ESTABLISHMENT OF AN EXPERIMENTAL SYSTEM TO STUDY p53 EFFECTS IN *Saccharomyces cerevisiae*

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

BY
TUBA DİNÇER
AUGUST 1997

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I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Prof. Mehmet Öztürk

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ABSTRACT

Establishment of an experimental system to study

p53 effects in *Saccharomyces cerevisiae*

Tuba Dinçer

M. S. in Molecular Biology and Genetics
Supervisor: Prof. Dr. Mehmet Öztürk
August 1997, 82 pages

The aim of this work was to establish an experimental model to study human wild type and mutant p53 protein effects in yeast *Saccharomyces cerevisiae* cells.

Wild type p53 was previously shown to be a DNA damage response gene that controls the genome stability in mammalian cells. We are interested in using yeast cells to study human p53-mediated cellular events after DNA damage. In this study we established an experimental model as an initial step. For construction of this system pAK31 plasmid expressing a human mutant p53-248W as well as control plasmid pL3 were used to obtain two different yeast cell populations, one expressing the mutant p53 248W protein and the other without p53 expression. Wild type p53 expression vector was avoided because of its known growth inhibitory effects in yeast cells.

In this experimental system initially p53 expression at pAK31 transformed yeast cells was shown. Then, the effect of mutant p53-248W expression to growth rate of yeast cells was analysed and no growth rate difference was detected between the cells expressing and non-expressing mutant p53-248W protein. To test the participation of mutant p53-248W protein in DNA damage response in yeast cells, cells were exposed to DNA damaging agents; UVC and cisplatin that were reported to induce wild type p53 protein in mammalian cells. Codon 248 is a common site of 'hot spot'mutation and the arginine residue that corresponds to codon 248 encoded by the wild type p53 sequence is in the DNA interacting face of the p53 protein. Mutant p53-248 (arg->trp) is defective in specific DNA binding and it has lost the ability to act as a transcription factor. Although there are not many reports about mutant p53-248W response to DNA damaging agents, it was shown that mutant p53-248W exhibit decreased repair of active genes upon UV radiation. In this study cell survival was analysed in mutant p53-248W protein expressing yeast cells in parallel to mutant p53 protein levels following to DNA damage and no effect of mutant p53 248W expression on cell survival upon DNA damage was detected. Also no difference was detected in mutant p53 protein levels following DNA damage.

ÖZET

p53 proteinin *Saccharomyces cerevisiae* mayasındaki etkilerini incelemek için deney sisteminin oluşturulması

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Yüksek Lisans Tezi,Moleküler Biyoloji ve Genetik
Bölümü Tez yöneticisi: Prof. Dr. Mehmet Öztürk
Ağustos 1997, 82 Sayfa

Bu çalışmanın amacı, *Saccharomyces cerevisiae* maya hücrelerinde insan p53 proteininin ve mutant formunun etkilerini görmek için deneysel bir modelin oluşturulmasıdır.

p53 proteininin DNA hasarına karşı tepki gösteren ve bu şekilde genom dengesini kontrol eden bir protein olduğu memeli hücrelerinde daha önce gösterilmişti. Biz DNA hasarına karşı insan p53 proteininin maya hücresinde gösterdiği etkilerle ilgilendik ve bunun için de ilk olarak mayada deneysel bir sistem kurduk. Bu deneysel sistemin oluşturulmasında, mutant p53-248W proteinini ekspres eden pAK31 plasmidi ve kontrol olarakta bu proteini ekspres etmeyen pL3 plasmidi kullanıldı. Bu şekilde mutant p53 ekspres eden ve etmeyen iki farklı maya hücre populasyonu oluşturuldu. p53 proteininin hücre çoğalmasını engellediğini gösteren raporlardan dolayı çalışmalarda p53 proteini kullanılmadı. Bu sistemde ilk olarak pAK31 vektörü ile transforme edilen maya hücrelerinde, p53 proteinininin ekspresyonu gösterildi. Daha sonra mutant p53-248W proteininin, maya hücrelerinin büyüme hızına etkisi analiz edildi ve mutant p53 ekspres eden ve etmeyen maya hücrelerinin büyüme hızında hiçbir fark görülmedi. Mutant p53-248W proteininin maya hücrelerinde, DNA hasarına karşı bir tepki gösterip göstermediğini kontrol etmek için maya hücreleri DNA hasarına yol açan, UVC-ışınlarına ve cisplatine maruz bırakıldı. Memeli hücrelerinde p53 proteininin DNA zararına tepkisi daha önceden UVC-ışınları ve cisplatinle gösterilmişti. p53 geninin arjinin amino asidine denk gelen 248. kodonu en sık rastlanan mutasyon noktası olmasının yanısıra p53 proteininin DNA ile etkileşen kısmıdır. Mutant p53-248' in (arg->trp) DNA ile etkileşimindeki özgüllüğünden yoksun kalması dolayısıyla transkripsiyon faktörü olarak işlev görme özelliğini kaybeder. Mutant p53-248W proteininin DNA hasarına gösterdiği tepki hakkında çok fazla rapor bulunmamakla beraber, bir rapor da UVC-ışını karşısında mutant p53-248W proteininin aktif genlerin tamirini azalttığı gösterilmiştir. Bu çalışmada mutant p53-248 proteini ekspress eden maya hücrelerinin, DNA hasarı sonucunda hücrelerin canlı kalmasına olan etkisine paralel olarak, bu hücrelerde mutant p53 protein seviyeleri de incelenmiştir. DNA hasarı karşısında mutant p53-248W proteininin canlı kalan maya hücresi sayısına etkisi belirlenmediği gibi p53 protein seviyesinde de bir farklılık görülmemiştir.

ACKNOWLEDGEMENT

It is my pleasure to express my deepest gratitude to my supervisor Prof. Dr.Mehmet Öztürk for his guidance, encouragement and invaluable efforts throughout my thesis work. I have not only benefited from his knowledge but also learned a lot from his superior academic character.

I would like to address my special thanks to Lütfiye Mesci, Birsen Cevher and Esma Yolcu for sharing their experiences with me. I would also like to thank Dr. Akbar Kuchkartayev for the plasmids pL3 and pAK31, and yeast strain.

I would like to thank my housemate Hilal for her loveliness.

My dear friend Tolga, who shared many of hard days with me throughout our six years, thank you for your friendship, the life, is easier with you.

I would like to thank Burçak, Cemaliye and Reşat for making a joyful environment and extending their helping hands whenever I needed.

My sincere thanks to my parents for their moral support and thanks to my brother Captain Emre for his being that make the life easier and funnier. Last but not least, I thank Zekoş for her incredible patience and lovely home.

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ABBREVIATIONS

bp	base pair
BCIP	5-bromo-4-chloro-3-indoyl phosphate disodium salt
bisacrylamide	N', N', methylene bis-acrylamide
cisplatin	cis-diamminechloroplatinium(II)
C-reminus	carboxy terminus
cDNA	complementary deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphate
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
EDTA	diaminoethane tetra-acetic acid
EtBr	ethidium bromide
kDa	kiloDaltons
LB	Luria-Bertani media
LiAc	lithium acetate
min	minute

MW	molecular weight
NBT	nitro blue tetrazolium chloride
N-terminus	amino terminus
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PBS	phosphate buffered saline
PCI	phenol: chloroform: isoamyl alcohol
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride paper
Rpm	revolutions per minute
SC	synthetic complete media
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBE	tris-boric acid-EDTA
TEMED	N, N, N', N'-tetramethyl-1,2-diaminoethane
Tris	Tris (hydroxymethyl-)methylamine
UV	ultraviolet
YPD	yeast extract-peptone-dextrose media

1. INTRODUCTION

The p53 is a 53 kDa nuclear phosphoprotein that suppresses abnormal cell proliferation and plays an important role for protection against cancer. p53 has been named as "guardian of the genome" by researchers to indicate its dictatory role in control of the genomic integrity. Recent studies indicate that the p53 protein is involved in gene transcription, DNA synthesis and repair, senescence, genomic plasticity, and in programmed cell death.

The p53 gene and protein product have become the center of study ever since it become clear that more than 50 % of human cancers contain mutations in this gene. These mutations were detected in more than 50 different cell lines and tissue types (Hollstein *et al.*, 1994). The nature of these mutations in cancer cells is most commonly a missense mutation in one allele, producing a faulty protein that is than observed at high concentrations in these cells. Deletions or chain termination mutations in the p53 gene were obtained rarely. On the other hand, p53 "knockout" mice (no p53 expression or p53 -/-) were shown to be prone to different tumor development (Donehower et al., 1992).

p53 is a component of biochemical pathways central to human carcinogenesis (Harris, 1993). p53 protein alterations due to mostly missense mutations and rarely loss of p53 protein by nonsense or frameshift mutations provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells. There have been some suggestions that the missense mutant producing a faulty p53 protein could contribute to a "gain of

function" phenotype in addition to the abrogation of p53 function (Dittmer et al., 1993). p53 protein levels are mostly regulated by posttranslational mechanisms (Finlay et al., 1988). Degradation of p53 may involve ubiquitin-dependent proteolysis; most mutations of p53 disturb the degradation pathway, resulting in particularly high levels of the mutant protein (Scheffner et al., 1990).

The mutational spectra at the p53 locus in different tissue types indicates a strong role for diverse environmental mutagens with a set of tissue preferences (Bressac et al., 1991; Hsu et al., 1991; Brash et al., 1991). In addition there is strong selection for subset mutations localised predominantly in the DNA binding domain of p53 (Lin et al., 1995). Thus both selection and a strong set of environmental mutagens combine to produce mutations in the p53 gene in human cancers.

2.GENERAL INFORMATION

2.1. p53 and cancer

Tumour suppressor genes maintain tissue homeostasis by controlling cellular proliferation, terminal differentiation and apoptosis (Weinberg *et al.*, 1991). The p53 tumour suppressor gene has become a hot topic of cancer research because it is commonly mutated in human cancers and spectrum of p53 mutations in these cancers

provide clues about the etiology and molecular pathogenesis of cancer (Harris *et al.*, 1993 ; Hollstein *et al.*, 1991). Statistic value indicates that, of the ~6.5 million cancer cases worldwide each year, 2.4 million tumours are estimated to contain a p53 mutation. About 50 % of human cancers contain p53 mutations, including the cancers of the breast, cervix, colon, liver, prostate, bladder, and skin and these cancers are more aggressive, more prone to metastasise, and more fatal (Harris *et al.*, 1996).

2.2. Historic landmarks of p53

The nuclear phosphoprotein p53 was originally discovered in extracts of transformed cells, reacting with antiserum from animals with tumours induced by Simian Virus 40 (SV40) (Linzer and Levine , 1979; Lane and Crawford , 1979). The protein was found to form an oligomeric complex in SV40 transformed cells with the SV40 oncogene product; the large T antigen. It was known that large T antigen is required for maintenance of transformed phenotype. p53 protein was also coprecipitated with E1B-55 kDa protein that cause a transformed phenotype (Sarnow *et al.*, 1982). At this time p53 was classified as a tumour antigen. The levels of p53 in cells containing those complexes were approximately 100 fold higher than in the non transformed cells and the half life of p53 is correspondingly extended from 20 min to 24 hr (Reich *et al.*, 1983 ; Oren *et al.*, 1981). The results of further studies have supported the hypothesis that increased levels of p53 effect phenotype of the normal cells; overexpression of p53 resulted in the immortalization of rodent cells (Jenkins et al., 1984 and Rovinski and Benchimol,1988) and in conjunction with an activated *ras* oncogene in rodent cells, p53

induced tumourogenic phenotype formation (Eliyahu et al., 1984, Parada *et al.*, 1984). However all of these studies were performed with a expressing a mutant forms of p53 (Hinds *et al.*, 1989, Finlay et al., 1988, Eliyahu et al., 1988). When wild type p53 was introduced in to the cells, it suppressed the transformation of cells in culture by other oncogenes like *ras* (Finlay *et al.*, 1989). DNA screened from colon cancer patients revealed that p53 mutations occur with usually high frequency in tumour tissue (Baker *et al.*, 1989). It is frequently altered by somatic mutations in a wide variety of human neoplasms (Hollstein *et al.*, 1991). Beside spontaneous human cancers, germline p53 mutations are found at Li-Fraumeni syndrome in which affected family members develop cancers at a young age (Malkin *et al.*, 1990). High incidence of tumours in p53 knockout mice is detected by six mounts of age (Donehower *et al.*, 1992). All these results confirmed that p53 is a tumour suppressor gene that requires loss of function mutations for tumour formation but some mutant forms of p53 exert a transdominant negative effect on normal p53 that result in gain of function for carcinogenesis (Milner et al., 1991, Srivastava *et al.*, 1993).

2.3. Structure of p53

2.3.1. p53 domains: structure-function relationship

p53 is a phosphoprotein of about 393 amino acids which can be divided into five domains. p53 has been shown to possess a transcriptional activation function and transcriptional activation domain has been mapped to the 1-42 amino acid residues at N-terminus that interacts with the basal transcription machinery (Lin *et al.*, 1994).The

sequence specific DNA binding domain of p53 localised between amino acid residues 102 and 292, the central part of the protein referred as core domain. The crystal structure of a complex containing the core domain and DNA has been determined at 2.2 A° resolution (Cho *et al.*, 1994). More than 90 % p53 of mutations occur in this core domain and 40 % of the missense mutations are localised to residues R175, G245, R248 R249, R273, and R282 which play a role in the structural integrity of this domain (Cho *et al.*, 1994). p53 is a nuclear protein , a nuclear localisation sequence was found between amino acid residues 316-325.(Shaulsky *et al.*, 1990). The native p53 protein is a tetramer in solution. C terminal domain between amino acid residues 323 and 355 in human p53 was mapped as oligomerization domain. Crystal structure of the tetramerization domain has been determined at 1.7 A° resolution (Jeffrey *et al.*, 1995). The tetrameric form of p53 protein that is a dimer of a dimer, binds to four repeats of consensus DNA sequence 5'- PuPuPuC(A/T)-3'and this sequence is repeated in two pairs, each arranged in inverted repeats such as→ ←→ ←, where → is the sequence written above. This consensus sequence was identified in the promoter and/or an intronic regions of the genes that are transactivated by p53 (El- Deiry *et al.*, 1992). The highly basic extreme C terminus which is connected to oligomerization domain with a flexible linker between the residues 368-387 can bind DNA sequence non-specifically and regulate non DNA binding-latent form of p53. This region negatively regulates p53 sequence specific DNA binding and deletion, phosphorylation by protein kinase C or casein kinase II, binding of antibody Pab421 to this sequence all activate latent state of p53 and provides its sequence specific DNA binding (Hupp and Lane *et al.*, 1994). The fifth domain defined approximately by amino acids 61-94 of human p53 contains five repeats of amino acid sequences PXXP (P

designate proline and X designate any amino acid). The fundamental importance of PXXP sequence, is supported by the fact that these motifs have been found in all proteins known to bind directly to SH3 domain which have important roles in signal transduction. A p53 cDNA deletion mutant (Δpro AE) which lacks the entire fifth domain has been shown to be completely dispensable for transcriptional activation. On the other hand a deletion of this p53 proline rich domain impairs p53 ability to suppress tumour cell growth. In addition to transcriptional activation domain, prolin rich domain may play a critical role in the transmission of downstream signal (Walker and Levine, 1996). Domains of p53 protein are shmematically presented in Fig.1.

2.3.2. Structural aspects of the p53 protein in relation to gene evolution

According to sequence analysis of various p53 proteins five highly conserved blocks were identified (Soussi *et al.*, 1990). Four of these five blocks (II to V) are found in the central domain and block I is located in the N-terminal.

Starting with vertebrates, a large number of p53 genes from different species have been characterised. Interestingly p53 was also identified in invertebrates such as *squid* (Ishioka *et al.*, 1995). Despite existence of p53 in diverse organisms, up to now no protein homologous have been identified in lower eukaryotes like *Drosophila* and yeast.

