two locations within the human MDM2 molecule each are potential sites of phosphorylation within the carboxy-terminus (Maya, R., *et al.*, 2001). The 2A10 epitope contains the phosphorylation site serine 395 which is a major site on MDM2 that is phosphorylated by ATM (Maya, R., *et al.*, 2001). The 2A10 epitope also contains a zinc-finger structure which is involved in forming interactions with DNA (Coleman, J. E., 1992; Chen, J., et al, 1993; Rhodes, D., *et al.*, 1993). 2A10 is not reactive with the phosphorylated form of MDM2 (Maya, R., *et al.*, 2001). We observed in replicating ML-1 cells treated with Zeocin that full-length p53 remain stably high and an increase in truncated forms of p53. This induced truncated form of p53 was shown to be deleted of its C-terminus by reactivity to p53 antibodies DO-1 & 1801 only, and not 421 (chapter 5). In replicating cells, we only observed an unphosphorylated form of MDM2 (based on reactivity to 2A10) in Zeocin treated samples while a phosphorylated form was present in Cpt treated samples. In response to damage, the MDM2 2A10 form (unphosphorylated at serine 395) may allow full–length p53 levels or activity to remain high and trigger for or contribute to the increased levels of truncated forms of p53 (p 50 kDa or p40-43 kDa) in Zeocin-treated samples where p53 transactivational function is much needed in transcribing

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genes necessary to arrest cells and repair damage. However, in Cpt-treated cells, we observed that full-length p53 levels all but disappeared while there was an increase in the levels of truncated forms of p53. This induced truncated form of p53 was shown to be deleted of its extreme N-terminus by reactivity to p53 antibodies 1801 & 421 only and not DO-1 (chapter 5). In ML-1 cells treated with Cpt, the form of phosphorylated MDM2 (unreactive with 2A10) may allow for truncated forms of p53 and their activity to increase and a decrease in full-length p53 levels. This form of MDM2 also contains the zinc-finger motif that enables it to form interactions with DNA. A form of MDM2 reactive to 2A10 was also present in control samples but there is no damage so p53 levels do not accumulate within these cells. A study done by Yin and coworkers also observed after transient co-expression of wild-type p53 with mdm2 in negative human-lung carcinoma cell line H1299 a truncated form of p53 approximately 47 kDa and a full-length form of p53 by immmnumobloting (Yin, Y, et al, 2002). They're second observation was that the expression of the truncated form (p53/47) was induced by MDM2 while full-length p53 levels declined. We also observed in our data that p53 full-length protein decreased while other truncated phosphorylated forms became more prominent in association with the presence of either phosphorylated or unphosphorylated MDM2 after Cpt and/or Zeocin treatment.

In non-replicating cells, the unphosphorylated form of MDM2 (reactive with 2A10) was seen in both lower dose treatments of Cpt and Zeocin while the phosphorylated form of MDM2 (unreactive with 2A10) was seen at the higher dose treatments of both drugs. The unphosphorylated form of MDM2 correlated with the induction of both full-length

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and truncated forms of p53 while the phosphorylated form of MDM2 was associated only with the induction of truncated forms of p53 in replicating and non-replicating cells. In ML-1 cells where DNA replication is already halted, the degree and extent of damage caused by the lower doses of Cpt or Zeocin seem similar while the degree and extent of damage cause by the higher doses of Cpt and Zeocin were similar with Cpt causing the most damage. The p53 levels triggered in response to damage correlated with the increase in dUTPs incorporation or DNA repair synthesis. These data suggest that the unphosphorylated and phosphorylation of MDM2 may play a role in regulating the ratios of full-length (FL) p53 to truncated forms of p53. It also suggests that cells undergoing limited or minute amounts of damage increase p53 to arrest cells via induction of $p21^{WAF1}$ to allow for DNA repair. But when damage is extensive, there is a decrease in $p21^{WAF1}$ levels in cells undergoing apoptosis.

Even though a lot of studies have helped to delineate pathways initiated after UV and IR damage, the pathways initiated when DNA synthesis is stalled is still unclear. Goffredi and coworkers showed that phosphorylation of residues S15, S20 and S46 and acetylation of K382 occur both after gamma IR and when DNA synthesis is blocked for 24 hours. We believe that at 24 hours of treatment the cells are interpreting the accumulative effects of APH as damage and pathways similar to the ones induced by UV and IR are initiated. IR damage triggers the ATM kinase, Chk2 kinase, and other kinases that phosphorylate p53 at residues S15 or S20 while more extensive damage trigger ATR kinase, CKII kinase and other kinases that phosphorylate p53 at residues S46 or S392. Unlike our data where we see an induction of transcriptionally active p53 after both

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Zeocin and a lower dose of Cpt treatments, Goffredi observed a transcriptionally inactive p53 after gamma IR or when DNA synthesis was stalled. A study preformed by Gottifredi and coworkers reported that cells treated with the DNA polymerase inhibitor aphidicolin (APH) induced p53 stabilization within cells. They stated that the transactivation of p53 target genes such as $p21^{WAF1}$ and mdm2 is impaired even though post-translational modifications such phosphorylation as mentioned previously were observed and this impairment could not be reversed in the presence of DNA damage (gamma-irradiation). In our study, we performed a dose curve for APH to determine the earliest point at which APH block DNA synthesis in our cells. This resulted in a shorter treatment time with APH (4 hours as compared to the 24 hours used in the Gottifredi studies), reducing the time the cells had to be exposed to the drug. After 24 hours of treatment with APH, the cells may become aware or sense through some upstream signal pathway involving ATM, Chk2, ATR or DNA-PK that something is wrong (large accumulations of cells at the G1/S border and/or an extreme depletion of dNTPs at 24 hrs), thus leading to the induction of p53. The difference in data may be due to the cells interpreting the effects of APH at 24 hours, as not only damage but extensive damage (as indicated by the generation of a form of p53 phosphorylated at S46 known to be associated with apoptosis-related genes), thus impairing p53 transactivation function as we observed in our data when we treated cells with 20 uM Cpt.

