INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

P53-TFIID-DNA INTERACTION

Analysis by DNA affinity chromatography

by

Maria Patricia Molina

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

2002

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/29/02
Date

Chair of Examining Committee

1/30/02
Date

Horst Schulz
Executive Officer

Dr. LAUREL ECKHARDT

Dr. ROBERT DOTTIN

Dr. JAMES MANFREDI

Dr. THOMAS HAINES

Supervisory Committee

The City University of New York

Abstract

P53-TFIID-DNA INTERACTION.

Analysis by DNA affinity chromatography.

By

Maria Patricia Molina.

Adviser: Dr. Jill Bargonetti.

Previous immunoprecipitation experiments have shown that p53 has the ability to associate with the TATA binding protein and some of its associated factors, and that this association modulates p53 function. By DNA affinity chromatography we studied the binding of wild-type p53 and the mentioned proteins to the p53-binding sites present in the Promoter 2 (P2) of the MDM2 gene, in the Ribosomal Gene Cluster (RGC) and to the ideal p53-binding site called superconsensus sequence (SCS). We report that p53 can be purified by DNA affinity chromatography using the above-mentioned p53-binding sites. We also report that the p53-binding sites select for specific p53 subpopulations, which have different binding characteristics, "bindomers". Our results demonstrate that DNA affinity Chromatography is a promising technique to isolate these p53 subpopulations or ("bindomers") in order to perform further biochemical analysis. We also show that the mdm2 P2 binding p53 is able to induce the addition of other(s) factor(s)

to a TBP-TAFII60-TAFII40 complex. We show that other forms of wt p53 do not induce this addition.

Key words: p53; DNA affinity Chromatography; Ts Val 135; TBP.

AKCNOWLEDGEMENTS.

This work is dedicated to my sister Ruth who has always being willing to sponsor my dreams.

TABLE OF CONTENTS.

LIST OF FIGURES.

1. INTRODUCTION.

1.1. P53 IS A TUMOR SUPPRESSOR.

p53 is a tumor suppressor protein. When the cell is under stress it produces specific signals that activate protective mechanisms. One of those protective mechanisms is the activation of the protein p53. Activated p53 elicits a series of cellular responses that promote genomic stability, cellular growth arrest, or apoptosis. This growth suppressor end result of p53 activity protects the cell from giving rise to genetically altered progeny (Levine A.J.1991; Zambetti G.P. 1993; reviewed by Levine A. 1997; Schackelford R.E, 1999 and Bucholzt T.A. 1999). Because of this protective role p53 has been named "the guardian of the genome" (Lane D.P., 1992).

Results of many in vitro experiments as well as clinical evidence have suggested the tumor suppressor activity of p53. The tumor suppressor activity of p53 was evident in the results from experiments showing that wild-type p53 reduces the efficiency of transformation by cooperating oncogenes (Finlay, C. 1989; Eliyahu D. 1989). Besides, the re-introduction of wild-type p53 into cancerous cells reduces their plating efficiency (Baker, S.J. 1990; Diller L.1990; reviewed in Harris CC., 1993). At a clinical level, it was observed that inactivating mutations and deletions of the p53 gene are common events in human cancers (Rodriguez NR. 1990; Sidransky D. 1992); this fact is exemplified by the Li-Fraumeni syndrome (Malkin D. 1990). The tumor suppressor function of p53 was also demonstrated, in vivo, by the high incidence of tumors and early death of p53 knockout

mice (Donehower LA. 1992; Purdie CA., 1994; Jacks T., 1994).

1.2. PATHOLOGICAL INACTIVATION OF P53.

An important factor contributing to the development of many human cancers is the lack of function of p53 (Sherr CJ.,1996). There are several mechanisms that result in the pathological inactivation of p53. One of these mechanisms is the binding and inactivation of p53 by cellular or viral proteins (Piette J., 1997 reviewed by Hansen R., 1997; Levine A. 1997). For example, the association between p53 and MDM2 is a physiological mechanism to control p53 activity, but in cells where MDM2 is overexpressed an abnormal association between these two proteins takes place (Momand J. 1992; Oliner JD. 1992). As a consequence of this abnormal association p53 loses its transactivation ability and gets degraded (Oliner JD. 1993; Zauberman A. 1993). The end result then is a lack of p53 activity. The E6 protein of the Human Papillomavirus (HPV) induces the degradation of p53 through the ubiquitin pathway; the HBX Ag protein of Hepatitis B virus (HBV) inactivates the transactivation function of p53 by binding its N-terminal domain and the simian Virus 40 (SV40) Large T antigen blocks the binding of p53 to DNA, which is central to p53 activity (Bargonetti J. 1991; Tan TH.,1986; Howley PM., 1991; Oliner JD., 1992; Ueda H., 1995). The aberrant localization of p53 in the cytosol instead of the nucleus, is another way of losing p53 function. The aberrant localization is given by failure in the translocation of p53 from the cytosol to the nucleus or failure to block the nuclear export of p53 (Zaika A., 1999; Moll U., 1995; Sengupta S., 2000; Kim IS., 2000). Reduced transcription of the p53 gene has also been reported (Prokocimer M.