2.3.3. Highly conserved regions that coincide with the mutation clusters of p53 found in human cancers.

Mutations in p53 have been shown to occur at nearly every position, with a preferential occurrence in III, IV, V conserved blocks, like the mutations R175 found in IIIth block; R248, G 245 and R249 found in IV th conserved block; R273 and R282 found in Vth conserved domain (Fig 1). Those mutations are p53 somatic mutations that are frequently seen in human tumours and cell lines (Hollstein *et al.*, 1994).

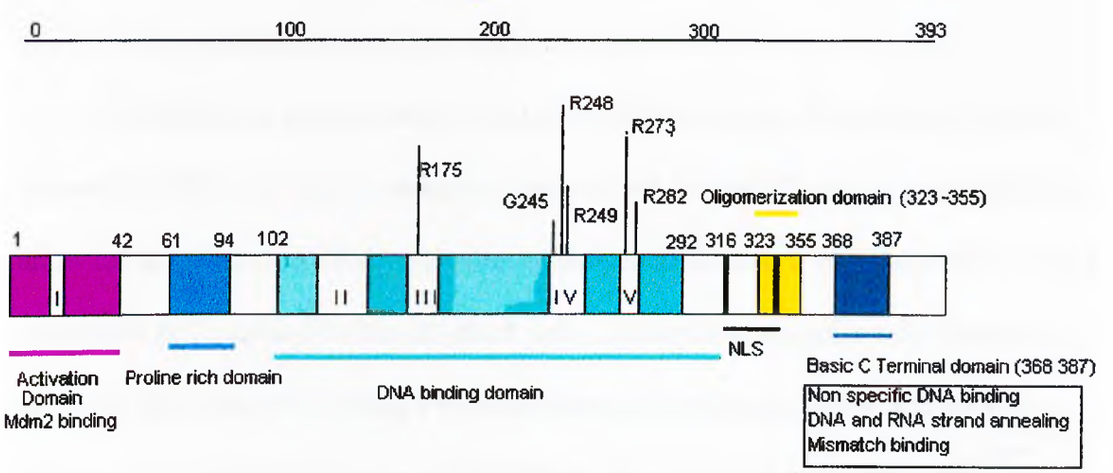


Fig.1. Schematic presentation of human p53. Several hot spots for mutations R175, G245, R248, R249, R273, R282 are also indicated

2.4. The cellular functions of p53

The high frequency of p53 alterations in human cancers suggests that p53 play a critical role in the control of the growth of normal cells. A number of biological and biochemical functions have been ascribed to wild type p53. Importantly mutant p53 proteins derived from human tumours are defective in some of these functions, suggesting that such functions like cell cycle arrest, apoptosis, transcriptional activation and repression, DNA repair and replication are relevant for p53 mediated growth control and tumour suppression

2.4.1. Mechanism leading to cell cycle arrest

All cells have mechanisms for coping with DNA damage. Most eukaryotic cells respond to DNA damage with transient delays in both G1 and G2 phases presumably to allow for repair prior to DNA replication or division of the cell. It was shown that tumour suppressor p53 required for the G1 check point function in mammalian cells (Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992). Fibroblasts from p53 nullizygous mice lack the G1 checkpoint function (Kastan *et al.*, 1992). Furthermore, normal human fibroblasts induce p53 and cause its accumulation in response to ionizing radiation (IR) whereas fibroblasts from the Ataxia talengiectesia (AT) patients are defective in this response (Kastan *et al.*, 1992). This response of p53 to DNA damage was also shown in many different cell types; cell lines having wt p53, exhibit G1 arrest following radiation, p53-null cells such as Saos-2 cells continue to progress through S phase (Kuerbitz *et al.*, 1992). Cells with mutant p53 enter S phase and amplify segments of DNA, Li-Fraumeni syndrome cells

containing wt p53 arrested in G1 and did not go gene amplification, the converse occur in cells lacking wt p53 (Yin *et al.*, 1995).

The amount of p53 in mouse cells increases 5-10 fold a few hours after UV irradiation or treatment with the DNA damaging drug 4NQO (Maltzman and Czyzyk. 1984).

In normal conditions p53 is a labile protein. By pulse-chase experiments half life of p53 protein in untreated cells found to be 20 min. Agents which damage DNA induce p53 to become very stable (Mosner *et al.*, 1995). The same group suggest that p53 is negatively auto-regulated by specifically inhibiting translation of its own mRNA in vitro. According to studies in hepatocytes; nuclei of untreated cells were always negative for immune detectable p53. In contrast, nuclei of UV damaged DNA stained positively for p53 (Sun et al., 1995). Additionally a novel p53 promoter that contains 80 bp was identified which is induced by genotoxic stress such as anti cancer drugs and UV (Sun *et al.*, 1995).

The ability of p53 to serve as a transcription activator suggests a mechanism for the induction of the damage responsive transcripts that might influence the cell cycle progression. A strong candidate is $p21^{CIP1/WAF1}$ (Nauba *et al.*, 1995; El-Deiry *et al.*, 1993; Harper *et al.*, 1993), which is activated by p53 and likely to play an important role in the p53-induced DNA damage because it encodes a potent cyclin dependent kinase inhibitor. By this way $p21^{CIP1/WAF1}$ prevents phosphorylation of the G1 cyclin/ CDK substrate pRB, thereby preventing the transition from G1 phase to S phase (Slebos *et al.*, 1994).

DNA damage is the triggering factor for the induction of p53 activation. Types of DNA damage that activate p53 were also studied. The experiments indicate that p53

dependent arrest mechanism can be activated by very few double strand breaks, and only one may be sufficient. In addition to double strand break, nuclear injection of linearized plasmid DNA, circular DNA with a large gap or single stranded phagemid DNA is sufficient to induce a p53 dependent cell cycle arrest (Huang *et al.*, 1996).

Beside G1 arrest, p53 has also been implicated in G2/M phase checkpoint. When mitotic spindle inhibitors are added to the cells with wild type p53, the cells are blocked in G2. In the absence of wild type p53, the cells re-initiate DNA synthesis (Cross *et al.*, 1995). If the radiation applied before G1 checkpoint, the fibrosarcoma cells undergo G1 arrest without subsequent G2 arrest and if the cells are irradiated after G1 checkpoint, cells undergo G2 arrest without subsequent G1 arrest. Furthermore in those cell lines which do not go radiation-induced apoptosis, the wild type p53 exhibit radioresistance in terms of clonogenic survival (Pellegata *et al.*, 1996).

2.4.2. Induction of apoptosis

p53 initiate apoptosis if the damage to cell is severe. This protects the organism from the growth of damaged cells. p53 plays a role in triggering apoptosis under different physiological conditions. Normal thymocytes will undergo apoptosis in response to DNA damage whereas thymocytes from p53 (-/-) mice don't undergo apoptosis in response to same stimulus (Lowe et al., 1993). A number of factors affect the decision of a cell to enter p53-mediated cell cycle arrest or apoptotic pathway; under conditions in which the DNA is damaged, if survival factors are limiting or an activating oncogene is forcing the cell in to a replicative cycle, p53 induced apoptosis prevails. Two of the genes that are regulated by p53 could influence the decision to commit to an apoptotic pathway; Bax

and IGF-BP3 (Miyashita and Reed, 1995; Bucbinder *et al.*, 1995). Bax regulate apoptosis by binding to BCL-2 that is a blocker of apoptosis and IGF induce apoptosis by lowering the mitogenic response of cell. Sequence specific transcription capacity of p53 is very important for induction of apoptosis like in the case of induction of Bax and IGF-BP3, but beside this, it was shown that a truncated p53 protein lacking sequence specific transcription capacity is able to induce apoptosis in He-La cells. This result indicated the existence of another p53-dependent apoptotic pathway of which occur without sequence specific transcription function of p53 (Haupt *et al.*, 1995).

2.4.3. Transcriptional activation and repression by p53

p53 is a potential transcription factor (Fields and Jang *et al.*, 1990) and once activated, it repress the transcription of one set of genes , like c-fos, c-jun, IL-6, Rb and Bcl-2. It was reported that only promoters containing initiator elements and lacking p53 binding sites are inhibited by p53 (Mack *et al.*, 1993). It stimulates the expression of other genes that have p53 binding sites, some of the target genes identified so far include; p21/Waf 1/CIP1 which is a cyclin dependent kinase inhibitor that combine with cyclins and PCNA and cause G1 arrest of cell cycle (El-Deiry et al., 1993; Harper et al., 1993) mdm-2 is a oncoprotein which is a cellular inhibitor of the p53 in that it can bind to transactivation domain and form an autoregulatory loop with p53 activity (Oliner *et al.*, 1992), Gadd-45 is induced when cells are subjected to DNA damage leading to arrest in G1 (Kastan *et al.*, 1992). It interacts with PCNA and inhibits DNA replication; cyclin G is a novel cyclin that is strongly activated in a p53-dependent manner in cells subject to DNA damage (Zauberman *et al.*, 1995), bax is a member of bcl-2 family that promotes

apoptosis (Miyashita and Reed, 1995), IGF BP3 is induced in cells after DNA damage, It is anti-mitogenic factor (Bucbinder *et al.*, 1995). Many effects of p53, like growth arrest, apoptosis and inhibition of carcinogenesis can be attributed to the biological functions of its downstream genes.

2.4.4. Involvement of p53 in cellular DNA replication and repair

In addition to the damage-induced p53 functions, evidence for a direct involvement of p53 in cellular DNA replication and repair has accumulated. The products of two different p53 target genes, $p21^{waf1/Cif1}$ and Gadd45 has been shown to interact with PCNA, a factor that is involved in DNA replication, both GADD45 and p21 discard PCNA from DNA replication via binding to it (Waga *et al.*, 1994; Smith *et al.*, 1994). These results suggest the idea that p53 involve in DNA replication regulation by inducing GADD45 and p21.

There are also evidences for a direct involvement of p53 in cellular DNA repair. Such an involvement can be deduced from several findings. In addition to sequence specific DNA binding, p53 protein binds non-specifically to double stranded (ds) and single stranded (sd) DNA (Kern *et al.*, 1991). Furthermore it has been described to reanneal both RNA and DNA (Balkalkin *et al.*, 1994). These functions might be important in the processes associated with DNA repair like recognition of DNA damage in the form of deletion insertion deletion mismatches (Lee *et al.*, 1995) and prevention of unscheduled recombination (Bertrand *et al.*, 1997). Another function of p53 is its Mg^{+2} dependent 3'-5' exonuclease activity that could be mapped to the central core domain of p53 protein, mammalian cells contain a variety of different exonucleases like PCNA

dependent polymerase δ and ε involved in DNA replication, recombination and proofreading repair (Mummenbrauer *et al.*, 1996).

In addition, physical interaction between the human RAD 51 (homologues of bacterial Rec A) and wt p53 but not mt p53 was found in vivo. According to results; it was shown that this interaction inhibit RAD 51 which catalyses three strand exchange and ATPase activity. It was suggested that this physical interaction might play a major role in controlling the extent and timing of homologous recombination which is so important for the genome stability (Sturzbecher *et al.*, 1996). Further investigations about p53 effect on homologous recombination in mammalian cells expressing a mutant p53 protein showed that mt p53 increase the intrachrosomal homologous recombination (Bertrand *et al.*, 1997).

Also there are some clues about the p53 involvement in nucleotide excision repair (NER) an important and versatile DNA repair mechanism, which is the major pathway for repair of UV-type lesions and damage by a variety of important carcinogens and mutagens. During the studies to determine the effects of p53 on cellular sensitivity to UV irradiation and on NER in primary human skin fibroblasts from patients of Li-Fraumeni Syndrome, it was found that p53 mutant cell lines were resistant to UV-induced cytoxicity and apoptosis but deficient in global repair (Ford *et al.*, 1995). According to in vitro data, host cell reactivation experiments with a reporter plasmid that is damaged with UV, mutant p53 containing cell lines showed reduced repair of UV induced DNA lesions (Smith *et al.*, 1995; Mckay *et al.*, 1997).

2.4.5. The events in the p53 activation and its response

The upstream events or signals flowing to p53 are mediated by several stressful situations. In addition to DNA damage, hypoxia is also able to stimulate p53 levels and activate p53 protein (Graeber *et al.*, 1996). Another signal that activates p53 is generated when ribonucleoside triphosphate pools fall below the critical value (Linke *et al.*, 1996). In the case of p53 activation, activated p53 may induce either growth arrest or apoptosis via transcriptional control or non-transcriptional control to maintain genomic integrity of cell. Figure 2 shows the major pathways leading to p53 activation and p53 mediated cellular changes.

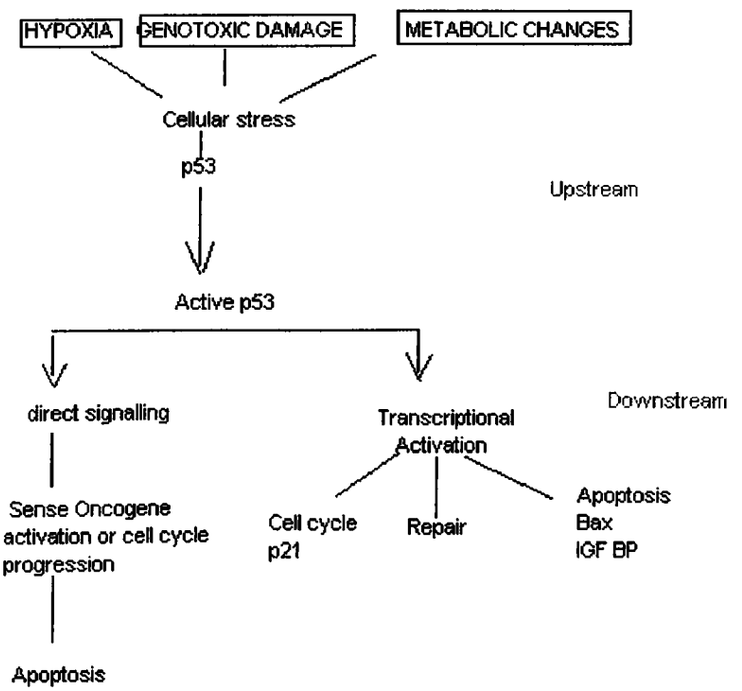


Figure 2. The p53 pathway

Levine, A. J. 1997

2.5.The p53 and yeast cells

Although the term tumour suppressor connotes a function attributable only to multicellular organism, the control of DNA damage and cell cycle progression is critical to all eukaryotes. Genes in which recessive mutations uncouple growth arrest signals from cell cycle progression can be considered as yeast paradigms of tumour suppressor genes. For example, in *S.cerevisiae*; Rad9, Rad17, Rad24, Mec3, involve in recognition of DNA damage and Pol2, Rfc5, Dpb11 involve in replication recognition of replication blocks; and the genes Mec1, Rad53 and Dun1 that transduce the growth arrest signals (Elledge *et al.*, 1996).