It was reported by Maya and coworkers that ATM activation after gamma IR correlates with a reduction of reactivity to MDM2-specific 2A10. This loss of 2A10 reactivity after gamma IR was also confirmed by Goffredi and coworkers. They observed that HU-treatment does not affect its reactivity with 2A10 suggesting that this

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stress does not activate ATM. But, when HU-pretreated cells were treated with IR, this reactivity to 2A10 was reduced. We observed similar results when we treated ML-1 cells with Zeocin vs Cpt. When ML-1 cells were treated with APH alone, this did not affect its reactivity with 2A10 and slightly with additional treatment with a lower dose of Zeocin. However in pretreated samples with APH, there was a reduction in reactivity to 2A10 after additional treatment with a lower dose of Cpt and a loss of reactivity with higher doses of both Cpt and Zeocin.

In replicating cells, both doses of Zeocin did not affect (reduce) reactivity 2A10. But, when cells were treated with both doses of Cpt there was a loss of reactivity with 2A10 and a faster migrating form reactive to 4B2 was observed. In non-drug treated samples, there was no affect with reactivity with b 2A10. This suggest that treatment with Zeocin initiated the onset of ATM activation while in replicating cells, the ATM pathway was fully activated or there may be some alternative pathway to phosphorylate p53 at residue S15. We can also assume based on our data that ATM is activated when cells are treated with Cpt. Non-drug treated samples do not activate ATM as this is predicted because ATM is a damage-inducible pathway.

One explanation for the incorporation of BrdU in cells treated with higher doses of Zeocin and Cpt when DNA synthesis was blocked was that the cells may be undergoing DNA replication coupled DNA repair. We also observed a decrease in $p21^{Waf1}$ when cells took up dUTPs but no cycling of cells was indicated by PI/ FACS analysis. Cpt and Zeocin cause nicks or breaks in the DNA and create bulky structures on DNA that are

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recognized by p53 via its C-terminus. p53 then triggers the initiation of a cascade of events that will lend to the recovery of from DNA damage.

The 75 amino acids of human and murine p53 have been shown to be necessary for recognition of DNA damage. The C-terminal tetramers can bind to ends of DNA and has a preference for single-stranded regions of DNA as shown with strand-transfer experiments (Reed, M., *et al*, 1995). These interactions of p53 with DNA (damaged) together with the interactions of p53 with proteins involved in DNA repair such as p53R2, TFIID and RPA implicate p53 as a part of the DNA repair pathway. p53R2 is a ribonucleotide reductase gene that is necessary for the production of dNTPs that is essential for both DNA replication and repair. This gene is directly targeted by p53 and is involved in a p53-dependent cell-cycle checkpoint that senses DNA damage (Nakano, K., *et al*, 2000; Tanaka, H., 2000). p53 can interact with components of the transcription factor TFIID transcription complex that is involved in nuclear excision repair (Sancar, A., *et al*, 1994).

Not only can p53 be directly involved in the DNA repair process by several biochemical activities including DNA damage recognition by its non-sequence specific DNA-binding activity via its C-terminus but by its 3'-5' exonuclease activity (Cheng, Chung-Hui , and Kuchta, R. D., 1993). The intrinsic 3'-5' exonuclease activity of p53, localized in the core domain could be an important player in repair activities of p53. Exonucleases are required for DNA repair and often enhance the fidelity of this process. Mutant p53 is exonuclease-deficient and cells expressing mutant p53 are defective in NER, this suggest a possible role of p53 exonuclease activity in DNA repair. An important type of error avoidance mechanism that requires exonuclease activities is the

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mismatch repair pathway and nuclear excision repair. Since p53 recognize DNA bulges caused by insertion/deletions mismatches (Lee, S., *et al.*, 1995), it might be particularly suited to excise them via its 3'-5' exonuclease for p53 may act as a proofreader for errors produced by cellular DNA stress.

p53 also has the ability to functionally synergize with c-JUN after its activated in response to UVR to regulate the transcription of mismatch repair gene hMSH2 (Scherer, S. J., *et al*, 2000). So we postulate that damaged DNA (type vary) is recognized by p53, it becomes activated and stabilized. Then p53 initiates the other cascade of events involving p53R2, TFIID, hMSH2 and RPA. p53 exonuclease activity is also activated (or a form of p53 with 3'-5' exonuclease activity gets enhanced) and excises the damage DNA, aiding in the DNA repair synthesis performed by DNA polymerase-beta (Wood, R. D., *et al.*, 1997; Zhou, J., *et al*, 2001). DNA polymerase-beta fills the gap using dNTPS induced by p53R2 (APH does not affect DNA polymerase-beta but it does affect all the other polymerases including viral DNA replication by inhibiting viral induced polymerases) (Cheng, Chung-Hui, and Kuchta, R. D., *et al*, 1993) and during this process some of the BrdU gets incorporated and this could account for the uptake of BrdU in cells when DNA synthesis is blocked and cells show no signs of cycling.

These data lends support to the idea that p53 guards the cell against damage that attacks the cell's many mechanisms of actions and when one of these mechanisms are affected, p53 is called into action to correct the damage (some mechanisms have priority over ours). We observed when cells were replicating, limited amounts of DNA damage (nicks & breaks) as the case with Zeocin treatments (repairable) induced a form of p53 that initiated DNA repair mechanisms while any damage (Cpt/Topo/DNA cleavable complex

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= bulky adduct) that interfered with the replication process as in the case of Cpt treatments induced a form of p53 that sensitizes cells for or aids in the apoptotic pathway. However in ML-1 stalled for DNA synthesis already, the amount of damage induced by higher doses of Cpt and Zeocin seem to induce the form of p53 that was induced in replicating cells after Cpt treatments. This suggests that the cell can still sense not only damage but the degree of damage when DNA synthesis was stalled.