1987; Stuart, ET. 1995). Finally, there might be defects in the p53 pathway, which will give as a result, a syndrome similar to inactivation of p53 (Raman V., 2000; reviewed in Vogelstein B., 2000).

A very common way of p53 functional abrogation is the mutation of the p53 gene. About 50% of all human cancers exhibit mutations in the p53 gene (Holstein 1991; Harris CC., 1993; reviewed in Soussi T. 1994; Ko LJ. 1996 and Levine AJ.1997). The high incidence of tumor associated mutations in this protein demonstrates that the lack of p53 function is an important factor contributing to the development of many human cancers.

1.2.1. p53 mutants.

The observed mutations in the p53 gene are mainly, missense in nature and tend to cluster in the region of the p53 protein engaged in the sequence-specific DNA binding function or core domain (Hollstein, 1994; Beroud, 1998; Walker DR., 1999; reviewed in Monique G.2000). Some of these mutations give rise to nonfunctional proteins that do not bind, or bind poorly, to the p53 response elements (Hollstein M. 1994; Prives C. 1994). The sequence-specific DNA binding ability of p53 is key to its function and will be discussed later. The lack of sequence specific DNA binding activity exhibited by some p53 mutants is due in some cases to a mutation in one of the amino acid residues that make contact with DNA, as defined by the crystal structure analysis of p53 (Cho Y. 1994). Examples of these so-called "contact mutants" are R248 and R273. There is another type of mutation in the p53 gene producing a protein in which the overall conformation is

changed and as a result, the binding of p53 to its cognate site is disrupted. These mutants are called "conformational mutants" and include R175, G245, and R249 among others (reviewed in Selivanova G. 1998; Sigal A. 2000).

In general, p53 mutants do not bind to the consensus sequence for p53 and/or lose their sequence specific transactivation function (loss of function mutants) (Finlay CA. 1989; Scharer E.1992; Farmer G.1992). As a consequence, these mutants lose their ability to induce growth arrest and/or apoptosis. Nevertheless, some p53 mutants behave as growth stimulators. These mutant p53 proteins have gained an oncogenic ability and are referred to as gain of function mutants (Hollstein M. 1994; Hollstein M. 1991, Dittmer D. 1993; Zambetti GP. 1993; reviewed in Vogelstein B. 1992; Sigal A. 2000; Monique G., 2000). The oncogenic activity of these mutant p53 proteins is due, in some cases, to a dominant negative effect of the mutant protein over the wild-type p53. In some other cases, it is due to a wild-type p53 independent gain of function of the mutant protein (Gottlieb E. 1994; Tang H. 1998; Wolf D. 1984; Lanyi A. 1998; reviewed in Sigal A. 2000). Amongst the growth advantage characteristics conferred by the p53 gain of function mutants are the increase in the cellular proliferation and growth density; tumorigenicity and invasiveness (Chen Y., 1994; Muller B., 1996 Wang X-J.1998; Shaulsky G., 1991; Cardinali, M., 1997; Hsiao M., 1994). The evidence suggests that most of the gain of function effects of the mutant p53 proteins are mediated by an increase in the expression of genes that facilitate cellular growth. Some of these genes are the proliferating cell nuclear antigen, multiple drug resistance, the growth factor genes EGFR, VEGF, bFGF as well as the oncogenes c-myc and c-fos (Deb S., 1992; Frazier M., 1998; Ludes-Meyer JH. 1996;

Kieser A., 1994; Ueba T., 1994; Kawamura M., 1996).

1.3. PHYSIOLOGICAL REGULATION OF P53.

1.3.1. Down-regulation.

Integrity of the genome and tight regulation of cell division are key to preventing tumor formation (Hartwell, L. 1992. reviewed in Pavlovich AG. 1997). Given that p53 activation leads to cellular responses that ultimately will prevent cellular division, it makes sense that, under non-insult conditions, p53 is tightly controlled and down regulated (reviewed in Woods DB. 2001; Selivanova, G., 1998; Levine, A. 1997). This down regulation is exerted by mechanisms such as controlling transcription as well as translation of the p53 gene but, predominantly via post-translational modifications to the p53 protein.

One of these mechanisms is the degradation of the p53 protein by the ubiquitin pathway. Under non-insult conditions the p53 protein has a very short half-life and is barely detectable by Western blot (Haupt, 1997; Kubbutat, M.1997; Fuchs, SY.1998; Maltzman W., 1984; reviewed in Selivanova G. 1998). The p53 amount is kept low, in part, via a feed back loop mechanism formed by p53 and the MDM2 protein. p53 activates transcription of the mdm2 gene and the MDM2 protein then binds to the amino terminal domain of p53 (Lin J., 1994; Picksley S.M. 1994; Kussie P.H., 1996). This binding blocks the transactivation function of p53 and induces its degradation by the ubiquitin

pathway (Momand J., 1992; Wu XW. 1993; Bar-Or L., 2000; Lozano G., 1998; reviewed in Prives C. 1999; Woods BD. 2001). MDM2 acts as a RING finger E3 ligase for the ubiquitin degradation system (Honda R., 1997; Fang S., 2000; Midgley CA., 2000). There is also evidence that the MDM2 protein shuttles p53 from the nucleus to the cytosol for degradation (Roth J., 1998; Boyd SD., 2000; Geyer RK., 2000). The importance of the p53 down-regulation by MDM2 is evident in the results from *in vivo* experiments with knockout mice. Mice null for MDM2 exhibit a high embryonic lethality that is rescued in the double null phenotype MDM2-/-, p53 -/- (de Rozieres S., 2000; Montes de Oca Luna R., 1995; Jones SN., 1995). In addition, the introduction of antibodies or synthetic peptides that disrupt the MDM2-p53 interaction into the cells, increases p53-dependent gene expression (Blaydes J.P., 1998; Blaydes J.P. 1997; Bottger A. 1997).