Although homologous of p53 protein has not been identified in yeast, there are several studies that demonstrate similarity between the effects of wild type and mutant p53 expression in yeast and human cells. Experiments in *S.cerevisiae* and *S. pombe* with human p53 indicates that p53 can alter the growth of lower eukaryotic cells. According to studies in *S.cerevisiae*, the difference among colony growth between the cells expressing wild type human p53, mutant p53; mutant (Ala-143), mutant (His-273); and no p53 was shown. Colony growth difference was detected among the cells expressing exogenous wild type p53, mutant p53, and no p53 (vector with or without insert). Colonies expressing wild type p53 were considerably smaller than the control colonies. Cells producing the Ala-143 mutant grew almost as well as the control cells, while cells expressing the His 273 mutant exhibited an intermediate colony site. Colonies containing vector alone (no p53) had a doubling time of 4.4 hr. The doubling time of cells containing

the wild type p53 was increased to 11.6 h and the doubling time of the strains containing the his-273 mutant was 6.5 hr. Also phase of the cell cycle of these arrested cells were determined by flow cytometry. Strains containing wild type p53 exhibited G1 and G2/M peaks. In those experiments, it was also observed that coexpression of p53 with the human cell cycle-regulated kinase, but not the yeast homologue CDC28, severely inhibiting cell growth and *S.cerevisiae* expressing both p53 and CDC2H accumulated largely in G1 phase. P53 was phosphorylated in *S.cerevisiae* but differences in phosphorylation did not account for the apparent growth rate observed in various strains containing p53 and CDC2H2 (Nigro *et al.*, 1992). The same effect of p53 to the growth of fusion yeast; *S.pombe* was also observed (Biscoff *et al.*, 1992). Wild type and two mutant polypeptides His 273, His 175 were expressed to similar levels in *S.pombe* and after 24 hr expression, although wild type p53 caused *S.pombe* to stop dividing, two mutant forms continued to divide slowly compared to non p53 expressing cells. In mammalian cells, p53 is predominantly a nuclear protein (Shaulsky et al., 1990). The p53 cellular localisation was also studied in *S.pombe* and it was shown that wild type protein was localised to the nucleus, the mutant polypeptides exhibited more extranuclear staining than wild type p53 did. p53 is highly phosphorylated in mammalian cells. It was shown that wild type p53, when expressed in *S.pombe*, was phosphorylated on serine 315 and serine 392 by casein kinase II and cdc2 kinase. It was shown that phosphorylation at Ser-315 and Ser-392 doesn't effect p53 growth inhibitory function in *S.pombe.* Also an *S.pombe* strain was constructed to identfy dominant mutant alleles of p53. Several different mutant alleles of human p53 were introduced to wild type p53 expressing *S.pombe* strain to release it from the growth inhibitory effects of wild type p53. The His-

175 that has been shown dominant to the wild type p53 in mammalian cells, were also detected as dominant in this assay (Biscoff et al., 1992).

p53 is a transcription activator (Fields and Jang *et al.*, 1990). It was shown that mammalian wild type p53 expressed in *S.cerevisiae* was able to activate transcription of a reporter gene under the control of a CYC1 hybrid promoter containing the 33 base pair p53 binding sequence whereas three mutants commonly found in human tumours, 175H, 248W, 273H were unable to activate transcription (Scharer *et al.*, 1992). Based on this function continuity of p53 in yeast, assays have been developed to detect p53 mutations. Dominant negative p53 mutations were selected by yeast transcriptional assay, using URA3 as a reporter gene under the ADH1 promoter that contain p53 consensus DNA binding sequence. According to this assay dominant-negative p53 mutations that suppress wild type p53 transcription activity were screened according to their Ura^- Foa^+ phenotype (Brachmann *et al.*, 1996). Also the effect of p53 mutations in the carboxy terminal to the transcription activity of p53 was investigated using yeast transcriptional activity (Ishioka *et al.*, 1995). For detection of p53 mutations in blood samples, in tumours and in cell lines, yeast transcription assay was developed (Flaman *et al.*, 1995).

During the studies to understand the mechanism of p53 transcription activity regulation in yeast *S. cerevisiae*, mutants were generated by EMS and UV mutagenesis that are defective for p53 transcription activity. To study sequence specific transcription activity (SST), *S cerevisiae* was transformed by two vector, one of them was p53 expressing plasmid and the other contained a reporter gene under the control of URA3 promoter that had specific p53 DNA binding site. Following cDNA screening to find a gene that complements SST p53 activity in yeast, PAK1 gene was identified. It was found

that all these mutants were complemented by PAK1 gene, which is a ser/thr kinase. Expression of PAK1 was associated with an increased specific activity of p53 in DNA binding assay and corresponding increased in transactivation (Thiagalingam *et al.*, 1995).

In an attempt to identify the p53 like a protein in the yeast *S.cerevisiae*, a mutant yeast cell that require wild type p53 for its viability was isolated. The mutant, rft-1, is defective in cell cycle progression and arrests before mitosis in the deficiency of p53. According to genetic and biochemical studies, it was shown that p53 complement the activity of rft-1 by forming a protein-protein complex (Koerte *et al.*, 1995).

Although there are homologues of mammalian genes in yeast involved in DNA damage and checkpoints like the *S.cerevisiae* genes; MEC1, TEL1, POL2 which are the homologues of human genes; ATR, ATM, Polε respectively, there is no homologue of another cell cycle control p53 gene (Elledge, 1996). But expression of wild type p53 affects yeast cell growth (Nigro et al., 1992). Additionally p53 conserve its sequence specific transcription activity in yeast (Shrarer et al., 1992) and a yeast gene that complement this activity was found (Thiagalingam et al., 1995). All these results encourage the further studies to investigate other p53 effects in yeast.

p53 response to DNA damage in yeast has not been studied yet. According to studies in mammalian cells upon DNA damage, p53 is activated, it is stabilised, accumulated in the nucleus and induce downstream genes to keep genomic stability.

In this study an experimental system was established to study p53 response in yeast *S.cerevisiae* upon DNA damage. Considering the difficulties to use wild type p53 due to it's growth inhibitory effect in *S.cerevisiae* (Nigro et al., 1992), mutant p53 was preferred to establish this experimental system. As mutant, R248 (CCG→TGG) which is

one of the most common p53 mutation detected in human tumours (Cho et al., 1994) was preferred. One of the DNA damaging agents used in this study was UVC (254 nm); the amount of p53 in mouse cells increases 5-10 fold a few hour after UV radiation (Maltzman and Czyzk, 1984). UV induced nuclear accumulation of p53 was also detected in primary human fibroblasts (Yamaizumi and Sugano, 1994). Beside p53 accumulation upon UV type DNA damage, it's involvement in repair of UV type DNA damage was also shown (Smith et al., 1995).

In studies about p53, It was shown that p53 response might differ according the status of DNA damaging agents (Zhang *et al.*, 1996, Vasey *et al.*, 1996). To confirm the p53 effects upon DNA damage in yeast another DNA damaging agent was used. In this case as direct DNA damaging agent cis-diamminedichloroplatinium(II) (cisplatin) was preferred. Cisplatin is an important anticancer drug which destroys tumour cells by forming covalent adducts on DNA, blocking polymerases, and preventing replication, transcription and cell division (Bruhn *et al.*, 1990). It was shown that treatment of mouse teratosarcoma cells with this chemotherapeutic agent led to an increase of p53, 3 h after addition of the drug (Kupper *et al.*, 1994). In *S. cerevisiae* cisplatin was also used as a DNA damaging agent to identify specific protein that bind cisplatin-DNA adducts (McA' Nulty and Lippard, 1996).

In this study an experimental system was developed to study the effects of wild type and mutant p53 protein following DNA damage.

3. Materials and Methods

3.1. Materials

3.1.1. Reagents

All of the chemicals obtained SIGMA except cisplatin that was obtained from BELLON RHONE-POULENC RORER.

3.1.2 Enzymes and enzyme buffers:

3.1.2.1. PCR enzyme

5u/µl Ampi-Taq DNA polymerase (PERKIN ELMER)

3.1.2. 2. Restriction Enzymes and buffers

25 u/µl Msp1 (STRATAGENE)

10x Assay Buffer #2 (For Msp1) (STRATAGENE)

10 u/µl Hae III (BOEHRINGER MANHEIM)

10x SuRe/ Cut Buffer M (For Hae III) (BOEHRINGER MANHEIM)

3. 1. 3. Markers

ϕx174/ Hinf I digested 0.5 μg/ml (MBI Fermentas)

100 bp DNA ladder 0.13 μg/μl (PROMEGA)

3.1.4. Commonly used buffers and stock solutions

3.1.4. 1. Buffers and solutions used in PCR

10x PCR buffer-$MgCl_2$ (PERKIN ELMER)

25 mM $MgCl_2$ solution (PERKIN ELMER)

5 u/μl Ampi-Taq DNA polymerase (PERKIN ELMER)

10 mM dNTPs mix (MBI)

3.1.4.2. Stock solutions required for bacterial transformation

1 M $CaCl_2$ stock solution.

54 g $CaCl_2.6H_2O$ was dissolved in 200 ml of dH_2O. The solution was filter sterilised using 0.22 μm filter sterilization unit (COSTAR).

3.1.4.3. Stock solutions required for yeast transformation

1.0 M LiAc stock solution, pH 8.4-8.9

10.2 g of lithium acetate was dissolved in 100-ml water, pH is adjusted to 8.4-8.9.

The solution was filter sterilised with 0.22 μm filter sterilization unit. Solution was stored at room temperature.

PEG stock solution, 50 % (w/v)

50 g PEG 3350 was weighed out and transferred to graduated beaker, water was added until the level just below the100 ml mark, solution was stirred with magnetic stirrer until PEG dissolved. Dissolved solution was transferred in to the measuring cylinder and the volume was completed to 100 ml. It was filter sterilised with 0.22 µm filter unit and stored at RT in a tightly sealed container.

100 mM LiaC solution

500 µl of 1.0-M stock solution was diluted in to 4.5 ml of sterile water.

PEG/LiAc solution (40 % PEG, 100 mM LiAc)

500 µl of 1.0 M LiAc stock solution in 500 µl of sterile water and 4.0 ml PEG 50 % (w/v) stock solution. Solution was vortexed vigorously for proper mixing.

3.1.4.4. Buffers and stock solutions for DNA isolation and Analysis.

3.1.4.4.1. Buffer for isolation of yeast genomic DNA

Breaking Buffer

2 % Triton X-100

1 % SDS

100 mM NaCl

10 mM Tris.Cl pH 8.0

1 mM EDTA.

3 M Sodium acetate(1 Lt)

408-g sodium acetate.3 H_2O dissolved in H_2O, pH is adjusted to 5.2 with 3M Acetic acid.

3.1.4.4.2. Buffers and solutions used for analysis of DNA

TBE electrophoresis buffer 10x stock soln. (1 Lt)

108 g Tris Base

55 g Boric acid

40 ml 0.5 EDTA, pH 8.0

0.5 M EDTA (1 Lt)

186.1 g $Na_2EDTA.2H_2O$ was dissolved in 700 ml dH_2O, pH was adjusted to 8.0 with 10 M NaOH (~50 ml), volume was completed to 1 Lt.

EtBr 10 mg/ml

0.2 g ethidium bromide was dissolved in 20 ml H_2O and stored in dark.

Loading buffer 6x (stored at 4°C)

0.25 % Bromophenol Blue

0.25 % xylene cyanol

40 % sucrose in water

3.1.4.5. Buffers and stock solutions used for selection and testing for p53 expression

3.1.4.5.1. Buffers used for cell lysis and protein extraction under denaturing conditions

Laemni lysis buffer

50 mM Tris.Cl pH: 6.8 (1.0 M Tris.Cl pH:6.8)

100 mM DTT (1 M stock solution)

2 % SDS (10 % SDS stock solution)

20 % Glycerol (v/v)

5 mM EDTA (0.5 M EDTA, pH: 6.8)

1 mM PMSF (10 mM PMSF) Additionally added as protease inhibitor.

3.1.4.5.2. Solutions used for SDS-PAGE

10 % polyacryamide resolving gel (40 ml)

15.9 ml deionized water

13.3 ml 30 % Acryamide mix

10 ml 1.5 M Tris (pH:8.8)

0.4 ml 10 % Ammonium per sulfate.

0.0016 ml TEMED

5 % stacking polyacryamide gel (10 ml)

6.8 ml deionized water.

1.7 ml 30 % Acryamide mix

1.25 ml 1.0 M Tris. Cl pH: 6.8

0.1 ml 10 % SDS

0.1 ml 10 % Ammonium per sulfate

0.01 ml TEMED

Tris-Glycine electrophoresis buffer (1 Lt)

25 mM Tris

250 mM Glycine pH: 8.3

0.1 % SDS

2x SDS gel-loading buffer (Store at 20° C)

100 mM Tris-Cl (pH 6.8)

200 mM DDT

4 % SDS

0.2 % bromophenol blue

20 % Glycerol

3.1.4.5.3. Buffers for Western blot analysis.

Transfer buffer for Western blot (1 Lt)

5.82 g Tris-base

3.75 ml 10 % SDS

200 ml methanol

2.93 g Glycine

Ponceau S solution (10x) (10 ml)

0.5 g Poncue S was dissolved in 1-ml glacial acetic acid and volume was adjusted to 10 ml

Blocking solution:

5%(w/v) non fat dry milk powder

0.2 % Tween-20 in PBS

Washing solution

0.3 % Tween 20 (v/v) in PBS

PBS 10x stock solution (1 Lt) pH 7.3

80 g NaCl

2 g KCl

11.5 g $Na_2HPO_4.7H_2O$

2 g KH_2PO_4

Chromogenic visualisation solution;

NBT stock solution (stored at 4°C)

0.5 g of NBT dissolved in 10 ml of 70 % dimethylforamide.

BCIP stock solution (stored at 4°C)

0.5 g BCIP dissolved in 10 ml100 % dimethylformamide

Alkaline phosphatase buffer:

100mM Tris.Cl pH 9.5

100 mM NaCl

5mM $MgCl_2$

3.1.5. Commonly used media

3.1.5.1. Media for bacteria

LB media (1 Lt)

10 gr Trptone

5 gr Bactoyeast extract

10 gr NaCl

LB-Amp media

100 μg/ml Amphicillin in LB media

3.1.5.2 Media for yeast

YPD (rich media) (1 Lt)

10 g yeast extract

20 g peptone

20 g dextrose

20-g bactoagar was added for solid media.

SC media (1 Lt) pH: 5.6-6.0

6.7 g yeast nitrogen base (without amino acids)

20 g glucose

20 g bacto agar for solid media

3.2 Methods

3.2.1. Strains and Media

3.2.1.1. Bacterial strains and media

E.coli strain DH5α; *F /endA1 hsdR17 ($r_k^-m_k^+$) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacIZYA argF) U159 deopR (ϕ80dlacΔ9lacZ)M15)* was used for bacterial transformation assay. DH5α were grown in standard LB media. Transformants were selected and grown in LB-Amp (100 μg/ml amphicillin) media.

3.1.1.2. Yeast strains and media

S.cerevisiae strain AKY28 (*Matα, leu 2-3,112, his 3-11, 15, pho3*) which is a derivative of GRF18, was obtained from Dr. Akbar Kuchkartaev (Bilkent University, Department of Molecular Biology and Genetics, Ankara, Turkey). Matα is the mating type locus where α mating type genes, Matα1 and Matα2 are expressed (McKay and Manney 1974). leu 2-3, 112 is the non-reverting mutant allele of LEU2 that express β-isopropylmalate dehydrogenase (Hsu et al., 1982). his3-11, 15 is the non-reverting allele of the HIS3 that express imidazoleglycerol-P dehydratase (Struhl, 1982). pho3 is the mutant form of the PHO3 that encodes acid phosphatase that is involved in thiamine biosynthesis (Nosaka, 1990). AKY28 strain was grown in rich YPD media.

Transformed AKY28 cells containing p53-expressing plasmids were selected in synthetic minimal (SC) media. Specific media was prepared for the induction of

gene expression. p53 cDNA was located under the PHO5 inducible promoter. Several genes encode acid phospatase in a repressible form but the primary gene product is derived from the PHO5 gene. PHO5 gene encodes a secreted acid phosphotase whose transcription is repressed when yeasts are grown in high concentration of inorganic phosphate (Bajwa et al., 1984). It is induced up to ~ 1000 fold in response to Pi starvation (Oshima *et al.*, 1982).

The Pi of normal synthetic SC media is 11.2 mM (1500 mg/liter) is sufficent for complete repression . A Pi of 0.22 mM (20 mg/ml) is the lowest initial Pi consistent with a reasonable growth rate and yield (O' Connell *et al.*, 1992).

High Pi and low Pi medium were prepared for repression and induction of the PHO5 promoter. SC media can also be used as high Pi media.