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Chapter 5

DNA Binding activity of p53 in ML-1 cells: different forms of p53 exist that bind to different p53 REs

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Introduction

p53 is a transcription factor that binds in a sequence–specific manner to target genes that contain the p53 consensus sequence 5'-Pu Pu Pu C(A/T) (T/A)G Py Py Py-3'. A number of target genes (approximately 20) have been identified that contain p53 REs. p53 binds in its tetrameric form to two adjacent repeats of the above sequence. The p53 protein through the transcriptional regulation of a number of genes has been implicated in cellular responses such as grow arrest and in certain cell types apoptosis (Levine, A. J 1997, Lane, D. P., 1992). How p53 decides which gene to target and transcribe under different physiological conditions still needs to be understood. Some studies point to the phosphorylation at S46 within the proline rich region as a potential regulator of apoptosis (Oda, K et al., 2000). However others suggest that not only phosphorylation but cleavage (fragmentation through degradation) or a combination of both may generate different p53 products that preferentially target growth arrest or apoptotic-related genes.

In order to investigate the mechanism of p53's selectivity of target genes, we looked to see if and how p53 was modified (if different forms or products of p53 exist) in the present of two drugs Zeocin and Cpt that induce different types (forms) of damage via different mechanisms of action which may favor different cellular outcomes such as G1 arrest or apoptosis, depending on drug dose. To detect products of p53, we compared Western blots probed with antibodies directed against different epitopes located in the N- or C-terminus and/or phosphorylated forms of p53. By using EMSAs we examined if these different fragments or forms of p53 had preferential binding to particular target genes (oligonucleotides) and were specific to different phases of the cell cycle. We

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observed that Cpt and Zeocin induce different phosphorylated forms or products of p53 that preferentially target different genes such as p21^{WAF1} and gadd45, suggesting that their signaling pathways differ. We also observed that two different p53 DNA binding species exist that preferentially bind to specific p53 target oligonucleotides during the cell cycle. These two different binding species may represent two different products of p53, full-length (latent form) p53 and truncated or an alternatively spliced form of p53 (p50, C-terminally spliced p53, active form).

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Results

Zeocin and CPT induce different phosphorylated forms of p53

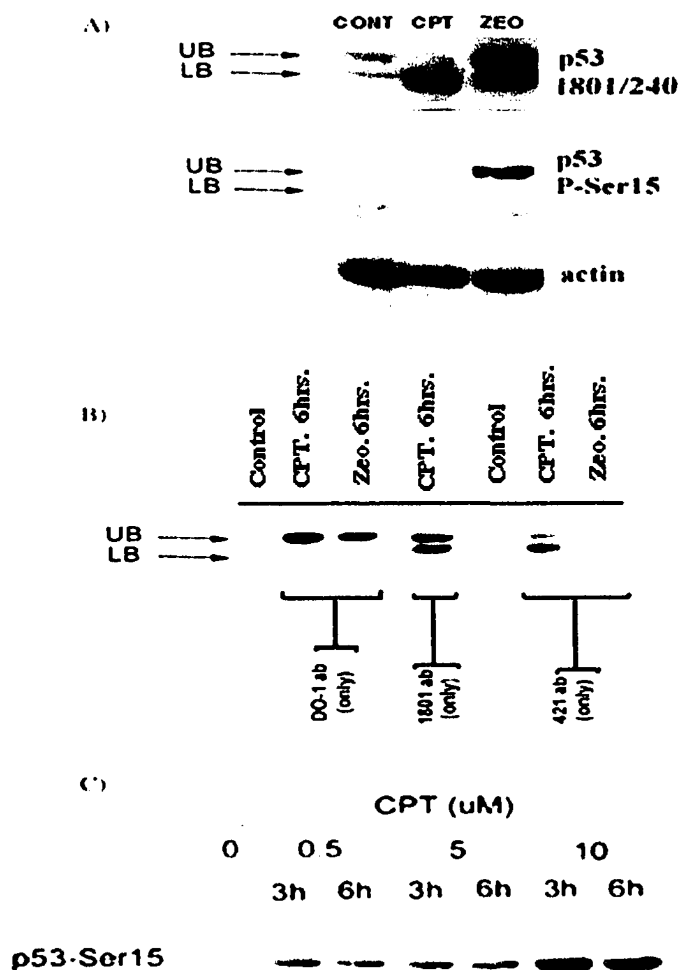

Figure 5.1: Zeocin and Cpt treatments generate different p53 fragments recognized by different anti-p53 monoclonal antibodies. (A) ML-1 cells were treated with 20 uM camptothecin (CPT) or Zeocin (ZEO) and nuclear extracts were prepared after 6 h treatment. 95ug of nuclear extract was used for western blotting with a rabbit polyclonal antibody specific to serine-15 phosphorylated p53 (upper panel) and then with a mixture of monoclonal PAb 1801/240 antibodies (middle panel). UB stands for upper band and LB

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stands for lower band. The blot was also blotted with an anti-actin antibody to check equal loading in each lane (lower panel). Experiment was repeated several times. (B) Nuclear extracts from ML-1 cells containing protease inhibitor Leupeptin were treated with 50 ug/ml of Zeocin or 20 uM of CPT for 6 h. 95ug of nuclear extract was electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose membrane and analyzed with a) anti-p53 antibody PAb 1801 only (specific to the N-terminus, aa 46-55), b) anti-p53 antibody PAb DO-1 only (specific to the extreme N-terminus, aa 18-30), and c) anti-p53 antibody PAb 421 only (specific to the extreme C-terminus, aa 371-380). Experiment performed twice. (C) Time and dose curve for CPT treatment. Protein extracts containing protease inhibitor Leupeptin from ML-1 cells treated with 0.5 uM, 5uM and 10 uM CPT for 3 and 6h. 95ug of nuclear extract was electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose membrane and analyzed for phosphorylated p53 at serine 15. Experiment *performed* twice.