The degradation of p53 by the ubiquitin pathway involving the action of MDM2 is the best-understood mechanism of p53 down-regulation but is not the only one. p53 has been reported to be degraded by the protease calpain (Kubbutat MHG., 1997; Pariat M., 1997; Zhang W., 1997). There is also evidence suggesting that p53 can be kept in check by maintaining the protein in a latent form. p53 can be maintained in its latent form by the interplay of several post-translational modification mechanisms and/or protein-protein interactions. For example, the C-terminal domain of the p53 protein inhibits its sequence specific DNA binding activity and this inhibition can be relieved by allosteric modifications to this region (Hupp, 1992; Haapajarvi, T., 1997; Chernov, MV.,1998; reviewed in Ko, LJ. 1996; Levine, A., 1997). Recent evidence suggests the involvement

of the N-terminal domain of p53 in regulating its dissociation from DNA as well (Cain C., 2000).

There is also evidence suggesting control of p53 activity at the level of transcription of the p53 gene, the translation of the p53 mRNA and the cellular localization of the p53 protein (Webster GA., 1999; Raman V., 2000; Fu L., 1996; Shaulsky G., 1991;Stommel JM., 1999; Gaitonde S.V. 2000; reviewed in Vousden KH., 2000).

1.3.2. Up-regulation.

Intracellular p53 levels can increase in response to different stresses e.g. DNA damage, metabolic deprivation, hypoxia, oncogene expression, hyperthermia and defects in chromosome segregation. This increase is accomplished mainly via stabilization of the p53 protein and a decrease in its degradation (Kastan M.B., 1991; Maltzman W., 1984; Eller M.S., 1987; Valenzuela M.T., 1997; Sablina .A., 1998; reviewed in Prives C. 1999; Salles-Passador I. 1999; Ljungman M. 2000; Hupp T.R. 2000)

The stabilization of p53 is accomplished by preventing the interaction between p53 and MDM2, abolishing in this way the targeting of p53 for degradation (Haupt R., 1997). The interaction between p53 and MDM2 is prevented by several mechanisms. One of these mechanisms is the phosphorylation of the amino terminal domain of p53, which physically hinders the binding site for MDM2 present on the p53 protein (Shieh SY. 1997; Unger T., 1999). Several kinases activated by cellular stress such as Ataxia

Tanlangiectasia Mutant (ATM) and Checkpoint Kinase 2 (Chk2) have been reported to induce phosphorylation of p53 at its N-terminal domain within the MDM2 binding site (Shieh S-Y. 2000; Hirao A., 2000; Meek D.W. 1998; Lakin N.D. 1999). There is evidence that this same goal can be accomplished by ATM-dependent phosporylation of the MDM2 protein (Khosravi R., 1999). Another way of preventing the p53-MDM2 interaction is by the intervention of the mouse p19 ARF or human p14 ARF protein, the product of the p16INK4A locus, which binds to MDM2 (Midgley C.A., 2000). The p19 ARF protein is up regulated as a response to proliferative signals mediated by oncogenes. p19 ARF prevents the MDM2 dependent targeting of p53 for degradation by binding to MDM2, thus making it unavailable for its interaction with p53 (Pomerantz J., 1998; Honda R., 1999; Weber J.D., 1999; reviewed in Prives C. 1999; Ashcroft M., 1999; Lowe S.W., 1999). The p53-MDM2 interaction is also down-regulated by the degradation of MDM2 by the protein caspase 3 or by inhibiting the expression of the mdm2 gene (Pochampally R., 1998; Pochampally R., 1999; Freedman D.A., 1999; Ashcroft M., 2000; Ma Y., 2000). Post-translational modification of MDM2, phosphorylation and/or sumoylation is yet another way of stabilizing p53 (Mayo L.D., 1997; Buschmann C.J., 2000). Finally, the binding of other cellular proteins such as p300 and/or TAF II31 to the N-terminal domain of p53 has also been reported as another way of preventing the p53-MDM2 interaction (Yuan Z.M., 1999; Lambert P.F., 1998).

There is some evidence that suggests an increase in the translation of the p53 mRNA by relieving a repressor mechanism (Sun X., 1995; Mosner J., 1995; Fu L., 1999; and reviewed in Reisman D.1998). There are also reports of an increase in the transcription of

the TP53 gene, for example when there is an over expression of c-myc or NF-*k*B (Kirch H.C., 1999; Reisman D., 1993; Sun X., 1995).