For high Pi and low Pi medium preparation, all the ingredients of SC media were used except bacto-yeast nitrogen base, which contains vitamins, trace elements and salts. To obtain high Pi and low Pi medium vitamins, trace elements, and salts were prepared separately and filter sterilized with 0.22 μm filter (Costar).

Table 1. Ingredients of specific media

	Amount/Lt
Carbon source	
Dextrose	20 g
Nitrogen source	
Ammonium sulfate	5 g
Vitamins	
Biotin	20 μg
Calcium Pantothenate	2 mg
Folic acid	2 μg
Inositol	10 mg
Niacin	400 μg

p-Aminobenzoic acid	200 µg
Pyridoxine hydrochloride	400 µg
Riboflavin	200 µg
Thiamine hydrochloride	400 µg

Trace elements	
Boric acid	500 µg
Copper sulfate	40 µg
Potassium iodide	100 µg
Ferric chloride	200 µg
Manganese sulfate	400 µg
Sodium molybdate	200 µg
Zinc sulfate	400 µg

High Pi salt		Low Pi salt
Potassium phosphate monobasic	1500 mg	20 mg
Magnesium sulfate	500 mg	500 mg
Calcium chloride	100 mg	100 mg
Sodium chloride	100 mg	100 mg
		Potassium chloride 1500 mg

This synthetic medium is based on media described by Wickersham and is marketed as 'Yeast nitrogen base without amino acids' (Fink and Guthrie, 1991a).

Vitamins and trace elements were stored at 4° C. Salts were stored at room temperature. AKY28 is a histine auxotroph. The specific medium also consists histidine amino acid. 100x stock solution of histidine with a concentration of 50 µg/ml was prepared and sterilised using 0.22 µm filter sterilisation unit and stock solution was stored at 4 ^{0}C. During the medium preparation, ammonium sulfate, distilled water and agar added for solid medium were autoclaved together at 122°C for 30 Min. Vitamins, trace elements, glucose, salts and amino acid histidine were added separately to the autoclaved ammonium sulfate and distilled water under the hood. Final solutions named as either high Pi solution or low Pi solutions were also kept at 4° C.

3.2.2. Plasmid constructs

All the plasmid constructs were obtained from Dr. Akbar Kuchcartaev (Bilkent university, Department of Molecular Biology and Genetics, Ankara, Turkey). Two plasmid constructs were used. pL3 is the construct that was used as a control; it doesn't contain p53 cDNA, its size is 7.76 kb. pAK31 has human mutant p53-248W cDNA and its size is 9.37 kb (Kuchkartayev, A. and Özturk, M., unpublished)

pL3 is 2 μm circle based episomal plasmid that has 20-40 copy number per cell. pAK 31 was derived from pL3 by insertion of mutant p53-248W cDNA between the PHO5 promoter and terminator. Plasmids contain an ampicillin resistance gene as bacterial selectable marker and LEU2 gene as yeast selectable marker. LEU2 is one of the most commonly used yeast selectable marker that complement leu 2-3,112 double frameshift mutation . The maps of the plasmids were shown in section 4 (Results) figure 3.

3.2.3. Bacterial transformation

E. coli DH5α was transformed with plasmids pL3 and pAK31, using calcium chloride method (Sambrook et al., 1989a). To prepare the competent cells, a single colony was obtained from plate that was freshly grown o/n at 37°C. Then it was inoculated to 20 ml LB and grown until its OD_{600} value became 0.3-0.4. The culture was kept on ice for 10 min. Cells were collected by centrifugation at 4800 rpm for 8 min. The pellet was resuspended in 1 ml ice-cold 0.1 M $CaCl_2$ and 200 μl of

competent cells were transferred in to eppendorf tubes. After plasmid DNA addition, they were incubated on ice for 40 min. For heat shock, they were transferred to a 42°C circulating water bath for 60 sec. They were then transferred to ice for 1-2 min. LB was then added and the cells were incubated for 1 hr in a 37°C water bath. 100 µl was taken from eppendorf tubes and spreaded on to LB-Amp plates to select cells that were transformed with plasmids; pL3 and pAK31. They were incubated at 37°C for 18 hr in incubator until the colonies become visible.

3.2.3.1Plasmid purification

Wizard Plus midiprep DNA purification system (PROMEGA)was used for plasmids purification from pL3 and pAK31 transformed DH5α cells.

3.2.4.High efficiency transformation of *S.cerevisiae*, AKY28 with LiAc.

AKY28 was transformed with pL3 and pAK31 using highly efficient LiAc transformation. (Gietz, D. R., and Woods, R. A. 1994). Strain AKY28 was grown overnight at 30°C in a shaking rotary incubator and 20 µl of this o/n culture was added to 20 ml of YPD (1/100 dilution) then grown in a shaking incubator until its OD value at 600 nm became 0.3-0.4. OD values were detected by spectrophotometer fixed wavelength program. (Du 640 spectrophotometer, BECKMAN). Cells were harvested by centrifugation at 1500 g for 5 min. Collected cell pellets were washed in 20-ml sterile water. After second centrifugation, cells were resuspended in 1.0-ml sterile dH_2O water and transferred to eppendorf tubes and recentrifuged at top speed for 10 sec. Collected cell pellets in eppendorf tubes were resuspended in 300 µl of 100 mM LiAc. Cells were incubated in a waterbath at 30°C for 15 min. After

vortexing briefly, 50-60 µl cell suspension were distributed to eppendorf tubes and 300 µl PEG/Li solution (40 % PEG, 100 mM LiAc) were added to each tubes. Incubation of transformation eppendorf tubes in a waterbath at 30°C for 30 min was followed by heat shock at 42°C for 20 min. PEG/LiAc solution was removed by microcentrifugation at high speed for 10 sec. The cells were washed by 1.0 ml of sterile dH_2O and diluted 100 times. 100 µl samples of dilutions were spreaded on to the SC selective medium without amino acids. Plates were incubated for 3-4 days at 30°C in incubator. After transformed yeast strain were obtained, immediately their glycerol stocks were prepared. For glycerol stock preparation 1 ml of transformed yeast cells were grown in selective media (SC) until saturation or late log phase, and 1 ml 30 % glycerol were mixed with the saturated cell suspension. Stocks were stored at –80° C.

3.2.5. Analysis of purified plasmids from bacteria and from genomic yeast DNA to test the state of p53 with PCR and restriction enzymes.

p53 mutant-248W containing plasmids purified from plasmid transformed bacteria DH5α and AKY28 yeast cells that were transformed with those plasmids were analysed by PCR and restriction enzymes to confirm the state of p53.

For analysis of p53 state from pL3 (without p53 cDNA insert) and pAK31(p53 mt-248W p53 cDNA insert) transformed AKY28 cells, total yeast genomic DNA was isolated from transformed yeast cells (Ausubel et al., 1987b). For total yeast genomic DNA isolation, 2-ml culture of AKY28 cells carrying the plasmids pL3 and pAK31 were grown to stationary phase in SC media, the liquid should be visibly turbid and pellet of the cells should have accumulated at the bottom

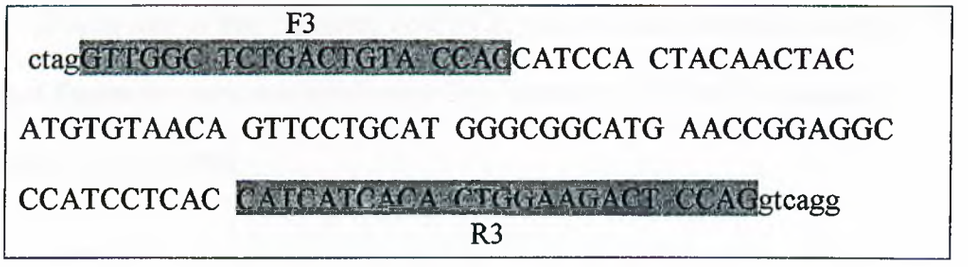
of the tube. 1.5 ml of the saturated culture were transferred in to a microcentrifuge tube and centrifuged 5 sec at room temperature. The supernatant was poured off and cells were resuspended in 200 µl breaking buffer. The crystal beads were added just below the meniscus of the cell suspension and the mixture of beads and cell suspension were mixed by vortexing at max speed for 60 sec, tubes was placed on ice for 1-2 min on ice. This step was repeated three times. Then 200-µl of 25:24:1 phenol/chloroform/isoamyl alcohol (PCI) was added and tubes were mixed by vortexing at max speed like previously done. The DNA containing mixture was centrifuged at max speed for 5 min at room temperature. The upper aqueous phase was transferred to another tube and one more 200 µl PCI was added and mixed by vigorous vortexing for further purification from proteins and solute molecules. For phase separation the tubes were centrifuged at max speed for 1 min at room temperature. The top aqueous phase containing DNA was transferred to another eppendorf tube containing 100 % ethanol that contain 1/10 vol of 3 M sodium acetate (If 200 µl DNA aqueous phase was collected, 20 µl from 3 M sodium acetate was added). Mixture of DNA aqueous phase, 100 % ethanol and sodium acetate were mixed by vortexing and kept at –20° C for at least 30 min for precipitation of DNA. After precipitation step the eppendorf tubes were centrifuged at max speed for 10 min which was followed by aspiration of 100 % ethanol, either with pipetting or pouring off. For washing step 1-ml of 70 % ethanol was added. Tubes were inverted several times and spinned in microcentrifuge tubes. Supernatant was aspirated carefully by pipetting. The pellet was dried under the lamp. After the ethanol completely removed, the pellet was dissolved in 40-µl dd H_2O. The concentration of obtained DNA was detected using the DNA/oligo quant, double strand DNA program of spectrophotometer (Du 640 spectrophotometer, BECKMAN)

To test the state of p53 at DH5α purified plasmids and plasmid transformed AKY28 yeast cells, 110 bp fragment that corresponds to exon 7 of mutant p53-248W sequence was amplified with PCR (PERKIN ELMER Gene Amp PCR system 2400).

3.2.5.1. Amplification of 110 bp fragment of exon 7 of p53 cDNA with PCR

To analyse purified plasmids and transformed AKY28, 110-bp fragment of exon 7 of the p53 cDNA sequence was amplified with PCR.

Table 2.The sequence of 110-bp fragment at exon 7 and localisation of primers on it.



Primers F3 (5' –GTTGGCTCTGACTGTACCAC-3') and R3 (5' -CTGGAGTCTTCCAGTGTGAT- 3') were used. The primers were synthesised at Department of Molecular Biology and Genetics of Bilkent university with oligosynthesizer (OLIGO 1000M BECKMAN). For 50 µl PCR solution; 5 µl 10x PCR buffer- $MgCl_2$, 1.5 mM $MgCl_2$ (25 mM stock solution), 200 µm dNTP mix (10 mM stock solution), 25 ng plasmid DNA, for amplification from yeast DNA, 100 ng total yeast DNA, 10 pmole from each primer, 1 unit Taq polymerase (5 u/µl) were used. PCR was performed by using Perkin Elmer Gene Amp PCR system 2400. PCR was programmed as; 30 cycle including; 95°C 30 sec for denaturation, 55 °C 30 sec

for annealing, 72 °C 30 sec for extension; initial cycle include one hold at 95°C for 3 min for initial denaturing and final cycle include three hold at 72° C for 10 min for final extension.

3.2.5.2. Purification of PCR products

Ingredients of PCR might effect the further assays like restriction enzyme analysis. To prevent this problem, PCR products were purified using QIA quick PCR purification kit (250).

3.2.5.3. Analysis of PCR amplified fragment in agarose/ NuSieve Agarose gel electrophoresis.

Due to size of the fragment, both PCR products and restriction enzyme digested fragments were analysed using 2% Agarose + 2 % Nu Sieve agarose dissolved in TBE buffer.

3.2.5.4. Restriction enzyme analysis of PCR amplified 110-bp p53 cDNA fragment that contains mutant p53 sequence (codon 248, R->W).

It is known that mutant p53 sequence (codon 248, R->W) can be differentiated from wild type by restriction enzyme analysis because a specific restriction enzyme site present on wild type is lost when there is a mutation at codon 248 or 249. 248th codon of the p53 corresponds to the nucleotide sequence CCGG, at mutant p53 sequence (codon 248, R->W), the nucleotide sequence change to CTGG. Recognition sequence of Msp I is CC↓GG. At mutant p53 sequence (codon 248, R->W) this site was disappeared and digestion of Msp 1 did not occur. After the presence of a mutant p53 sequence (codon 248, R->W) in pAK31 vector was

verified, this vector as well pL3 as control were transfected to AKY28 strain. AKY28 cells that were transformed with those plasmids were also analysed to test the existence of mutant p53 sequence (codon 248, R->W) with restriction enzyme analysis. Conditions for Msp 1 digestion was as following; for 25 µl total volume, 2.5 µl from 10x assay buffer #2, 8 µl from PCR product (amount that was loaded to agarose gel) 1 µl of 25 u/µl Msp 1 restriction enzyme, and 13.5 µl dd H_2O.

Beside Msp 1, Hae III enzyme was used. Hae III recognises the sequence GG↓CC that corresponds to the 249th amino acid of p53. The mutant p53-249(W) is one of the commonly detected p53 mutations in human cancers (Bressac et al., 1991). In the case of mutant p53 sequence (codon 249, R->S) mutation, that correspond to wild type p53 sequence, GGCC change to GTCC that loses the Hae III recognition site. Mutant p53 sequence (codon 248, R->W) that we used in this experimental system, conserves this sequence so beside wild type p53, Hae III is also able to digest mutant p53 sequence (codon 248, R->W). Conditions of Hae III digest for 25 µl total volume include; 2.5 µl of 10x SuRe cut buffer M, 13.5 ml of dd H_2O, 8 µl from purified PCR product, and 1µl of 10 u/µl Hae III restriction enzyme.

During the experiments pC53-SN (wild type p53 sequence insert) and pCMVp53-249 (mutant p53 sequence (codon 249, R->S) insert) were used as control plasmids. 110-bp fragment was also amplified from those pladmids with PCR and digested with Msp I and Hae III.

3.2.6. Selection and testing for p53 expression

p53 expression in yeast was analysed using Western blot analysis. For Western blot analysis, cells were lysed and proteins were extracted under denaturing conditions.

3.2.6.1. Cell lysis and protein extraction under denaturing conditions

For cell lysis and protein extraction (Louise et al., 1994); 15 ml of cells were precipitated and the pellet was washed with ice cold sterile water. Cells were resuspended in 150 µl Laemni lysis buffer. Crystal beads were added to the level of the meniscus and mixture of bead and cells were vortexed at max speed for 60 sec, tubes were placed on ice for 1-2 min on ice. This step was repeated five times or more, until nearly 90 % of the cells lysed. Lysis of cells can be examined under the light microscope with 10x magnification. By centrifugation, supernatant that contains crude cell extract was collected in to another tube. To obtain more cell lysate and to wash the beads, one more 150 µl Laemni lysis buffer was added and the steps were repeated. Collected supernatant was pooled in to eppendorf tubes (Loison, 1994b).

3.2.6.2. Determination of protein concentration

Due to interference of lysis buffer with Bio-Rad assay kit, protein concentrations were determined using the following formula (Marshak et al., 1996);

$$\text{Protein concentration (mg/ml)} = [1.55\ A_{280} - 0.76\ A_{260}] \times DF$$

DF: Dilution factor

3.2.6.3. Analysis of proteins in crude cell lysates by western blot

Western blot analysis was applied after solubilization of proteins and their separation by SDS-PAGE (Sambrook et al.,1989b)

Between the size range 14-66 kDa, optimal percent of acrylamide gel is 8-10%. For 53-kDa p53 protein 10% resolving gel was preferred and as stacking gel 5% polyacrylamide gel was used.

SDS-page was runned at 80 V for 12-18 hr and as running buffer Tris-Glycine electrophoresis buffer was used.