Induced N-terminal, C-terminal, and core cleavage products of p53 have been described *in vitro* (Okorokov, A. L., et al, 1997) and observed *in vivo* (Molinari, M., et al, 1996). As mentioned previously, incubation of p53 with dsDNA generates a cleavage product of 50kd, p50 (dN23) via specific cleavage of the protein between residues 23 and 24 lying within the MDM2 binding site of p53. Okorokov and coworkers propose that this product may accompany or favor conditions that lead towards either growth arrest or apoptosis. Cleavage products that lack the C-terminus, p50 (dC) and p40 (dC) have also been described. It has been shown that with the generation of a cleavage product similar to p50 (dC) from endogenous p53 *in vivo* there is an up-regulation of $p21^{WAF1}$ expression in ML-1 cells exposed to either adriamycin or cisplatin (Okorokov, M., et al, 1997) as seen in our data also comparing Zeocin and camptothecin treated ML-1 cells (Fig. 3.4B and C and comparing Figs. 5.1 B and 5.2). Deletion of the C-terminal 30 aa of human p53 is associated with activating p53 for DNA binding but it does not affect the ability of p53 to oligomerize. This deletion has been seen in many systems to impair the ability of p53 to efficiently induce a p53-dependent apoptosis while the G1-arrest response is not affected (Chen, X., *et al,* 1996; Wang, X., *et al,* 1996).

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We wanted to examine the different products induced by Zeocin and Cpt. Therefore, we investigated the ability of Zeocin and Cpt to induce N-and C-terminal products in ML-1 cells by comparing Western blots probed with antibodies directed against different epitopes located in the N- and C-terminals of p53 such as D0-1 (aa 18-33), 1801 (aa 46-55), and 421 (aa 371-380) (Fig. 5.1 A).

A lower band (LB) p50 was observed in cells treated with Cpt for 6 hrs. A lower (p50) and upper band (p53) were observed in samples treated with Zeocin for 6 hrs. with the upper band (UB) being more prominent (Fig. 5.1A, upper panel). When the UB was detected, phosphorylation at serine 15 was observed (Fig. 5.1A, middle panel). After observing that Cpt had only generated a LB of 50 kb while Zeocin-induced damage generated a LB (50 kDa) and a UB (53 kDa), we then examined if the UB produced in the presence of Zeocin was absent in Cpt due to degradation or masking by interaction with some protein.

First, we looked to see if an UB could be detected in Cpt treated samples in the presence of a protease inhibitor Leupeptin. We found that we could detect an UB in ML-1 lysates in the presence of a protease inhibitor and that this UB was able to react with the DO-1 antibody (aa 18-30), like the Zeocin induced UB. We observed that both bands, a prominent UB and a LB induced by Cpt, were reactive with PAb1801 antibody (aa 46-55) as seen with both Zeocin-induced bands (Fig. 5.1 B, my extracts were run on a gel and probed by Magali Oliver). However, only Cpt induced a form of p53 (LB) that was reactive with the PAb421 antibody (aa 371-380). The UB and LB observed in Zeocin-treated samples (Fig. 5.1A, upper panel) were not reactive to PAb421 (Fig.5.1 B) and only to the PAb1801 antibody (aa 46-55). These data also suggests that with the addition

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of a protease inhibitor, the turnover rate of the UB was slowed due inhibition of the protease degrading p53. The UB that was retained in Cpt treated samples in the presence of a protease inhibitor retained its ability to be phosphorylated at serine 15 (Fig.5.1C; Cpt dose curve done by Magali Oliver).

We conclude that Cpt can induce a form of p53 that does not react to PAb DO-1 antibody (specific to the extreme N-terminus, at least the first 30 aa) and is phosphorylated at serine 392 while Zeocin can induce a form of p53 that does not react to PAb 421 antibody (specific to the extreme C-terminus, at least the last 22 aa) and is phosphorylated at serine 15. We can also conclude that this UB may have been degraded because in the presence of a protease inhibitor, it can be detected by a phospho-specific antibody at serine 15.

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Zeocin and Cpt induce forms of p53 that have preference for transactivating different target genes

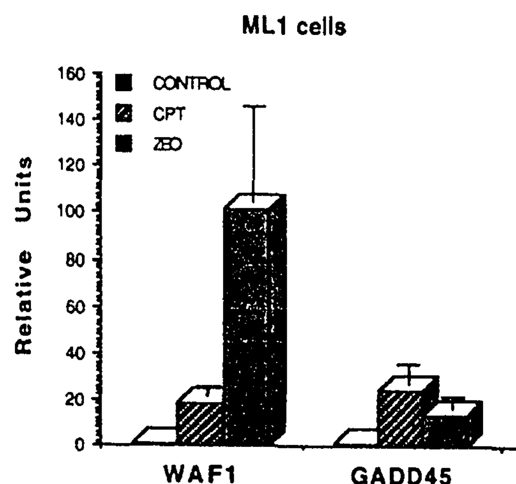

Figure 5.2: Zeocin-induced p53 induces p21^{WAF1} mRNA levels in ML-1 cells. Cytoplasmic RNA was extracted from ML-1 cells treated for 3 h with either 50 ug/ml of Zeocin or 20 uM CPT at 37°C. 0.5ug of total RNA were used for each RT-PCR reaction in the presence of Waf1, and Gadd45-specific molecular beacons. Values for Waf1 and Gadd45 were normalized with GADPH. Results were expressed as percent of control (untreated cells). Experiment *performed* twice.

We next examined the functional significance of these various p53 products for their ability to (1) transactivate downstream genes and (2) show a preference for transactivating one particular target as opposed to another. Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed to analyze the p21^{WAF1}and gadd45 mRNA levels. The mRNA levels of these genes were detected by RT-PCR in the presence of molecular beacons specific to primers p21^{WAF1} and gadd45.