1.3.3. Modulation of p53 activity.

Increase in stability and as a result, in the cellular amount of p53 after insult, is a very important event in the activation of the p53 pathway but certainly not the only one. Another important event is the change in the conformation of p53 and the induction of its DNA binding activity (Hupp T.R. 1992).

Post-translational modification is one of the most versatile mechanisms to control the activity of p53, modifying its sequence specific DNA binding function as well as its interaction with other proteins (Meek D.W. 1999; Jayaraman L., 1999). Among the post-translational modifications on p53 are phosphorylation, acetylation, O-glycosylation and sumoylation (Gu W., 1997; Meel D.W. 1999; Prives C., 1999; Giaccia A.J., 1998).

p53 is phosphorylated by several protein kinases as a response to cellular insults. For example, DNA dependent Protein Kinase (DNA-PK), CAK, ATM, ATR and JNKs have been seen to phosphorylate p53 at its amino-terminal domain as a response to DNA damage (Siliciano JD. 1997; Woo RA., 1998; Tibbetts RS., 1999; Kachnic LA., 1999; reviewed in Caspari T., 2000; Colman MS.,2000). Two other DNA damage-inducible enzymes, Protein Kinase C (PKC) and Caseine Kinase II (CKII) phosphorylate p53 at its carboxy-terminus (Sakaguchi K., 1998; Shaw P., 1996; reviewed in Prives C. 1999). The

physiological meaning of these phosphorylation events is being investigated. Phosphorylation of p53 at Ser15 and/or Ser 20 prevents the interaction of p53 with MDM2 and induces the stabilization of the p53 protein (Craig A.L. 1999; Craig A.L.(b) 1999; Unger T., 1999; Shie S.Y., 2000; Chehab N.H., 2000) . In vitro experiments have shown that phosphorylation of p53 at its C-terminal domain (Ser392) by CKII, activates the latent DNA binding activity of p53 and phosphorylation of the equivalent site in the murine protein may regulate the transactivation function of p53 (Hupp T.R., 1995; Hao M., 1996). In general, it seems that covalent modifications to the C-terminal domain of p53 increase the p53 mediated gene expression. This increased transactivating function of p53 may be the result of induction of the latent sequence-specific DNA binding ability of p53 or reduction of its function as a non-sequence specific DNA binding protein. These two activities are controlled by the C-terminal domain of p53 (Hupp T.R., 1994; Anderson M.E., 1997; Hoffmann R., 1998; Selivanova G. 1998). Among the modifications at the C-terminal domain of p53 is its acetylation by p300/CBP and/or PCAF (Sakaguchi K., 1998; Gu W., 1997; Liu L., 1999). The acetylation of p53 correlates with an increase in p53 dependent transcription, and in agreement with this result, its deacetylation has been reported to result in reduction of such transcription activity (Juan L.J., 2000; Kobet E., 2000). p53 can be sumoylated and this event also increases p53 transactivation activity (Gotissa M., 1999; Rodriguez M.S., 1999; Muller S. 2000). Finally the glycosylation and ribosylation of p53 may also affect the stability and activity of p53 (Vaziri H., 1997; Kumari S.R., 1998).

Another way to modulate its activity is the association of p53 with cellular and viral

proteins such as BP1, BP2, SP1, MDM2, TFIIH and SV40 T antigen (reviewed in Hupp T.R., 2000; Prives C. 1999; Levine A. 1997). The physiological importance of all these associations is actively being investigated. Probably, one of the best understood is the interaction of p53 with MDM2 as discussed previously. Even more, the binding of MDM2 to the amino terminal domain of p53 prevents the association of p53 with the TATA Binding Protein (TBP), which is key for the transactivation function of p53 (Wu X., 1993; Lozano G., 1998). The association between p53 and the transcriptional co-activator p300 Creb Binding Protein (CBP) is important for p53 dependent gene expression and apoptosis (Avantaggiati M.L., 1997; Gu W., 1997). Noteworthy, there are also reports that show that phosphorylation at Ser 15 facilitates the interaction between p53 and p300 and the subsequent acetylation of p53 (Lambert P.F. 1998; Fiscellla M., 1993; Unger T., 1999). The interaction of p53 with Ref-1 and HMG-1 activates p53 function (Jayaraman L., 1998; Jayaraman L., 1997). Increased p53-dependent transcriptional activation has also been observed as a result of interaction between p53 and other tumor suppressors like Wilm's Tumor–1 (WT-1) and Breast Cancer 1 (BRCA1) (reviewed in Sionov R.V. 1999). Some other cellular proteins bind to p53 and modulate its DNA binding affinity; these interactions will be discussed under DNA binding activity of p53. Finally, the p53 mRNA can be spliced in at least two different ways, giving rise to p53 proteins with different sizes and possibly with different functions (Kulezs-Martin MF., 1994; Wolkowicz R., 1995). These results suggest that the effective regulation of p53 may require the simultaneous or consecutive interplay of several control mechanisms.

The modulation described before increases the DNA binding activity of p53 and might give the p53 molecules promoter selectivity. Nevertheless, the latent form of p53 is not devoid of function. This form of p53 exhibits some functions related to DNA repair i.e. 3'-5' exonuclease activity and is able to recognize ssDNA as well as unpaired bases. (Janus F., 1999; Janus F. 1999a).