For Western blot analysis, proteins on the SDS-PAGE were electrophoretically transferred in a semi-dry electrophoretic transfer cell. Before blotting, resolving gel and PVDF were equilibrated at room temperature for 15-30 min in transfer buffer.

The semi dry system was placed as follows; two 3MM whatman papers prewetted in transfer buffer were inserted at the bottom which was cathode part of the semi-dry unit and PVDF paper was placed on them, gel is placed on top of the membrane, the transfer stack was completed by putting two 3MM whatman paper on it. Air bubbles were prevented between the gel, membrane and 3MM whatman papers by gentle rolling with a test tube. The cell were runned for 1 hr at 12 V. After the transfer was complete, PVDF paper was removed and washed with Ponceau S solution for 5 min at room temperature. Ponceau S staining was followed by destaining with water to mark the sites of molecular weight standards. For quantification of immobilised proteins probed with specific antibodies, PVDF paper was immersed in blocking solution for 30 min to 1 hr at room temperature with constant agitation to fill all protein binding sites. For probing of p53 proteins with

specific antibody, blocking solution was replaced with 1000x diluted primary antibody HR231 (ascites fluid) (a gift from T. Soussi, Institude Curie, Paris, France) in blocking solution and was incubated at room temperature for at least 1 hr with constant agitation. For additional studies the following anti-p53 monoclonal antibodies against different epitopes of the p53 protein were used; 7F4, 9E4, pAb240, H53C1, H53C2, H53C3, and pAb122 (ATCC). Hybridoma supernatants were used at a 1/10 dilution and ascites fluids were used at a 1/1000 dilution. The blot was washed with four changes of washing solution for 5 min each. Then anti mouse alkaline phosphatase antibody conjugate was added which was (SIGMA) diluted in blocking solution (1/35000 dilution ratio). This step followed by 1 hr incubation at room temperature with constant agitation. After following washing step that included four changes of washing solution for 5min each, the PVDF membrane was placed in 5 ml alkaline phosphatase buffer containing BCIP/NBT (33 µl NBT from NBT stock solution, 17 µl BCIP from BCIP stock solution) for visualisation of the p53 protein (Harlow and Lane, 1988).

3.2.7. Application of DNA damaging agents

3.2.7.1. UV-C treatment

UVC (254 nm) irradiation was carried out using UV crosslinker (stratalinker, STRATAGENE). For UV irradiation of cells; 10 ml liquid medium like YPD, SC or specific high Pi or low Pi medium was inoculated with a freshly subcloned sample of the yeast strain to give approximately 1x 10^5 yeast cells/ml that corresponds to 1/100 dilution. The culture was incubated until it's OD value at 600 nm became 0.5 (nearly mid-log phase which is the phase most sensitive to DNA damage due to high cell

division rate). When absorbance of cell suspension reaches to 0.5 at OD 600 nm, there are about 1.5x 10^7 cells/ml. The cells were washed in sterile water and they were irradiated either on plates or in liquid suspension, according to the assay type. For cell survival assay, irradiation was exposed on solid media after serial dilutions of cells were applied to the surface of agar media in petri plates and for p53 protein level analysis upon DNA damage, irradiation in cell suspension was preferred (Ausubel et al., 1987a; Fink and Gutrie, 1991b). The optimal dose of UV was selected according to survival assay of yeast cells and this dose was applied for further protein assays.

3.2.7.2. Cisplatin treatment

As a second DNA damaging agent, cisplatin was used. Cisplatin as DNA damaging agent in *S.cerevisiae* was used at previous studies (McA' Nulty et al., 1996a, McA' Nulty et al., 1996b). The protocol of cisplatin treatment was derived from these studies.

Experimental procedure including cell growth, collection of cells and washing with water followed by UV-C treatment was nearly same for cisplatin treatment. Only the exception is, the cells were resuspended in cisplatin containing specific liquid medium for p53 protein response upon DNA damage which was detected by Western blot and, for cell survival assay, cells were grown in cisplatin containing plates.

100 mM cisplatin stock solution was prepared, by dissolving cisplatin (MW 300 g/mole) in dH_2O. Small amount of aliquots was prepared for daily use and they were kept at 4°C. The optimal cisplatin concentration was found according to cell survival assays and this dose was applied for further protein assays.

3.2.7.3. Cell survival assay

After cells were grown in specific media (either high Pi salt containing or low Pi containing media) up to the their OD_{600} value became nearly 0.5, cells were pelleted and resuspended in water to a density of 2×10^7 cells/ml. Tenfold serial dilutions of each pL3 transformed and mutant p53-248W transformed AKY28 yeast cells were prepared and 10-µl aliquots of the undiluted and diluted samples were spotted on plates.

For UV treatment after spotted colonies were incubated on plates for a time period (incubation time before UVC treatment optimised), the plates were exposed and unexposed as a control to the different doses of UVC light using a UV crosslinker that change between 50 j/m^2-150j/m^2. The controls were exposed to UV light at all. Plates were photographed after 2 days and 3 days of growth at 30°C incubator.

For cisplatin treatment, 10-µl aliquot of the undiluted and diluted samples were spotted on plates containing different concentrations of cisplatin, ranging from 0.5 to5 mM.

4. Results

Our aim was to establish an experimental model to study wild type and mutant human p53 protein effects in yeast cells. As yeast cells do not produce p53 protein, we based our experimental system on yeast cells stably expressing human p53 proteins. We decided to use mutant form of p53-248W for the initial step of this long-term project. 248(R->W) is one of the most frequently altered residue in the p53 protein, result in defective contacts with the DNA and loss of the ability of p53 to act as a transcription factor (Cho et al., 1994). Yeast expression vectors allowing regulated expression of p53 were constructed prior to our work. These vectors contain human p53 coding sequence under the control of Pi regulated PHO5 promoter. In this work we used pAK31 plasmid that express human mutant p53-248W. As a control pL3 with no insert was used. We decided not to use a wild type p53 expressing vector during the establishment of this experimental system because of the reported growth inhibitory activity of p53 protein in yeast cells (Bischoff et al., 1992; Nigro et al., 1992).

As a first step, we verified the presence of a mutant p53 sequence (codon 248) in pAK31 vector. Then this vector as well as pL3 control were transfected in to AKY28 strain yeast cells and stable colonies were selected in the Leu^- medium. These cells were first tested for the expression of mutant p53-248W protein.

After demonstrating Pi regulated expression of mutant p53-248W protein, we then compared the growth rate of cells transfected with pAK31 and pL3. Next, different cell lines (expressing and non-expressing mutant p53-248W) were tested for UV-C and cisplatin induced DNA damage and cell survival. p53 protein levels in UVC and cisplatin-treated cells.

Human mutant p53 (codon 248, R->W) cDNA sequence together with sequences including nucleotides from –17 to +12 (relative to tranlation start site and stop codon respectively) between the Pi repressible PHO5 promoter and terminator was subcloned in to the Sal I and Hind III site of the pL3 plasmid for the construction of pAK31 plasmid (Fig 3) (Kuchkartaev, A.and Özturk, M.unpublished)

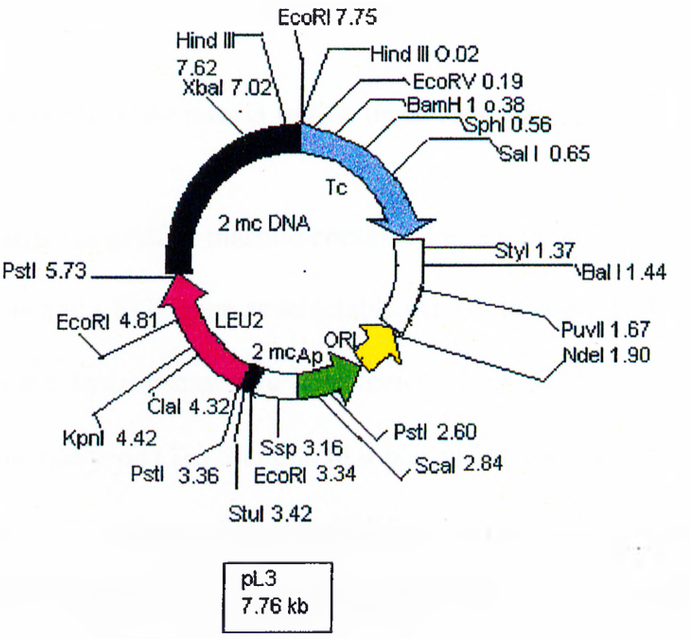


Fig. 3. Map of the control plasmid pL3

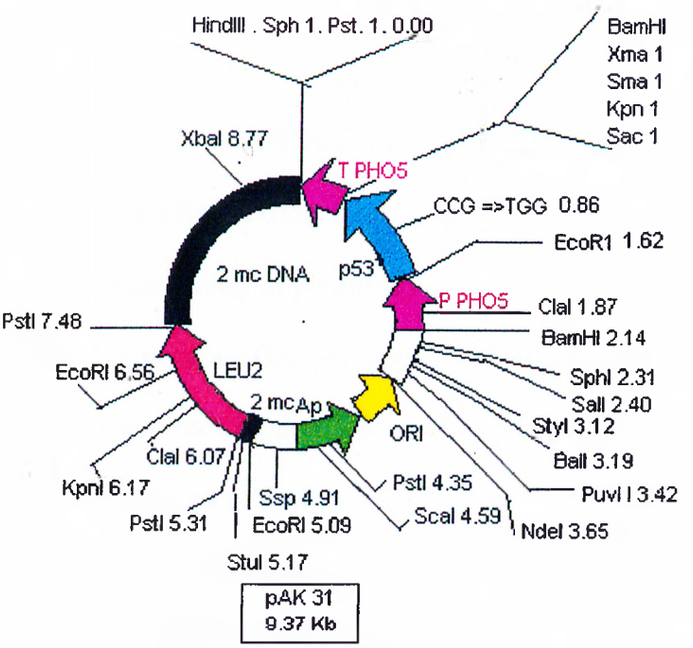


Fig.3. (Cont'd). Map of the plasmid pAK 31 (mutant p53-248W cDNA)

The constructed pAK31 plasmid contains ampicillin^r gene as selectable marker in bacteria and LEU2 gene as selectable marker in yeast. LEU2 gene encodes β-isopropylmalate dehydrogenase that complements Leu 2-3, 112 mutation. The tetracycline resistance gene (Tc) found in pL3 plasmid is not found pAK31 due to insertion of mutant p53 sequence between PHO5 promoter and terminator. Transcription from the PHO5 promoter is repressed when cells are grown in high Pi medium and depressed in low Pi medium.

4.1. Verification of mutant p53 sequence (codon 248) in pAK31 vector and pAK31 transfected AKY28 yeast cells.

Plasmids pL3 (without p53 cDNA insert) and pAK31 (mutant p53-248W cDNA insert) were purified from DH5α transformed cells using Wizard midi prep purification kit. The concentration of plasmids were determined using the DNA/oligo quant single strand DNA program (Du 640 Spectrophotometer BECKMAN). For analysis of mutant p53 sequence (codon 248), a 110-bp fragment of p53 exon 7 that contains 248 mutant site was amplified with PCR. After PCR amplification the PCR products were purified using QIA quick PCR purification kit. Purified PCR products were analysed in 2 % agarose/ 2% Nusive agarose gel (Fig. 4)

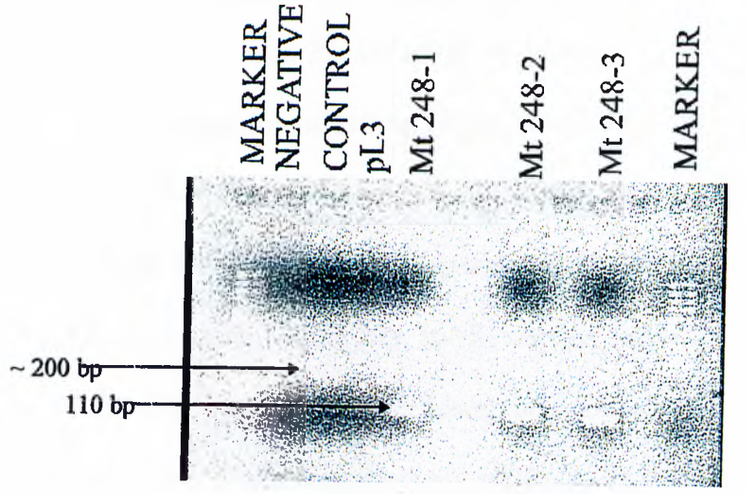


Fig. 4. Results of PCR amplification of 110-bp fragment obtained from pAK 31 plasmid. 110 bp DNA marker was used. Negative indicates the PCR reaction without DNA, Control is the PCR amplified 200 bp fragment, Mt-248-1,2,3 indicates pAK 31 isolated from three colonies of transformed DH5α bacteria cells.

The amplified 110-bp fragment obtained from mutant p53 sequence (248 codon, R->W) was digested with restriction enzymes. The nucleotide sequence at 248 th codon of wild type p53 corresponds to CCGG which is the sequence

recognised by the restriction enzyme Msp 1 (C ↓CGG). In the mutant p53 sequence (codon 248, R->W), the second nucleotide is replaced by a T nucleotide that destroys Msp 1 recognition site. Thus Msp 1 is able to digest the wild type p53 c DNA but unable to digest the mutant p53 sequence (248 codon, R->W). In addition to Msp 1, Hae III was also used for digest analysis. Hae III recognises the sequence GGCC. The mutant p53 sequence (codon 249, R->S) is one of the commonly detected p53 mutations in human tumours (Bressac et al., 1991). In the case of this mutation GGCC changes to GTCC. The recognition sequence of Hae III enzyme (GG↓CC) is destroyed in the mutant p53 sequence (codon 249,R->S). Hae III digests both the wild type p53 sequence and the mutant p53 sequence (codon 248, R->W) that conserves recognition sequence of the enzyme. Msp 1 digestion of 110-bp fragment amplified from wild type p53 sequence yields 68-bp and 42-bp fragments and Hae III enzyme digestion yields 75- and 35-bp fragments.

Msp 1 digestion of 110-bp fragment amplified from the mutant p53 sequence (248 codon, R->W) plasmid, pAK31, yielded an undigested 110-bp fragment. Hae III digestion yielded 75- and 35-bp fragments. As control wild type p53 sequence and mutant p53 sequence (codon 249, R->S) were amplified from control plasmids pC53-SN (wild type p53 sequence insert) and pCMVp53-249 (mutant p53 sequence (codon 249, R->S) insert) respectively were also digested with Msp 1 and Hae III (Fig.5).

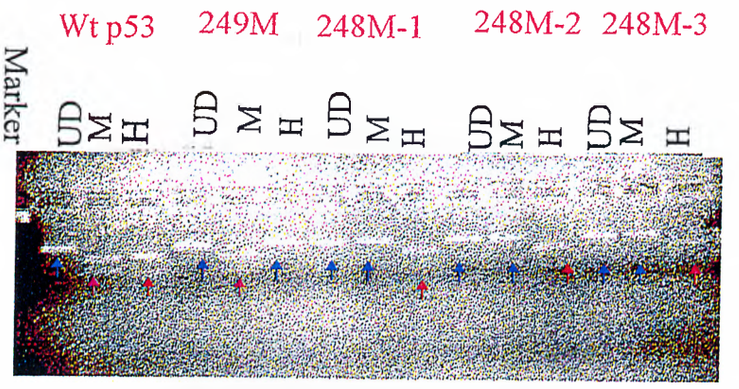


Fig. 5. Msp 1 and Hae III digestion of 110-bp fragment amplified from mutant p53 sequence (codon 248, R->W) in pAK 31vector. As control pL3, 249M, and Wt p53 were used. Wt p53 and M249 were amplified from wild type p53 and mutant p53 sequence (codon 249, R->S) containing plasmids pC53-SN and pCMVp53-249 respectively. UD represents undigested, 110 bp fragment. Blue arrow indicates undigested 110 bp fragment, pink arrows indicate Msp I digestion products 68 bp and 42 bp fragments, red arrow indicates Hae III digestion products 75 bp and 35 bp fragments.

pAK 31 yeast expression vector containing the mutant human p53-248W and control vector pL3 were used for the transformation of AKY28 (Matα, Leu 2-3,112, pho3) yeast strain. High efficiency LiAc transformation protocol was performed (Johnston, 1994). After transformation with the LEU2 containing plasmids, pL3 (without p53 insert) and pAK31 (mutant p53 sequence (codon 248, R->W) insert), AKY28 cells were grown on Leucine deficient SC medium. The transformed colonies were obtained after 3-day incubation at 30 °C. Three colonies were selected from transformed AKY28 for restriction enzyme analysis. Total yeast DNA was isolated using the rapid DNA isolation protocol (Sambrook *et al.*,1989b) to analyse the p53 state in the transformed AKY28 cells. The 110 -bp p53 sequence was amplified from the total yeast DNA using the same primers and PCR conditions. PCR products were analysed in 2% agarose/ 2% NuSieve agarose gel. As marker, ϕx174/ Hinf III was used (Fig.6).