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Zeocin induced a p21^{WAF1} mRNA level that was 4-fold higher than that seen in Cpt-treated samples (Fig. 5.2). However, Cpt induced a higher gadd45 mRNA level compared to Zeocin treated cells. p21^{WAF1} mRNA levels were lower than gadd45 mRNA levels in Cpt treated cells and the reverse was seen in Zeocin-treated samples. This suggests that the p53 products induced by Cpt or Zeocin were selective when transactivating downstream target genes. We suggest that when Zeocin-induced damaged DNA signaled to p53, it generated products of p53 (a UB, p53, reactive with PAb1801, and DO-1; a LB, p50, reactive with PAb1801 not DO-1 or PAb421) that selectively transactivates the p21^{WAF1} genes and not the gadd45 genes. When Cpt-induced damage signaled to p53, it generated products of p53 (no detectable UB; a LB, p50, reactive with PAb1801 and PAb421) that selectively transactivated gadd45 over p21^{WAF1} genes and overall had little ability to transactivate either one. This could be due to cleavage at the N-terminus of both p53 fragments. Another band at approximately 40-42 kb was also observed in response to Cpt-induced damage that was phosphorylated at serine 392 (Fig. 3.4C) and reactive with PAb240 antibody (specific to the central or core domain of p53, aa 212-217) (Fig. 3.1A) this reactivity was not detected in Zeocin-treated samples.

p53 DNA binding species exists that bind to different p53REs

The human p21^{Waf1} gene contains two p53REs in the promoter located -1.3kb and -2.2kb upstream from the first exon with no nucleotides in between (El-Deiry, W. S., et al., 1995). It has been shown that the p53 binding characteristics to these sites are

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different (Renick-Silverman, L., et al, 1998). The gadd45 gene contains one p53RE in its third intron (Hollander, M.C., et al., 1993) and the mdm2 gene has two tandem p53 REs in its first intron, 10 bp of the TATA box (Zauberman, A., et al., 1995). The bax gene contains three imperfect p53 REs and one perfect RE (Miyashita, T., et al., 1995; Reed, J. C., et al., 1997). They are located 70 bp 5' of the TATA box. Each of the genes contains base pair sequences with high homology to the p53 consensus sequence, varying in only a few base pairs. Bax has two p53-binding sites containing eight mismatches per 35 base pairs (Miyashita, T., et al., 1995). Mdm2 has two p53-binding sites containing five mismatches per 40 base pairs (Zauberman, A., et al., 1995). Waf 1 has one p53-binding sites containing two mismatches per 20 base pairs (El-Deiry, W. S., et al., 1995). Gadd45 has one p53-binding sites containing one mismatches per 20 base pairs (Hollander, M.C., et al., 1993). Its p53RE has the highest homology to the p53 consensus sequence. p53 binds to these different p53 REs containing different versions of the p53 consensus binding sequence. p53 post-translational modifications seem to selectively affect its binding properties for the genes gadd45, mdm2, $p21^{WAF1}$, bax, and RGC that all have a specific p53 binding site sequence at different locations from the transcription start site.

A gel shift assay with competive DNA was carried out to examine the specificity of p53 binding to various p53-RE oligonucleotides from non-elutriated and elutriated samples treated with 0.5 uM Cpt for 3 hrs (Figs. 5.3 and 5.4). EMSAs were preformed with oligonucleotide sequences cooresponding to the selected p53 binding elements. The binding assays were performed in the presence of monoclonal PAb 421 (recognizes a C-terminal epitope) activates p53 DNA binding (Hupp and Lane *et al,* 1995; Shaw, *et al.,* 1996). Nuclear extracts were omitted in the sample designated " no protein" and used as a

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negative control to see if anything else super shifted with the PAb 421 antibody besides p53. We observed in competition experiments using SCS, gadd45, $p21^{WAF1}$ (p53-RE upstream of promoter), $p21^{WAF2}$ ($p21^{WAF1}$ with the p53-RE downstream of promoter), mdm2 bax, mutant $p21^{WAF1}$ and mutant RGC that the p53 DNA binding species (band 1), which was supershifted with PAb 421 antibody, was competed with the super consensus sequence (SCS) and $p21^{WAF1}$ throughout the cell cycle (Fig. 5.4). Band 2, which was not supershifted with PAb 421 antibody, was present in only non-elutriated (Fig. 5.3 and 5.4) and G1 and seemingly G2/M cell populations (Fig. 5.4). It was not competed with $p21^{WAF1}$ but with SCS, $p21^{WAF2}$, mdm2 and somewhat with gadd45. Mutant p53 binding site oligonucleotides, mutant $p21^{WAF1}$ or mutant RGC, were unable to compete away ban1 or 2. This shows that both EMSA bands are probably the result of p53 binding. This suggests that there are two p53-dependent gel shift species induced and that the first p53 dependent species (band1) appears throughout the cell cycle after drug treatment while the second p53-dependent species (band 2) is specific to G1 and perhaps G2/M phases and appears in control and nondrug-treated samples. It also suggests that the two bands may have selective functions because of their specificity to different oligonucleotides derived from p53-targeted genes and to be activated by different mechanisms the abilityto be supershifted with PAb 421, which mimics C-terminal activation by phosphorylation and/or acetylation. These two bands were also observed in fig. 5.3.