1.4. DOWNSTREAM EFFECTS OF P53 ACTIVATION.

p53 downstream effects depend on the cell type and also on the kind and magnitude of the insult as evidenced by gene profiling experiments (Zhao R., 2000; Midgley C.A., 1995; Chen X., 1996; reviewed in Kumaravel S., 2000; Prives C.., 1999 ; Agarwal ML. 1998). Besides the gene profiling type of experiments, analysis of phosphorylation of p53 at different amino acid residues reveals that different stimuli can induce phosphorylation of different residues in the same cell type (Webley K., 2000).

Transcriptional activation, transcriptional repression, and protein-protein interactions mediate the downstream effects of p53 activity. Among those downstream events are growth arrest and/or apoptosis. Cells with high proliferative characteristics such as colon epithelium and hemopoetic ones require a strong control of cell growth and p53 generally induces apoptosis in these type of tissues compare to its induction of growth arrest in tissues with lower proliferative activity (reviewed by Chumakov PM., 1999) . The p53-induced growth arrest can be G1, G2 or post-mitotic (Kastan, MB.,1992; Agarwal, ML., 1995; Cross, SM., 1995 ; reviewed in Agarwal ML. 1998; Schwartz D. 1998; Rotter V.

1998; Levine AJ. 1997; Teyssier F. 1999). There is also evidence for the involvement of p53 in the pre-meiotic checkpoint (reviewed in Schwartz D., 1998). The growth arrest gives the cell time to repair the damaged DNA. The DNA damage repair is also orchestrated in part, by p53 (Kastan MB. 1991; Kuerbitz SJ. 1992; reviewed in Janus F. 1999; Ko LJ. 1996).

The p53-dependent G1 arrest is mediated mainly through the up-regulation of the cyclin-dependent kinase (CDK) inhibitor protein p21/wafl (Waldman T., 1995; Deng C., 1995; Brugarolas J., 1995). The p21 protein inhibits the phosphorylation of the Rb protein required for progression to the S phase of the cell cycle (Sherr C.J., 1998). In addition, the p21 protein also binds to the Proliferating Cell Nuclear Antigen (PCNA) and decreases the processivity of the DNA polymerase, helping to switch the cell from a replicative state to a reparative one (Waga S., 1994). The p21 protein is also implicated in the contribution of p53 to the G2 growth arrest (El-Deiry W.S., 1993; Agami R., 2000; Mailand N., 2000). Besides the p21 protein, there are other p53 effectors that intervene in the G2 growth arrest. Amongst these effectors are the gene products Reprimo, 14-3-3 sigma, B99 and GADD45 (Ohki, R. 2000; Chan T.A.1999; Utrera R. 1998). The 14-3-3-sigma gene product binds to the cdc25 phosphatase and prevents it from acting upon cyclin B/cdc2, an event required for the G2/M progression). The GADD45 gene product binds to and inhibits the cyclin B/cdc2 complex. The over expression of B99 leads to G2 growth arrest but its mechanism of action is not clear yet (Laronga C., 2000; Zhan Q. 1999; Hermeking H., 1997).

Another possible consequence of the activation of p53 is for the cell to undergo apoptosis (Jonish-Rouach E. 1991; Lowe S., 1993; reviewed in Schwartz, D. 1998; Gottlieb MT. 1998; Agarwal ML. 1998; O'Connell, MJ. 2000; Voudesn K.H., 2000). p53 induces apoptosis in response to unrepaired DNA and the apoptotic response seems to correlate with the levels of p53 , the extent of the DNA damage and the inhibition of DNA repair (Gao Y., 2000; Chen X., 1996; Beneke R. 2000). Although the transactivation function of p53 seems to be involved in its apoptotic response to DNA damage, p53–dependent apoptosis can be achieved without it (Caelles C., 1994; Wagner A.J., 1994; Haupt Y., 1995). There is evidence that suggests that the cellular p53-dependent apoptosis that do not require activation of transcription may be dependent on the ability of p53 to bind DNA in a non-sequence specific way and that it is linked to the DNA repair mechanism (Wang X.W., 1996; Spillare E.A., 1999). The transcriptional independent apoptotic activity of p53 is mediated by the activation of the caspase pathway and by translocation of Fas receptor molecules from the Golgi to the cell surface (Ding H.F., 2000; Bennett M., 1998).

p53 can also induce apoptosis by increasing transcription of some pro-apoptotic genes. One of these is bax; this gene belongs the Bcl-2 family and its gene product promotes the release of cytochrome c from the mitochondrion activating the caspase pathway, which ends in cellular apoptosis (Oltvai Z.N., 1994; Rosse T., 1998; Srinivasula S.M., 1998). Other pro-apoptotic proteins induced by p53 are Noxa and p53Alp1. p53 also up-regulates the death receptor proteins PIDD, KILLER/ DR5, and Fas (Oda E., 2000a; Lin Y., 2000; Wu G.S., 1999; Sheikh M.S., 1998; Muller M., 1997; Owen-Schaub L.B.,

1995; Selvakumaran M., 1994; Miyashita T., 1994). Besides activating transcription of pro-apoptotic genes, p53 also down-regulates the transcription of the anti-apoptotic protein Bcl-2, and of the survival signals IGF-II and IGF-1R (Haldar S., 1994; Findley H.W., 1997; Zhang L., 1998; Werner H., 1996).