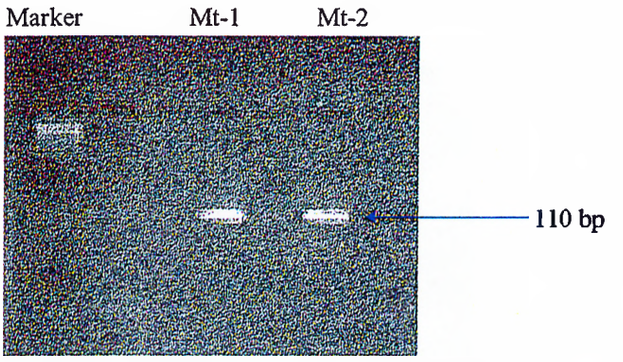


Fig. 6. Results of PCR amplification of 110 bp sequence from exon 7 of p53 cDNA obtained from mutant p53 expressing yeast cells. pL3 without insert was used as a control. Two colonies of pAK31 transformed AKY28 cells were selected for PCR amplification. mt-1 and mt-2 indicate 110 bp that was amplified from total DNA of those colonies.

After the110 bp fragment was amplified, it was digested with Msp 1 and Hae III restriction enzymes. The results of digestion were examined at 2 % agarose and 2 % NuSuive gel. As a marker, ϕx174/ Hinf I was used. As a control, Msp I and Hae III digested wt p53 cDNA sequence and mutant p53 sequence (249 codon, R->S) were amplified from control plasmids pC53-SN (wild type p53 sequence insert) and pCMVp53-249 (mutant p53 sequence (codon 249, R->S) insert). Following Msp 1 digestion of mutant p53 sequence (codon 248, R->W), an undigested 110 bp fragment was obtained. Hae III digestion of mutant p53 sequence (codon 248, R->W) yielded 75- and 35-bp fragments like the Hae III digested wild type p53 sequence (Fig.7).

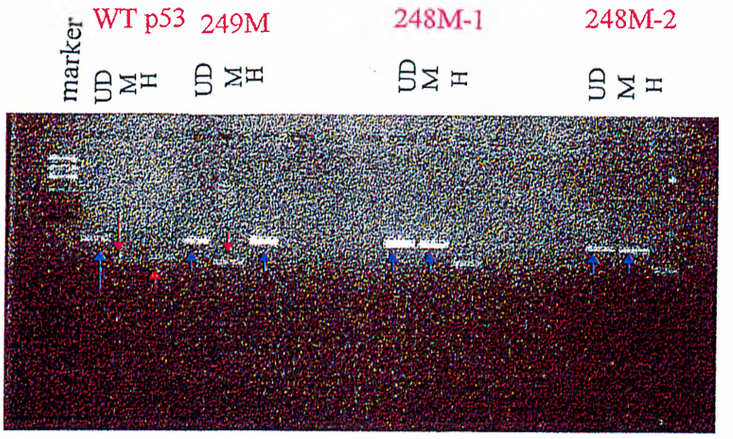


Fig 7. Msp 1and Hae III digestion of 110-bp fragment obtained from mutant p53 expressing yeast cells. 248M-1 and 248M-2 were digestion products of amplified mt-1 and mt-2. Wt 53 and 249M 110 bp DNA fragments were obtained from control plasmids pC53-SN (wild type p53 sequence insert) and pCMVp53-249 (mutant p53 sequence (codon 249, R-> S) insert) respectively. UD indicates undigested 110 bp fragment. Blue arrow indicates undigested 110 bp fragment, pink arrow indicates Msp I digestion product 68 bp and red arrow indicates Hae III digestion product 75 bp.

4.2. Western blot analysis of mutant p53-248W protein expression in AKY28 cells

Mutant p53-248W protein expression in AKY28 strain yeast cells was shown by Western blot analysis. Initially pL3 and pAK31 transformed cells were grown in high Pi (for PHO5 repression) and low Pi medium (for PHO5 induction) to stationary phase (~ 30 hr incubation at 30 °C) until the medium became visibly turbid and a pellet of cells accumulated at the bottom of the tube. Total protein was extracted under denaturing conditions from each cell suspension. The concentration (mg/ml) of proteins was calculated using the formula: 1.55 A_{280}- 0.76 A_{260}. For SDS/PAGE 150-µg protein was applied per well. The proteins were separated on an SDS-polyacrylamide gel, transferred to PVDF menbrane (Millipore), and then reacted with antibodies against different epitopes of p53 (Table 3) (Fig. 8)

Name of the Antibody	Human epitope location	References
HR231	C terminus	Bartek et al., 1993; Legros et al., 1993.
7F4	central	Yolcu. E., Yurdusev, N. and Öztürk, M. (unpublished)
9E4	central	Yolcu, E., Yurdusev, N. and Öztürk, M. (unpublished)
H53C1	central	Vaultzel, T. and Öztürk, M. (unpublished)
H53C2	central	Vaultzel, T. and Öztürk, M. (unpublished)
H53C3	central	Yolcu, E., Yurdusev, N. and Öztürk, M. (unpublished)
pAb 240	central	Gannon et al., 1990, Stephan et al., 1992
pAb122	C-terminus	Gurney et al., 1980; Wade, A. and Jenkins, J. R., 1985.

Table. 3. Murine monoclonal anti-p53 antibodies against different epitopes of p53.

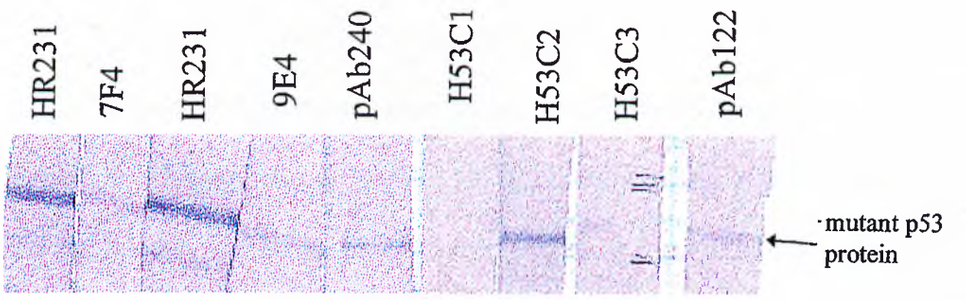


Fig. 8. Demonstration of p53-248W protein expression in yeast using different anti-p53 monoclonal antibodies.

Best results were obtained with HR231 and it was routinely used for Western blot analysis.

Mutant p53-248W was detected in yeast cells transfected with pAK31, but not pL3. p53 was detected cells grown in both high Pi and low Pi media. Although protein levels were higher cells in low Pi medium compared the high Pi medium, the expression was leaky under high phosphate condition, suggesting that PHO5 promoter was not totally suppressed in high Pi medium. (Fig.9)

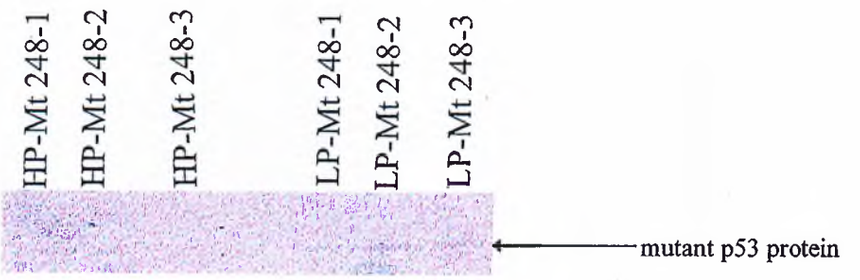


Fig .9. The results of western blot analysis, mutant p53-248W protein expressed in low Pi and high Pi medium. Mt-248-1,2,3 represents the mutant p53-248W protein that was purified from three AKY28 colonies that were transfected with pAK31.

PHO5 promoter induction is regulated by Pi concentration. For maximum induction of the PHO5 promoter, cells have to reach the post-exponential phase for Pi starvation (Cartwright *et al.*, 1994). The expression of p53 in yeast cells grown in high Pi and low Pi containing medium using different incubation times 24 hr, 48 hr, and 72 hr. We also measured Western blot analysis of p53 protein at different incubation times. The results were indicated that 24-hr incubation was sufficient for PHO5 promoter induction and subsequent p53 expression (Fig.10).

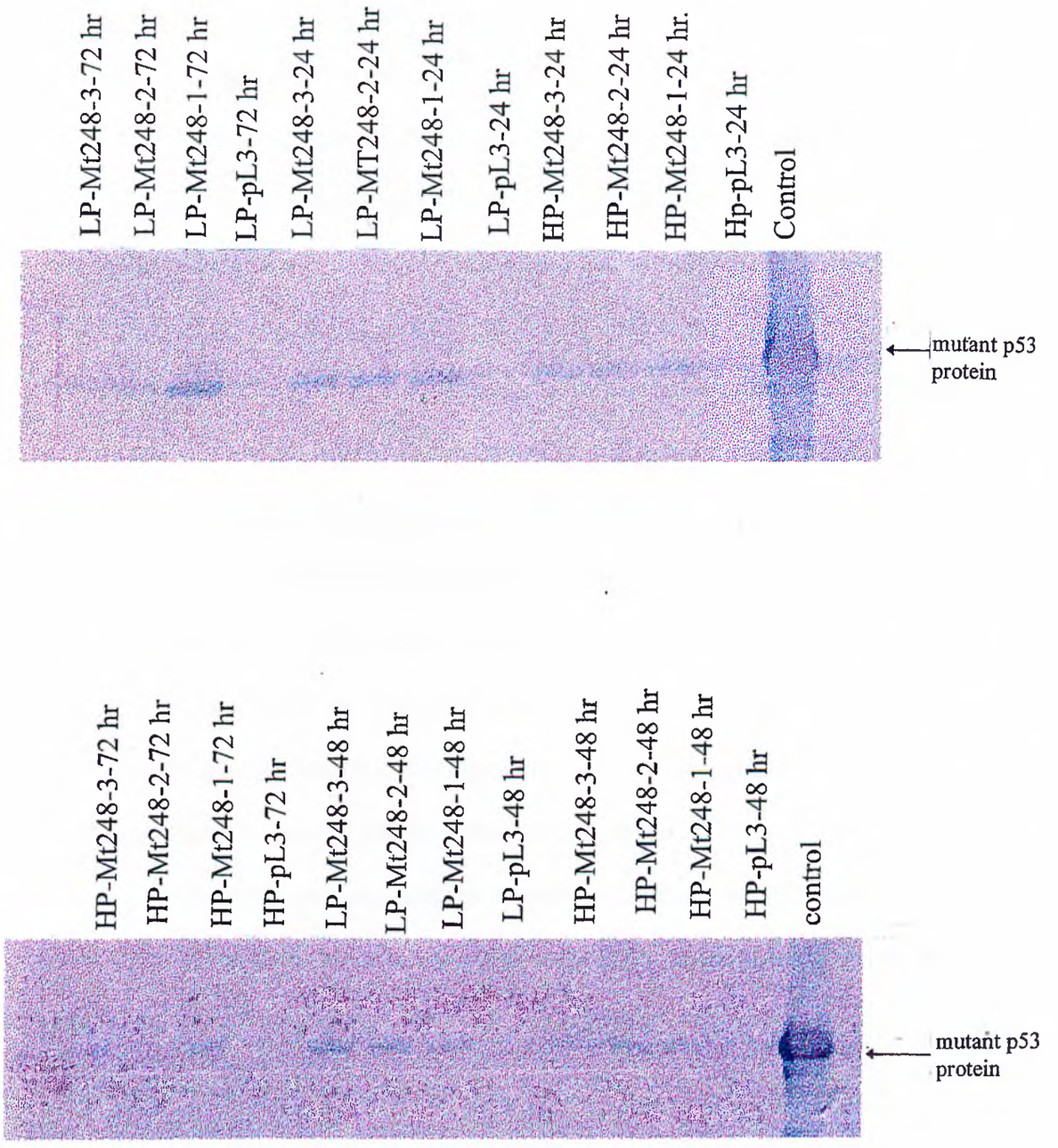


Fig.10. Analysis of p53 expression at 24th, 48th, and 72th hr incubation in both high Pi and Low Pi medium. Mt248-1, 2, 3 indicate mutant p53-248W extracted from three different colonies of pAK31 transformed AKY28 cells.

4.3. Effect of mutant p53-248W protein expression on growth rate of the yeast cells

After mutant p53 expression was shown in high Pi and low Pi medium, the effect of mutant p53 on yeast cell survival was examined. The growth-inhibitory effect of p53 has been examined previously. It was reported that colony growth differed among the cells expressing wild type p53, mutant p53, and no p53 (Bischoff etal., 1992). After transformation of yeast cells, no difference was detected in the colony size and growth rate of the cells expressing and control cells (Fig.11). An experimental system was designed to study mutant p53 growth effects in yeast. This system was also used for further studies including p53 growth effects upon DNA damaging agents in yeast. For p53 growth effect assay the pL3 and pAK31 containing yeast cells were grown at high Pi and low Pi medium until their OD value at 600 nm reach 0.5. The cells at this density were pelleted and resuspended in water to a density of $2x10^7$ cells/ ml. Tenfold dilutions of each were prepared and 10-µl aliquot of the undiluted and diluted samples were spotted on plates containing high Pi and low Pi agar medium. Then the plates were incubated for 3 days at 30° C. This assay was performed in one petri plate to examine p53 effects on cell growth clearly. According to this analysis no difference was detected in growth phenotypes between the yeast cells expressing mutant p53 protein and control cells.

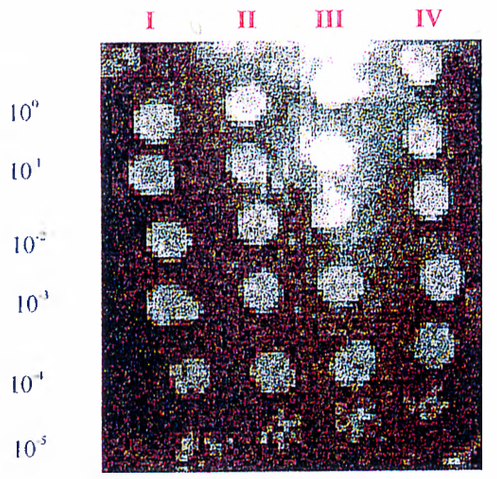


Fig.11. Effect of mutant-p53 expression on growth rate of yeast cells. I indicates non- mutant p53 expressing yeast cells, II, III, IV indicate mutant p53 expressing yeast cells.

4. 4. Establishment of an experimental model to evaluate the role of p53 in yeast after DNA damage.