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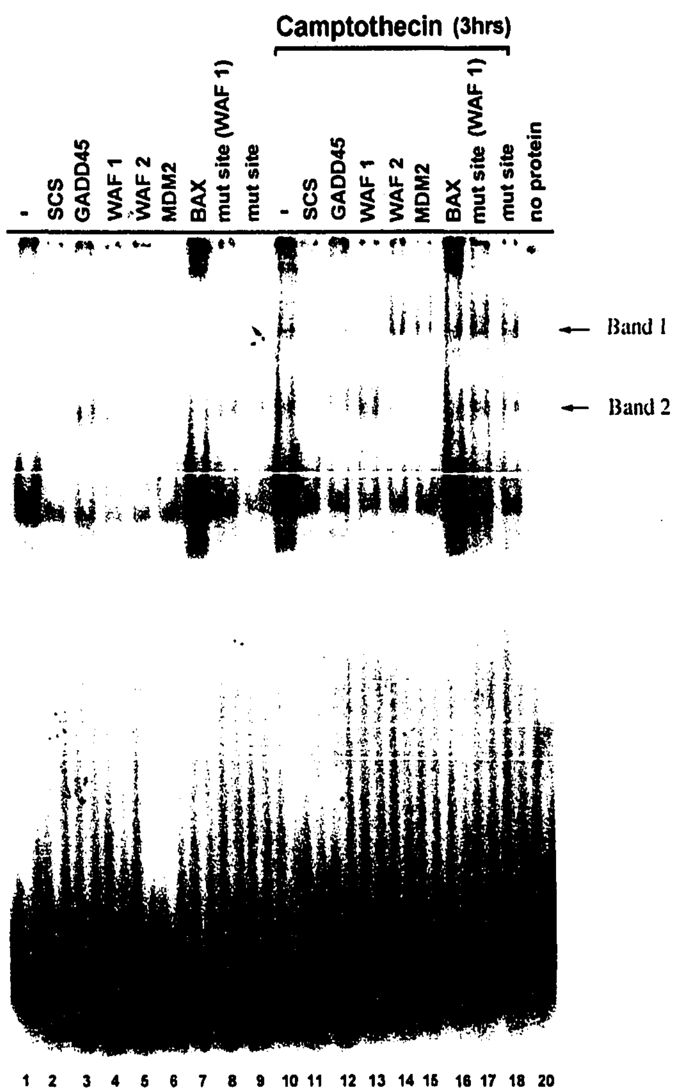

Figure 5.3 p53 DNA binding species exists that bind to different p53REs. Competition of p53 induced by CPT with oligonucleotides containing p53REs. Nuclear extracts from non-elutriated ML-1 cells isolated after 3 h treatment with 0.5 uM CPT were incubated with [P32]-labeled DNA corresponding to the p53 super consensus site and then electrophoresed on a 4% gel. Competition was carried out with 100 fold SCS, Gadd45, Waf1, Waf2, MDM2, Bax, mutant Waf and mutant RGC oligonucleotides. Mutant =defective p53 binding site. Reactions contain PAb 421 antibody. Experiment *performed* twice.

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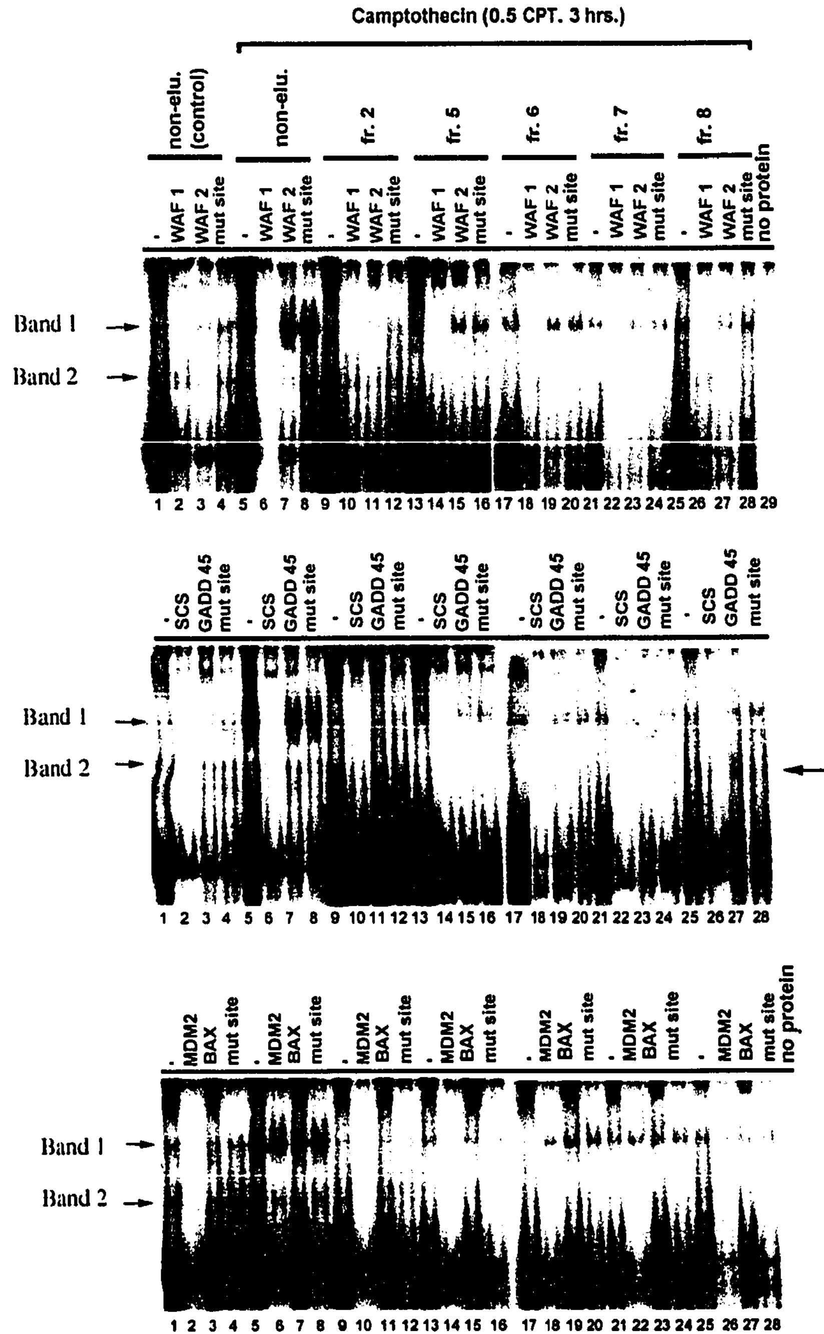

Figure 5.4: p53 DNA binding species exist that correlate within different phases of cell cycle. Competition of p53 induced by CPT with oligonucleotides containing p53-REs. Nuclear extracts from non-elutriated and elutriated ML-1 cells isolated after 3 h treatment with 0,5uM CPT were incubated with [P32]-labeled DNA corresponding to the p53 super consensus site and then electrophoresed on a 4% gel. Competition was carried out with 100 fold SCS, Gadd45, Waf1, Waf2, MDM2, Bax, mutant Waf and mutant RGC oligonucleotides. Mutant =defective p53 binding site. Reactions contain PAb 421 antibody. Experiment *performed* twice.