It is not clear what makes p53 induce either growth arrest or apoptosis, although there is evidence suggesting that the presence of some forms of the JMY protein, a co-factor for p300, determines, at least in part, whether p53 induces growth arrest or apoptosis. High levels of E2F also induce p53-dependent apoptosis and may be by overriding the growth arrest pathway. Finally, p53 regulates some redox enzymes like PIG-3, PIG-6, and PIG-12, which induce apoptosis by formation of reactive oxygen intermediates (ROI) (Shikama N., 1999; Sherr C.J., 2000; Hiebert S.W., 1995; Polyak K., 1997).

p53 activity has also been implicated in DNA repair and there seems to be a direct correlation between p53 and the nucleotide excision repair mechanisms (NER). p53 up-regulates the GADD45 and XPE gene products, which are involved in DNA repair (Ford J.M. 1998; Wang X.W., 1996; reviewed in van Steeg H., 2001; Yuangang L., 2001). Some of the biochemical features of p53 predict its involvement in DNA repair. For example, p53 can mediate ssDNA reannealing as well as mediate rejoining of DNA double strand breaks. In vivo studies show that p53 can inhibit chromosomal recombination. p53 has 3'-5' exonuclease activity (Bakalking G., 1994; Huang L.C., 1996; Tang W., 1999; Bill C.A., 1997; Wiesmuller L., 1996; Mekeel K.L., 1997; Mummenbrauer T., 1996). Also there is evidence that p53 participates in the base

excision repair pathway (BER) (Offer H., 1999; Zhou J., 2001).

1.5. P53 IS A TRANSCRIPTION FACTOR.

As discussed before, one of the consequences of p53 activation is the up-regulation of genes producing effector proteins. This up-regulation is exerted at the level of activation of transcription of the effector genes.

p53 belongs to the acidic activator class of transcription factors (Fields, S. 1990; Raycroft, L. 1990). In vitro and in vivo experiments have shown that p53 can activate transcription of reporter genes having the p53-binding site in their promoter region (Farmer G., 1992; Michalovitz D. 1990; Fields S. 1990; Raycroft L. 1990; Martinez J 1991; reviewed in Vogelstein B. 1992; Prives C. 1999). In addition, the transcriptional activity of p53 correlates with its up-regulation in response to DNA damage (Kastan M., 1991; Zhang W., 1994). The transactivation function of p53 is responsible, at least in part, for the observed responses to p53 activation that we described before, such as growth arrest, apoptosis and antiangiogenesis (reviewed in Ko LJ. 1996; Levine AJ. 1997).

p53 exerts its transactivation function by binding to p53 response elements located in regulatory regions of the genes seen to respond to p53 transactivation function. Many genomic p53-response elements have been identified, although their position relative to the promoter varies from gene to gene. For example, the mdm2 gene has two p53 consensus sites in its second promoter (P2), located in the first intron. The cd95 gene also has the p53 cognate site in its first intron while for gadd45, the p53 cognate site is located

in the third intron. The p21/waf 1 gene, the main effector of p53-mediated G1 arrest, has two p53 response elements in its promoter, and there is evidence that p53 selectively uses one or the other depending on the signaling pathway. Other genes having p53 response element in their promoters are the p53-dependent apoptosis mediators, bax and PIG-3 (Wu X., 1993; El-Deiry W.S., 1993; El-Deiry W.S., 1995; Resnick-Silverman L., 1998; Miyashita T., 1995; Polyak K., 1997). It has been proposed that DNA loop formation might be the mechanism for p53 transactivation function (Stennger J.E., 1994).

Besides activating transcription of genes that control cell growth, prevent angiogenesis or promote apoptosis, the tumor suppressor function of p53 also involves the repression of transcription of some cellular as well as viral DNA elements. Among the DNA elements repressed by p53 are: HIV LTR, the SV40 large T antigen gene and the cellular genes c-fos IL6, c-myc and insulin receptor which are involved in promoting growth by protein-protein interactions.(Gingsberg D., 1991; Sanhtanam U., 1991; Subler M., 1992; Morberg KH., 1992; Webster NJG., 1996). Finally, p53 inhibits transcription of some antiapoptotic factors including Bcl2 and RelA (reviewed in El-Deiry ES. 1998; Ko LJ 1999). The p53 repression of transcription seems to depend on the C-Terminal domain of the protein and its ability to interact with members of the basal transcription machinery like TBP and suppress the activity of TFIID (Liu X., 1993; Cairns C.A., 1998). The idea of transcriptional repression by p53 not depending on its sequence-specific DNA binding activity is now changing, and there are reports suggesting the existence of p53-specific DNA sites for p53 transcriptional repression (Li B. 2001; Krause K. 2001). Noteworthy is the fact that p53 also inhibits the transactivation function of other transcription factors

such as the TBP, SP1, HIF-1, and STAT5. This inhibition of transcription is mainly via protein-protein interactions. (Miu X., 1993; Bargonetti J., 1997; Blagosklonny M.V., 1998; Yu C.L., 1997).