For cell survival assay after DNA damage, pL3 and pAK31 containing yeast cells were grown at high Pi and low Pi medium until their OD value at 600 nm reach 0.5. Tenfold dilutions of each were prepared and 10-μ l aliquots of the undiluted and diluted samples were spotted on plates containing high Pi and low Pi agar medium. Before UVC treatment the plates were incubated for 24 hr at high Pi and Low Pi medium to allow expression of the mutant p53 protein. Then the cells were exposed to different doses of UVC light in the range between 50 j/m^2 and 150 j/m^2. After UV exposure, plates were incubated for 2 more days at incubator at 30°C and then they were photographed. The results of UV survival data showed no decrement of survival in any of these cell lines after 24-hr incubation when high doses were

applied. Following observation, cells were incubated for18 hr instead of 24 hr at 30°C before UV exposure (Fig.12).

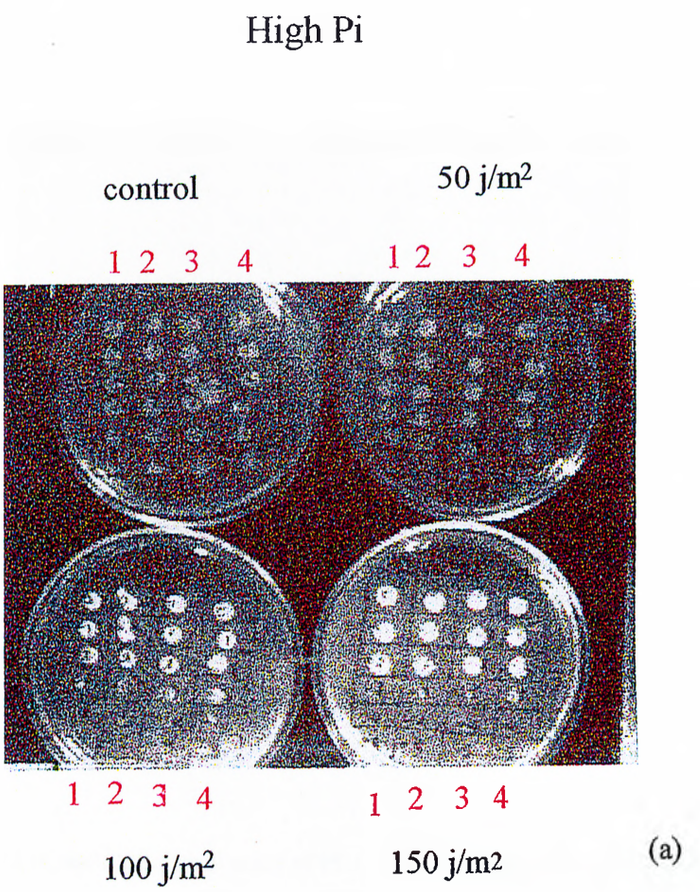


Fig.12. Survival assay of PL3, pAK31 transformed AKY28 yeast cells.
(a) AKY28 yeast cells transformed with PL3, pAK31 that grown on the high Pi medium **(b)** AKY28 yeast cells transformed with PL3, pAK31 that grown on the low Pi medium. UVC was applied after 18 hr incubation at 30°C. I indicates pL3 transformed yeast cells. II, III and IV indicate three colonies of pAK31 transformed AKY28 yeast cells.

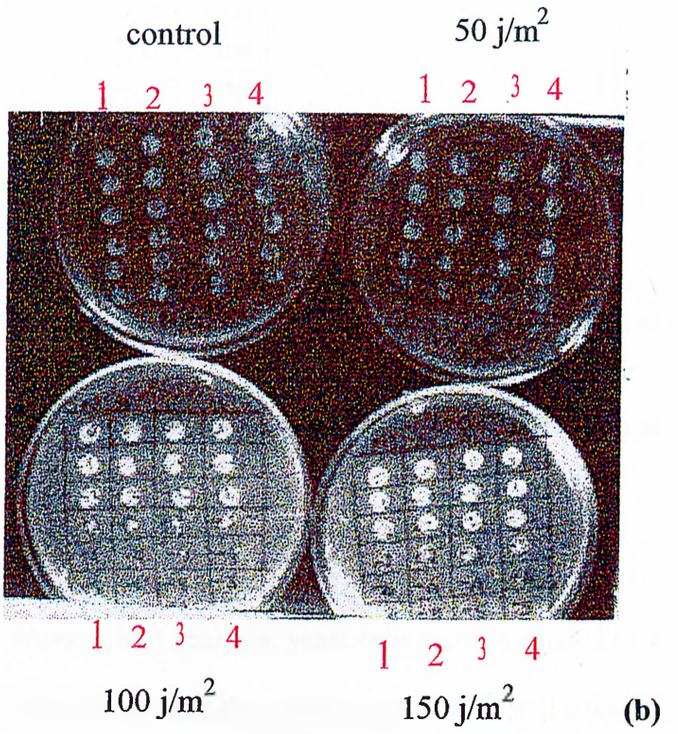


Fig. 12 (Cont'd). Survival assay of PL3, pAK31 transformed AKY28 yeast cells.

Yeast cell survival analysis indicated that 18 hr incubation prior to UVC exposure was sufficient to observe the killing effect of radiation. Survival curve of mutant p53-248W expressing yeast cells after UVC radiation are shown in Fig.13. The survival assay of mutant p53 expressing (pAK31 transformed) and non-expressing (pL3 plasmid transformed) AKY28 cells that were grown in both high Pi and low Pi medium did not exhibit any detectable difference so that survival curves

of mutant p53 expressing and non-expressing yeast cells were same. Both cells showed the same sensitivity to UVC-radiation. Also no growth difference was detected among the mutant p53 expressing and non-expressing cells that were not exposed to UVC radiation.

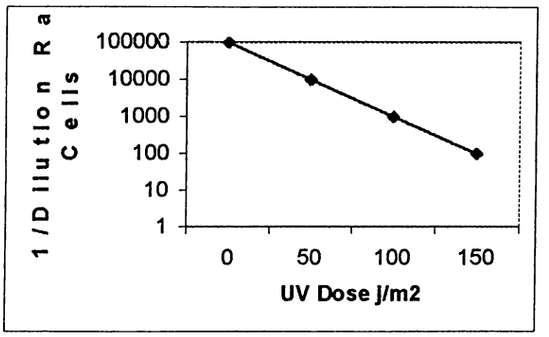


Fig.13. Survival curve of mutant p53 expressing yeast cells in low Pi medium.

4.5. Western blot analysis of mutant p53 expression after UVC radiation of yeast cells.

For Western blot analysis, yeast cells were irradiated in suspension rather than on solid agar. Depending on cell concentration, cell suspensions significantly absorb and scatter UV. Suspensions therefore need to be exposed to higher UV doses then cells on plates. For this reason, 100 J/m^2 UV was applied to the yeast cell suspension in 10-ml water in a plate. At various time points (4 h, 8 h, and 24 h) following irradiation, cells were examined for p53 expression. Immunprecipitation of mutant p53 protein with anti-p53-monoclonal antibody, HR231 did not show any difference in the p53 protein levels at those post-radiation time points (Fig.14).

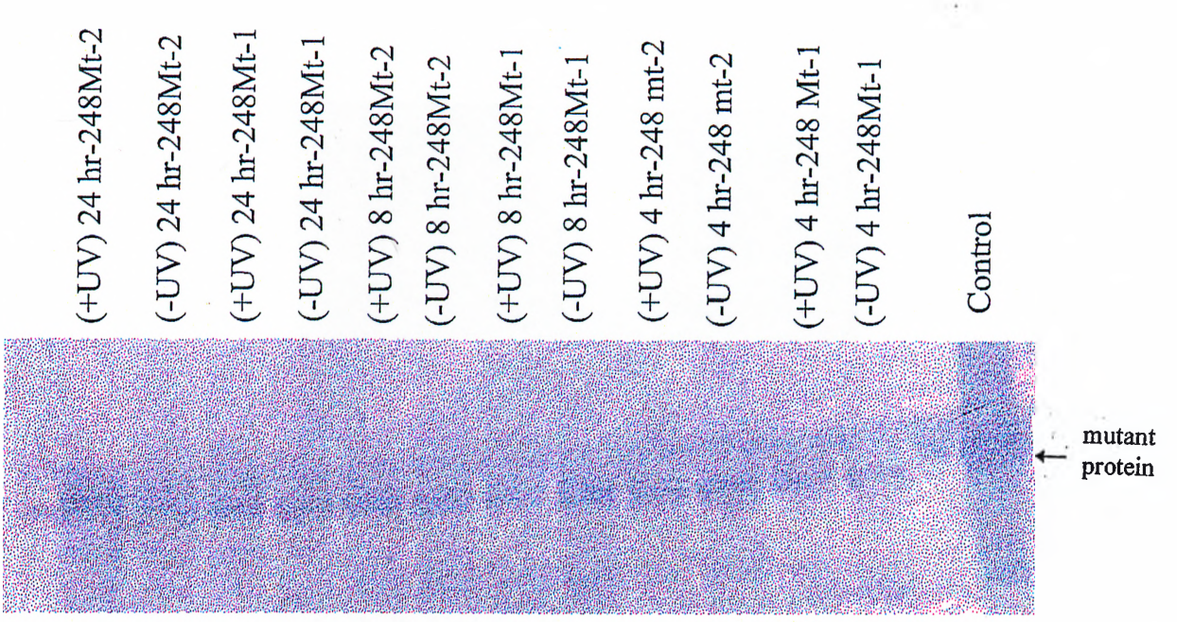


Fig.14. Western blot analysis of mutant p53 protein at various time points (4 h, 8 h, and 24 h) following UV-radiation. Yeast cells were grown in high Pi medium. 248 Mt-1, 2, and 3 indicate mutant protein obtained from three colonies of pAK31 transformed yeast cell

4.6. Effect of mutant p53 on yeast survival upon another DNA-damaging agent cisplatin treatment.

The effect of mutant p53 on yeast cell survival was analysed with another DNA damaging agent, cisplatin. For cisplatin treatment, cells were grown in low Pi medium to saturation. Tenfold serial dilutions were prepared and 10 µl aliquots of the diluted and undiluted samples were spotted on plates containing 0.5 mM, 1 mM, 3 mM, and 5 mM cisplatin dissolved in low Pi agar medium. Plates were incubated for 3 days at 30°C and then the plates were photographed using image analyser under the visible light. The survival effect of different cisplatin concentrations was analysed at the end of third day of incubation. The killing effect of cisplatin was

detected at 0.5 mM. At 3mM and 5 mM cisplatin concentrations, the killing effect was highly inreased (Fig. 15).

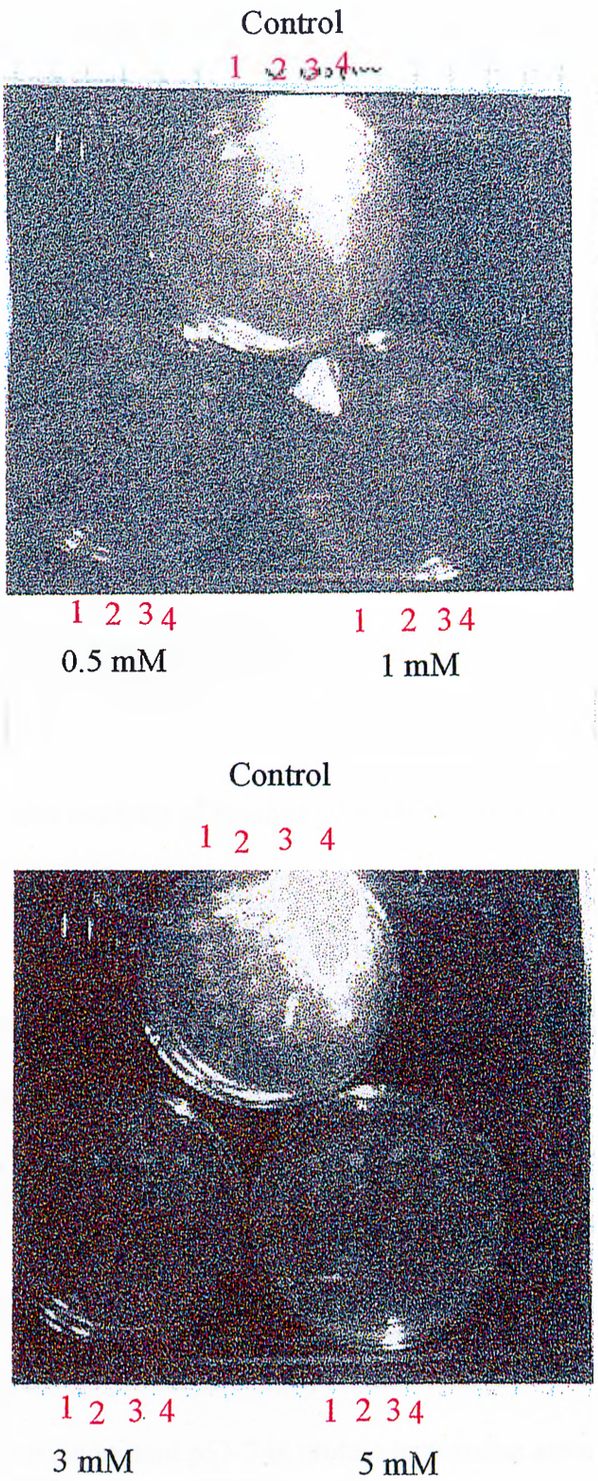


Fig. 15. Survival assays of mutant p53 expressing yeast cells upon different concentration of cisplatin, 0.5 mM, 1 mM, 3 mM, and 5mM in high Pi and low Pi medium. 1 indicates pL3 transformed yeast cells. 2, 3 and 4 indicate three different colonies of AKY28 cells that were transformed with pAK31.

According to survival curve of mutant p53-248W protein expressing yeast cells to different cisplatin concentrations (Fig.16), no difference was detected between mutant p53 expressing and control yeast cells. Both of them have the same survival curve.

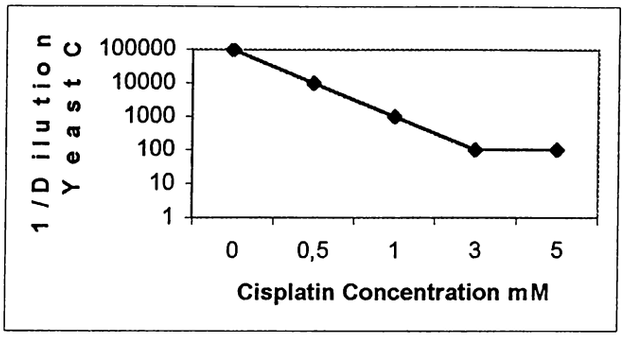


Fig.16. Survival curve for the mutant p53 expressing yeast cells. Cisplatin concentration in low Pi medium ranged between 0 mM to 5 mM intervals.

4.7. Western blot analysis of mutant p53-248W expression in yeast cells that were grown in different concentrations of cisplatin containing medium.

After testing the effect of mutant p53 on yeast cell survival after exposure to DNA damaging agent cisplatin, the mutant p53 protein levels were analysed. AKY28 yeast cells were grown in different cisplatin concentration (0.5 mM, 1 mM, 2.5 mM, 5 mM, and10 mM) containing low Pi medium for 24 h. The cells were lysed and total protein was extracted under denaturing conditions using Laemni lysis buffer. For Western blot analysis the primary antibody HR231 was used. No significant differences were detected in mutant p53-248 protein expression among the cells that were grown in different cisplatin concentration. More importantly, there was no

increase in mutant p53 protein level following cisplatin induced DNA damage (Fig.17).