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Discussion

The N-terminal domain of p53 is essential for the transactivation of its target genes and it is this region of p53 that interacts with cellular transcriptional machinery. The central domain is also important for transcriptional activation, because in order for p53 to function as a transcription factor it must sequence-specifically bind to DNA through this region. The core domain has to undergo precise folding to create the right orientation of residues that interact with specific DNA targets sequences (Cho, *et al,* 1994) and studies suggests that this is governed by C-terminal regulation (Hupp et al., 1992). The C-terminus recognizes and binds to sites of DNA damage non-sequence-specifically that are necessary for activation of the p53 response (Lee, *et al,* 1995). Once p53 is activated through different post-translational modifications at its N- and/or C-terminus like phosphorylation and/or acetylation, it is able to control multiple cellular pathways to induce cellular responses such as growth arrest. p53 mediates these responses through binding to and transcribing p53 target genes which contain different versions of the p53 consensus binding sequence that are located at different sites from the transcription start site (Rensnick-Silverman, L. *et al,* 1998; El-Deiry, W. S., *et al,* 1992; Hollander, M. C., *et al,* 1993; Macleod, K. F., *et al*, 1995; Zauberman, A., et al, 1995; Hermenking, H., *et al*, 1997). However, it is unclear how p53, once activated, selects which p53 target gene to transcribe and thus which cellular pathways (growth arrest and/or apoptosis) to undergo. Some studies preformed so far have suggested that the proline-rich region (residues 64-92) and a region in the N-terminal transactivation domain (residues 43-63) may govern p53 decision to undergo growth arrest or apoptosis by altering its binding

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affinities for certain growth arrest or apoptotic-related genes (Oda, K., et al, 2001). They observed that removal of either region resulted in the disruption of the apoptotic response.

We not only found that Zeocin and Cpt (each causes a different type of DNA damage) induced different phosphorylated forms of p53 but each induced a different product of the p53 protein and this affected p53 ability to be phosphorylated at serine 15. We believe that a portion of the protein is removed before the phosphorylation process of N-terminus occurs because with the addition of leupeptin, a p53 product was retained that could be phosphorylated at serine 15 by its respective kinase. The products may be cleaved with subsequent degradation of that cleaved portion and not masked through protein-protein interaction due to the following observations: 1) non-reactivity and reactivity to anti-p53 antibodies specific to different regions of p53 and 2) the ability of this p53 product to be retained in the presence of leupeptin. These phosphorylated products of p53 generated by Cpt and Zeocin bound differently to the $p21^{WAF1}$ and gadd45 target genes. Based on our results, we suggest that selective functions of p53 may be activated by phosphorylation via stress-activated kinases and proteolytic cleavage of the protein (which comes first is unclear) either within the N- or C-terminus of the protein. These modifications are governed by the type of DNA damage (ssDNA and/or dsDNS) that p53 encounters (Molinari, M., *et al,* 1996 and Okorokov, A. L., *et al,* 1997; Steegenga, W. T., *et al.,* 1996). Once p53 is cleaved by the initial interaction with sites of damage, fragmented products of p53 (core domain) may also be involved in auto proteolysis of full-length p53 (Molinari, M., et al, 1996). Our results and others cited

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(Molinari, M., *et al,* 1996 and Okorokov, A. L., *et al,* 1997; Steegenga, W. T., *et al.,* 1996) suggest that along with phosphorylation of p53 at specific sites, interaction of p53 with damaged DNA may trigger a proteolytic cleavage process that is involved in the activation of p53 protein. These processes may create post-translationally modified products of p53 with altered conformations that have preferential binding to specific p53REs. These specific phosphorylated products may carry out or contribute to different cellular responses within the cell. Studies done by Kulesz-Martin and coworkers suggest the existence of full-length (latent) and truncated (active) protein products of p53. They observed the presence of full-length and C-terminal alternatively spliced p53 (p53as) mRNA. The product generated from the p53as mRNA was suspected to be 9 amino acids shorter (approximately 43 aa) than p53FL and has 17 different aa at the C-terminus (Kulesz-Martin, M., et al., 1994; Wu, Y., et al., 1997).

We also observed the existence of two p53 DNA binding species that bind to different p53 target oligonucleotides during different cell cycle phases. Kulesz-Martin found the products of p53FL and C-terminally spliced p53 mRNA gave rise to protein products that both bound efficiently to DNA and that the p53as had a higher DNA binding ability possibly due to C-terminal activation. They also observe that p53FL was preferentially expressed in G1 while p53as was expressed during G2 and in cells greater than G2 DNA content (Han, K. A., et al, 1992 a & b;Wu, Y., et al, 1994; Liu, Y., et al, 2001). By using ectopically expressed p53FL and p53as proteins in p53-deficient mice, p53as was shown to have a concentration dependent repression of the p21 (WAF1/Cip1/Sip1) promoter (Huang, H., et al, 2002) as we observed in the case with Cpt-induced p53.