The number of genes that respond to p53 transcriptional regulation (activation/ repression) is increasing and estimated to be around 70 for each (El-Deiry W.S., 1998). Nevertheless, there are other cellular responses to p53 activation that do not depend on the function of p53 as a transcription factor; their mechanistic details are not clear yet (reviewed in Zhan QM. 1998).

1.6. P53 IS A DNA BINDING PROTEIN.

The transactivation function of p53 greatly depends on the ability of the protein to bind DNA, in a sequence specific manner, to the p53-response elements located within the p53 effector genes (reviewed in Levine AJ. 1997; Ko LJ., 1996). p53 is a sequence specific DNA binding phosphoprotein that binds as a tetramer to two adjacent copies of the palindromic consensus sequence Pu-Pu-Pu-C-A/T-T/A-G-Py-Py-Py, via a loop-helix-loop motif. The two copies have an intervening DNA segment between 0 and 13 base pairs long (Kern SE., 1991; Bargonetti J., 1991; El-Deiry WS., 1992 Jeffley PD., 1995; McLure KG., 1998; reviewed in Vogelstein B. 1992; Zambetti GP. 1993).

The sequence specific DNA binding activity of p53 increases upon cellular stress via post-translational modifications and protein-protein interactions. Although the region of

p53 that binds DNA in a sequence-specific manner is located in the middle of the protein, its C-terminal portion seems to regulate such activity. This C-terminal domain of p53 keeps p53 in a latent state for sequence specific DNA binding. The negative regulation of the binding activity of p53 can be overcome by modification to the C-terminal domain of p53. These modifications can be post-translational modifications, protein-protein interactions, proteolytic truncation or alternative splicing (reviewed in Hupp T.R., 1994; Ko L.J., 1996). For example, the product of the 14-3-3gamma gene, and the c-Abl protein increase the DNA binding activity of p53 by binding to its C- terminus, while the Replication protein A (RPA) has been shown to decrease such activity when bound to the N-terminal domain of p53. (Watermann M.J., 1998; Nie Y., 2000; Miller S.D., 1997). In general, modification to the C-terminal domain of p53 like its phosphorylation by Protein Kinase C (PKC) or cdk and its association with the PAb 421, increase p53 sequence-specific DNA binding activity (Takenaka I., 1995; Wang Y., 1995). Another modification shown to increase sequence-specific DNA binding activity of p53 is the acetylation of its C-terminal domain by p300/CBP. This in turn, which is facilitated by the phosphorylation of p53 at its N-terminus (Sakaguchi K., 1998). The binding of ssDNA to the C-terminal region of p53 also relieves the inhibition exerted by this domain on p53 DNA binding function (Jarayaman L., 1995; Bakalking G., 1995). The reduction state of p53 is another modifier of its DNA binding activity, due to the presence of seven cysteine residues in the DNA binding domain of p53. (Hainaut P., 1993; Sun Y., 1996). In agreement with this idea is the observation that thioredoxin and Ref-1 (an enzyme involved in the response to oxidative stress) are capable of stimulating p53 DNA binding (Ueno M., 1999). It seems that the DNA binding activity of p53 not only depends on the p53 status

but also on chromatin factors. For example the non-histone chromosomal protein High mobility group protein –1 (HMG-1) also increases such activity (Jarayaman L., 1998).

Besides its sequence-specific DNA binding activity, p53 has also been observed to bind DNA in a non-sequence-specific manner. p53 can bind ssDNA, as mentioned before, and this binding not only impinges upon the binding of p53 to its cognate sites but also has been seen to facilitate its annealing and strand transfer activities (Bakalking G., 1994). P53 can bind to several DNA structures that represent intermediates of DNA damage and repair. Examples of these structures are nicked or damaged DNA with free ends, mismatched duplex DNA, triple stranded DNA and Holliday junctions. (Reed M., 1995; Lee S., 1995; lee S., 1997; Dudenhoffer C., 1998). The non-sequence-specific DNA binding activity of p53 suggests a role for p53 in DNA repair, but the physiological meaning of this biochemical feature of p53 in still under investigation.

1.7. STRUCTURAL FEATURES OF P53.

The human p53 protein has 393 amino acids and structurally can be divided into three main domains. Each one of these domains is responsible for one or more of the biochemical activities attributed to the p53 protein and some of them can be further subdivided into functional sub domains.

Within the N- terminus of p53, which extends between amino acid residues 1 and 100, there is a region responsible for the transactivation function of the protein, amino acids 1-

42 (Fields S., 1990; Unger T. 1992; Miller CW. 1992). This transactivation domain interacts with several proteins involved in the regulation of the p53 function. Among these proteins are the members of the general transcription machinery TBP, TATA Associated Factors II40, II60, II31, II 70 (TAFII40) (TAFII60) (TAFII31)(TAFII70) and the p300/CBP co activator (Martin DW. 1993; Xiao H., 1994; Thut C.J., 1995; Lu H., 1995; Scolnick D.M., 1995; Lill N.L. 1997; Gu W. 1997). The amino terminal domain also plays a role in the degradation of p53 due to the fact that the interaction with MDM2 described before maps to this region (Momand G.P. 1992; Haupt R. 1997; Kubbutat M.H.G. 1997). The N-terminus of p53 is the target for several phosphorylation events that modulate its function by either changing the conformation of the protein or by blocking its association with other polypeptides (Shieh S-Y. 1997; Fuchs S.Y. 1998; Pise-Masison C.A. 1998). A proline rich sub-domain, amino acids 63-97, contains the PXXP element and is required for the full tumor suppressor activity of p53. This N-terminal proline rich sub-domain is characteristic of proteins that interact with peptides containing the SH3 domain and seems to participate in the p53 transcription-dependent and transcription-independent apoptotic response (Gorina S. 1996; Walker K.K.1996; Venot C. 1998; Sakamuro D. 1997).