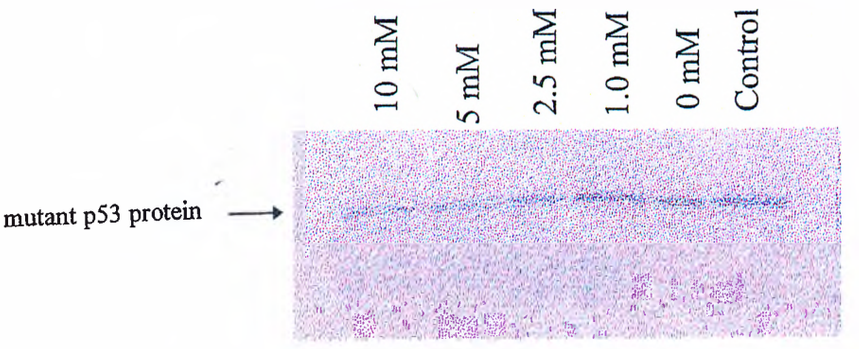


Fig.17. Western blot analysis of mutant p53 expression among the cells that were grown in different cisplatin concentration.

4. 8. Western blot analysis of mutant p53 expression upon UVC and cisplatin treatment among the AKY28 cells that were grown in high Pi medium.

Mutant p53 expression in yeast cells exposed to different doses of UV-radiation and cisplatin concentrations in low Pi medium was also analysed. Previous mutant p53 expression analysis indicates that constructed PHO5 promoter is a leaky promoter which is not completely suppressed in high Pi medium. Although mutant protein was also expressed under high Pi conditions, a small difference was detected compared to mutant p53 expression in low Pi medium. Considering this difference, mutant p53 expression was also analysed in high Pi medium upon UV-radiation and cisplatin treatment.

For Western blot analysis of mutant p53 expression after UVC and cisplatin treatment, the cells were grown in high Pi medium to the saturation and 10 ml of

those saturated cells were exposed to 100 j/m^2 UVC and 5 mM cisplatin. Cells were incubated at 30°C for 24 hr. After incubation the cells were lysed with Laemni lysis loading buffer. The proteins were fractionated by electrophoresis in a 10 % SDS polyacryamide gel and electroblotted to PVDF paper. p53 proteins probed with anti-p53 antibody HR231 and binding was visualised using colorimetric detection assay (Fig18).

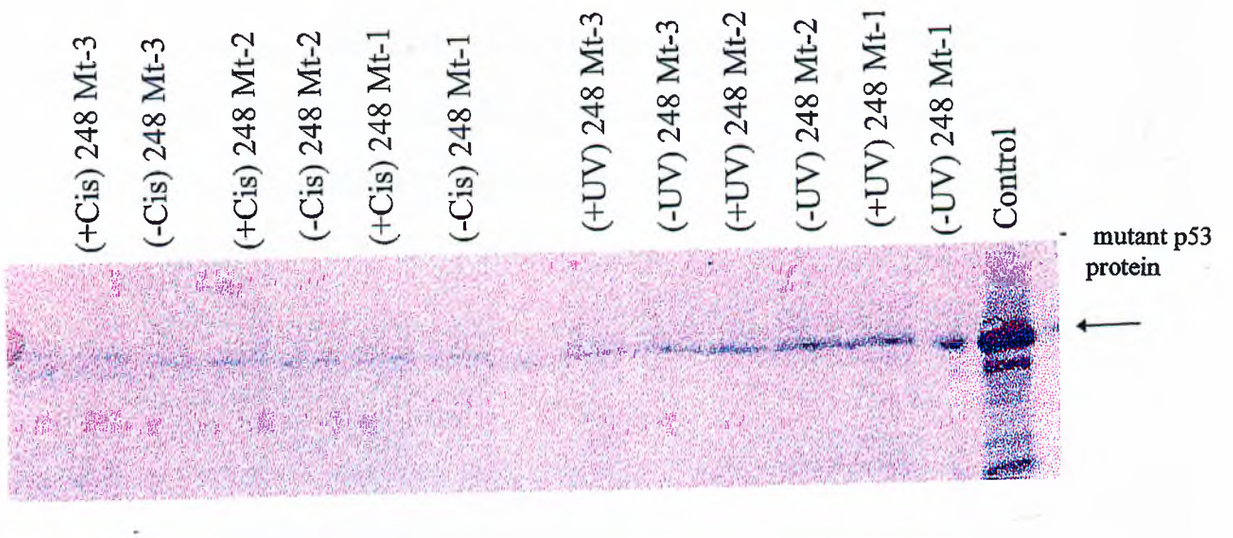


Fig.18. Western blot analysis of the mutant p53 expression in yeast cells grown in high Pi medium and exposed to UV-radiation or cisplatin treatment

In accordance with the previous Western blot analysis of mutant p53 protein extracted from cells grown in low Pi medium, no difference for mutant p53 expression was detected cells grown in high Pi medium

5. DISCUSSION

Existence of p53 homologue has not been demonstrated in yeast yet. The lack of demonstrable p53 homologue in yeast however doesn't discourage the further studies that use this organism to explore potential functions of p53 because human p53 might function directly or indirectly by interacting with other macromolecules of yeast that are functionally conserved between yeasts and mammals.

A growth inhibitory effect of p53 on *S.cerevisiae* was clearly demonstrated with wild type p53 (Nigro et al., 1992). This growth inhibition effect of wild type p53 was also demonstrated in fission yeast *S.pombe*. The results clearly show analogy with the p53 colony growth inhibition effect in human tumuor cells that were transfected with wild type p53 genes (Baker et al., 1990; Diller et al., 1990).

This is the first known study to evaluate the effects of p53 protein on yeast cell survival and response to DNA damaging agents. In this study yeast *S.cerevisiae*, AKY28 strain, was chosen as a model organism.

As a first step to study p53 effects in yeast, we established an experimental model. In this model, we used the expression vectors, pL3 and pAK31. pL3 was the original plasmid from which pAK31 was constructed and it was used as a control during the experiments. pAK31 was used as regulatable mutant p53-248W protein expressing vector. Regulation of p53 expression was under the control of Pi regulated PHO5 promoter. Instead of wild type p53, mutant p53-248W was used to establish this experimental system due to reported growth inhibitory effect of wild type p53 on yeast cells (Bischoff et al., 1992; Nigro et al., 1992). In this system,

initially, we verified the presence of mutant p53 sequence (codon 248, R->W) in pAK31 and in yeast cells that were transfected with this plasmid. After the presence of mutant p53 sequence in transfected yeast cells was confirmed, expression of p53 protein was analysed by Western blotting. The results indicated that p53 protein expression was not completely regulated by PHO5 promoter because p53 expression was detected in both high Pi and low Pi medium suggesting that PHO5 was not totally suppressed in high Pi medium. Although constitutive expression of transfected wild type p53 may inhibit yeast cellular proliferation but in this case because of mutant p53-248 expression in yeast cells, regulatable expression of p53 protein was not stringently required. After demonstrating mutant p53-248W expression in yeast cells, the growth rate of p53 expressing and control cells was analysed. Serial yeast dilutions ranged from 10^{-5} dilution to 10^{-1} dilution intervals were applied to agar plates and the growth rate of those cells was compared. No difference was detected between mutant p53-248W expressing and non-expressing (pL3 transformed) yeast cells.

The mutation in amino acid residue R248, one of the most frequently altered residues in the protein, results in defective contact with the DNA and loss of ability to act as a transcription factor (Cho et al., 1994). The mutant p53-248W exerts a trans-dominant loss of function effect on wild type p53 protein in human cells (Hollenstain et al, 1991). A report that analysed dominant-selective mutants in *S.cerevisiae* demonstrated that the p53 mutations in codon 248 was one of the most dominant-negative mutants that could interfere with one and two copies of wild type p53 (Brahman et al., 1996). Demonstrated mutant p53-248W effects on *S.cerevisiae* growth showed analogy to report that demonstrate the effect using mutant p53-143A expression (Nigro et al., 1992). Ala 143^{val} is a temperature sensitive mutant in

mammalian cells that shows a wild type conformation at lower temperature (32°C) (Zhang et al., 1994). This mutant was previously shown to have strong dominant-negative effects on p53 protein (Kern et al., 1992). According to report, the growth of *S.cerevisiae* was affected by the Ala-143 mutant much less dramatically than by wild type p53. Another mutant p53-273 H was shown to have recessive effect on p53 protein (Chen et al., 1990) and behaved in an intermediate fashion (Nigro et al., 1992). According to another report, mutant p53-273H and mutant p53-175H expression showed different effects on *S.pombe* yeast cell survival (Bischoff *et al.*, 1992). Although both mutants showed different growth rate effect compared to wild type p53, the mutant p53-175H showed the smallest effect and mutant p53-273H showed intermediate effect between wild type p53 and mutant p53-175H (Bischoff et al., 1992). It was previously shown that mutant p53-273H exerted recessive effect and mutant p53-175H exerted dominant effect on wild type p53 in both mammalian and yeast cells (Bischoff et al., 1992). These reports suggest that effect of the mutant p53 protein on yeast cell growth rate vary according to type of the p53 mutation. In general, mutants that show a dominant effect on p53 protein, exert a smaller effect on yeast cell survival than mutants that show a recessive effect. In the case of our study we demonstrated that mutant p53-248W which was previously shown to exert dominant loss of function effect on the wild type p53, doesn't effect the growth rate of yeast cells.

The effect of mutant p53 on yeast cell growth after DNA damage was also analysed. UVC and cisplatin were used as DNA damaging agents. Cell survival assay was performed using the same procedure that demonstrated p53 effect on yeast cell growth rate. No distinct mutant p53 effect on yeast cell survival was detected when mutant p53 expressing and non-expressing cells were compared. The effect of

p53 expression on cell survival upon exposure to UV-radiation was also studied in mammalian cells and it was reported that clonogenic survival of the mouse cells expressing wild type p53 following UV-radiation enhanced upon DNA damage compared to non-induced mutant p53-143V expressing cells (Yuan et al., 1995).

In parallel to the cell survival assay mutant p53-248W protein levels following cisplatin and UVC treatment were compared with untreated cells and no induction was detected. Similar analysis were also performed in mammalian cells and wild type p53 levels were shown to increase following γ-irradiation, UV-radiation and treatment of base adduct factors (Lane, 1993; Lu and Lane, 1993). Induction of p53 in mouse fibroblast cell line was found to enhance the clonogenic survival of the cells following UV-irradiation compared to p53-deficient parental mouse cells (Yuan et al., 1995).

In this study mutant p53-248W was used. 248R mutation is one of the p53 hot spot mutation in human cancers and its found in the DNA interacting face of p53. Mutations in codon 248 result in defective contacts with DNA and loss of ability as transcriptional factor (Cho et al., 1994; Scharer and Iggo et al., 1992). According to following reports in mammalian cells that study p53 response to DNA damage, mutant p53 248W exerted loss of function phenotype compared to wild type p53 function. The p53-248W mutant cells were more resistant than the wild type p53 cells to UV cytotoxicity and exhibited less UV-induced apoptosis (Ford and Hanavalt, 1995). It was demonstrated that wild type p53 is required for heat shock and ultraviolet light enhanced repair of a UV-damaged reporter gene in normal diploid fibroblasts however this inducible DNA repair response was absent in Li-Fraumeni syndrome (LFS) fibroblasts which are heterozygous for mutant p53-248W

and immortalized LFS cell sublines, which express only mutant p53-248W (Mckay et al., 1997).

The obtained results were not unexpected because of reported dominant-negative effect of mutant p53-248W in yeast and mammalian cells. It is possible that mutant p53-248W is defective for the functions of p53 that provide its induction and following effect on cell survival upon DNA damage. But at this step we are not able to speculate about results without considering and comparing the wild type p53 effects with demonstrated mutant p53-248W results obtained in this established experimental system.

6.CONCLUSION

We established an experimental system to study p53 effects in yeast upon DNA damaging agents. Mutant p53-248W was used in studies. Although p53 effects on mammalian cell growth and p53 protein response was previously studied in mammalian cells, this is the first report about mutant p53 effects upon DNA damage in yeast. In this study it was initially shown that mutant p53-248W didn't effect the growth rate of yeast cells in normal conditions. This result was also confirmed by the previous reports that demonstrated nearly same effect (Nigro et al., 1992). We also demonstrated that mutant p53-248W expression did not effect yeast cell survival upon DNA damage and mutant p53-248W protein didn't show any response like increase or decrease to those agents. The unresponsive effect of mutant p53-248W on yeast cell survival upon DNA damage, is not an unexpected result because mutant p53-248W that is one of the p53 mutations commonly found in human tumours, lose some of the biological functions of p53 in yeast that was reported previously in yeast *S.cerevisiae* (Schrarer and Iggo 1992).

This study based on an establishment of experimental model to study p53 effects in yeast. It shows that mutant p53-248W in yeast cells has no demonstrable effect on yeast cell growth after DNA damage.

7.PERSPECTIVES

We have established an experimental system to study the effects of human wild type and mutant p53 in yeast cells. Mutant p53-248W expression vector, pAK31 was used to establish this system.

Next we will study the effects of wild type p53 in this experimental system. Since growth inhibitory effects of wild type p53 in yeast have already been shown, it will be more important to focus on the possible involvement of wild type p53 in DNA damage response in yeast cells. As the expression system used here doesn't allow full suppression, it may be necessary to construct another wild type p53 expression vector under the control of other inducible promoters. Because constitutive expression of transfected wild type p53 can inhibit cellular proliferation in yeast, we chose the mutant form of p53 protein to construct this experimental system. We can overcome this obstacle by using alternative regulatable p53 expressing vectors.

After an appropriate wild type p53 expression vector is obtained, there are many different studies to performed in our experimental system that provides an easy system for studying the mechanism of action of human p53 in yeast upon DNA damage.

Induction of p53 leads to either cell growth arrest or cell death. Apoptosis and growth arrest provide mechanisms by which p53 functions to control DNA damage, protecting cellular descendants from accumulating excessive mutation. Many reports that provide information about factors involved in each of these

responses. However, there are still many questions to be answered (Shiomura et al., 1996). Wild type p53-mediated growth responses like cell cycle arrest and apoptosis can be studied in yeast cells after DNA damage using this experimental system.

Wild type p53 is normally present in extremely small quantities. Induction of p53 results in an increase in the levels of p53. DNA damage induces p53 and causes its accumulation (Kastan et al., 1991). This response of p53 can be studied in yeast cells exposed to DNA damaging agents.

Other strategies can be developed to understand action of p53 in yeast cell upon DNA damage. One of the strategies can be identification of yeast and mammalian proteins that can modify p53-mediated growth control in yeast cells. Using in vitro systems, p53 protein interacting peptides can be isolated from a random peptides library. Also the in vivo yeast two-hybrid system provides high sensitive detection of this interaction without any biochemical modifications. The yeast two hybrid system can be used to screen a library of random peptides fused to a transcriptional activation domain in order to identify peptides capable of binding to the DNA damage induced p53 protein.

Generation of p53 response defective mutant yeast strains by mutagenesis might allow identification of a yeast gene that complements p53 function induced upon DNA damage This yeast gene could be isolated from the mutant cells that were transformed with a library of yeast genomic cDNA and identified by transposon insertion mutagenesis of the cDNA expressing plasmid.

Also dominant-negative mutant p53 proteins that inactivate p53 function upon DNA damage can be determined using our established system by transfecting the mutant p53 protein in to the wild type p53 expressing yeast cells.

Two different conformation of p53 were detected based on their differential immunreactivity with specific antibodies: a "suppressor" conformation that inhibit cell division and a "promoter" conformation. The promoter conformation is generally characteristic of mutant p53s found in human cancers (Gannon et al., 1990). At present the mechanism responsible for this conformation is unknown. Using this established experimental system, those factors can be determined in response to DNA damaging agents that induce p53 activation.

There are also evidences for direct involvement of p53 in DNA repair but this function of p53 has not been clarified yet. Yeast is the commonly preferred organism to study DNA repair system and homologous genes involved in DNA repair have been found in yeast and mammalian cells (Sancar, 1996). Recent studies have suggested p53 involvement in DNA repair (McKay et al., 1997; Smith et al., 1995), so similar assays might be applied in yeast using this established system.

Strategies can be developed more and more because p53 tumour suppressor has been the hot topic and studied by many researchers since 1979s. In this study we preferred to investigate p53 functions in yeast and as initial step we established an experimental system that is open to further investigations.

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