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As mentioned previously, there are two p53REs in the human p21 promoter located -1.3kb and -2.2kb upstream from the first exon with no nucleotides in between (El-Deiry, W. S., et al., 1995). These two bands, band 1 (competed away with SCS and $p21^{WAF1}$) and band 2 (competed away with SCS, $p21^{WAF2}$ and mdm2) could be different subclasses of p53 that exist within the same cell. One form of p53 could require C-terminal activation and the other does not (constitutive). These two forms could have the potential to bind simultaneously or separately to the different $p21^{WAF1}$ promoter sites to transcribe different p21 transcripts leading to growth arrest and to the shut-off of PCNA-dependent replication in non-stressed and/or stressed cells. In non-stressed cells a form of p53 (band 2) was present that had binding preference to the $p21^{WAF2}$ p53-RE downstream of the p21 promoter. While in stressed cells, in addition to band 2, a form of p53 (perhaps a form similar to p53as containing the C-terminus) was induced (band 1) that had binding preference for $p21^{WAF1}$ p53-RE upstream of the p21 promoter that was able to interact with PAb421 antibody. This suggests that the binding to $p21^{WAF1}$RE requires C-terminal activation. Band 2 could be a form of p53FL (latent) that functions in cells not undergoing damage that is just involved in every day housekeeping within cells such as induction of cyclin D1 synthesis and/or binding to mdm2 to simulate cell proliferation, among other cellular processes.

We conclude that different forms of p53, full-length or truncated form either lacking the N or C-terminus were generated, after exposure to the different drugs, that have preferential binding to different downstream p53 targets that govern if a cell undergoes apoptosis, growth arrest, differentiation or proliferation. Based on our data and others cited, we proposed the following model for p53 activation.

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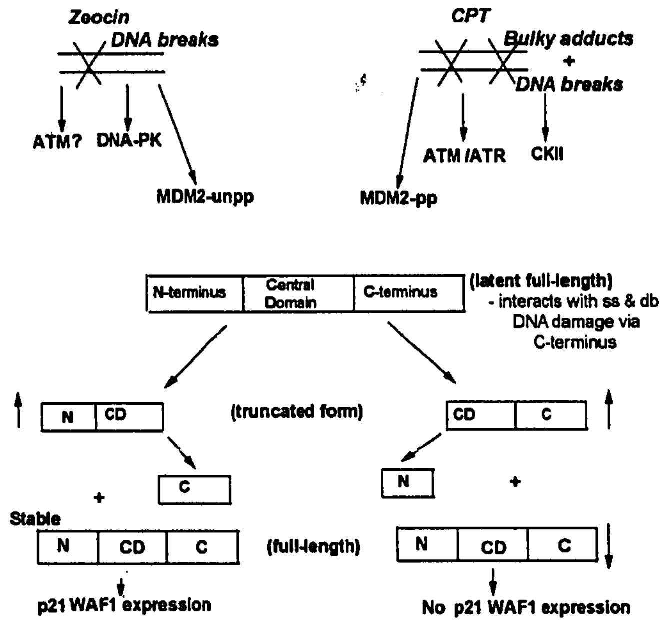

Figure 5.5: Model of p53 Activation

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Chapter 6

Appendix

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APPENDIX :

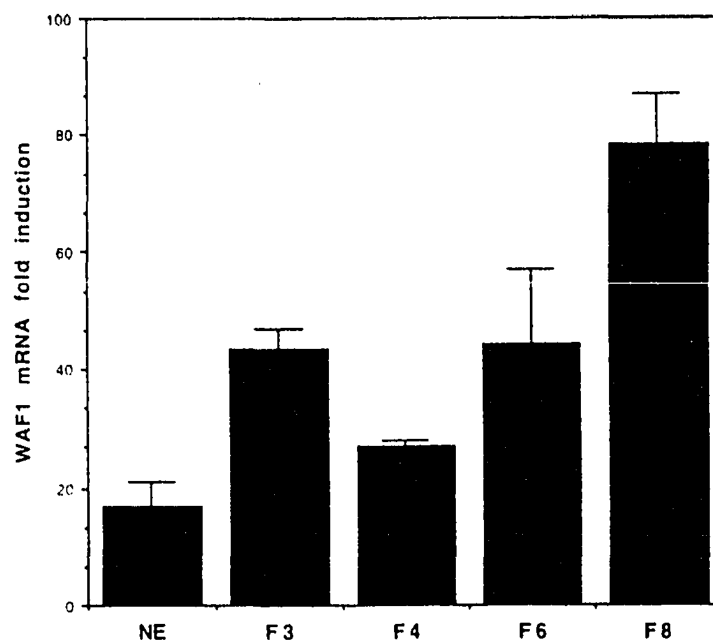

Figure A1: p21^{WAF1} mRNA levels from non-elutriated and elutriated ML-1 cells treated with 400 ug/ml of Zeocin. Elutriated and non-elutriated (NE) ML-1 cells were treated for 6 hrs. with 400 ug/ml of Zeocin (ZEO). Fractions **3** (mostly G1 population), **4** (cells going from G1 into S-phase), **6** (mostly S population), and **8** (mostly G2/M population) were used to represent cell populations from each stage of the cell cycle. From Ct obtained by Real-time RT-PCR, mRNA fold induction was calculated as follows: $2.23^{(Ct\ treated - Ct\ control)}$ gene/ $2.23)^{(Ct\ treated - Ct\ control)}$ gapdh..

Current studies in our lab are investigating how different types of DNA damage (induced by variable means) regulate the transcriptional activity of p53 over the course of the cell cycle and how the many different p53 target genes respond to this differentially activated p53. It has been reported that p21^{WAF1} interacts with cyclin A and B complexes during the later stage in the cell cycle. This association may influence (inhibit) cyclin B and A and their respective kinases to cause G2/M-arrest. p21 ^{Waf1} mRNA levels have also been observed in humans fibroblast in later stages of the cell cycle (Li, Y., et al, 1994;

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Poon, R. Y. C., et al., 1996; Adams, P. D., et al, 1996; Dulic, V. et al, 1998) This was also seen in elutriated Ml-1 cells treated with 400ug/ml of Zeocin (figure A1). We observed that the highest $p21^{Waf1}$ mRNA levels were in later cell cycle stages.

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Chapter 7

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