The central domain of p53, amino acids 100 to 300, is responsible for its sequence specific DNA binding activity and is highly conserved among vertebrates. This domain of p53 recognizes and binds to the consensus sequence described before (Bargonetti J., 1993; Pavletich N.P. 1993; Cho Y. 1994; Pietenpol J.A. 1994; reviewed in Milner J. 1995; Zambetti GP. 1993; Ko LJ. 1996). Most of the oncogenic mutants that have been

characterized result from point mutations in this central region (Reviewed by Vogelstein B., 1992) and these mutant forms of p53 do not bind DNA in a sequence specific manner. Thus, one important event in tumor formation seems to be the inability of p53 to bind to its cognate sites in the genome. Besides being responsible for the sequence-specific DNA binding activity of p53, this central domain also exhibits some degree of non-sequence-specific DNA binding activity (Selivanova G., 1996).

The carboxyl terminal domain, encompassed by amino acid residues 300 to 393, is responsible for several of the biochemical activities of p53. First, a locus between amino acids 305 and 323 has a nuclear localization signal (Sahulsky G.1990; Addison C.1990). Second, it has the oligomerization sub-domain between amino acid residues 323 –355. This oligomerization sub-domain exhibits an alpha sheet-turn-beta helix motif, which forms a tetramer composed by a dimmer of two dimmers and is linked to the DNA binding domain by a flexible hinge (Stenger 1992; Clore G.M., 1994; Lee W., 1994; Jeffrey P.D., 1995; Waterman J.L. 1995). Mutations in the tetramerization domain of p53 have been identified in human cancers; these mutations induce a dimmeric or monomeric configuration of p53 instead of the tetrameric one and exhibit reduced specific DNA binding activity (Clore G.M., 1995; Davison T.S. 1998; Lomax M.E.1998). Third, the non-specific DNA binding activity of p53 is also located within its carboxyl terminal. This activity of p53 maps to the last 30 amino acid residues of the protein, which is a very basic region. This sub domain of p53 participates in the DNA repair and allosteric regulation of the sequence-specific DNA binding function of p53 discussed before.

Fourth, the p53 dependent repression of transcription also maps to the C-terminal region of p53.

The C-terminal portion of the p53 protein participates in several protein-protein interactions and is the target of some of the post-translational modification described before (Wang Y., 1993; Lee S. 1995; Bakalkin G., 1994; Bayle J.H., 1995; Hupp T.R., 1994; Meek W.D. 1997; reviewed by Prives C. 1998). Some of the modifications to the C-terminus of p53 correlate with in vivo increase in rate of p53-dependent transcriptional activation (Hupp T.R. 1995; Abarzua P. 1995; Caron de Fromental, 1999).

1.8. HYPOTHESIS.

As mentioned in the background, ***in vitro*** and ***in vivo*** experiments have shown that p53 has the ability to associate with other proteins and that this association modulates p53 function. Some of these interactions chemically modify p53, as is the case of the interactions with kinases and acetylases. Other interactions decrease the activity of p53 by physically blocking its activation domain or by promoting the degradation of p53 e.g. p53-MDM2 interaction. The significance of other interactions between p53 and cellular or viral proteins is still under investigation. For example, the ubiquitous transcription factor SP1 associates with p53 and the Sp1-p53 complex binds DNA. Furthermore, there is an Sp1-p53 binding motif present in some regulatory DNA regions and Sp1 binding to DNA changes in the presence of a mutant p53 which is not able to bind DNA on its own (Borellini. F. 1993; Bargonetti J. 1997; Borellini. F. 1997; Macleod, M.C. 1993).

Of great importance because of its role in transcription, is the association between p53 and TBP as well as the TATA associated factors TAF II 40 and TAF II 60 (Seto E. 1992; Liu, X. 1993; Truant, R. 1995). Mapping experiments have shown that the interaction between p53 and TBP occurs at two different sites on the p53 protein. TBP binds to the amino terminal domain of p53, which is the part of the protein, required for activating transcription. TBP also binds to the carboxyl terminal domain of p53, which is involved in the tetramerization and negative regulation of its specific DNA binding activity. TAFII40 and TAFII60, two of the TBP associated factors, bind to p53 at the amino terminus as well. (Truant R. 1992; Liu X. 1993 Horikooshi N. 1995).

Parallel experiments of co- immunoprecipitation and activation of transcription (in vitro and co-transfection) comparing wild- p53 and some p53 mutants have shown that the mutant forms of p53 that do not associate with TBP also exhibit a deficiency in the p53 dependent transactivation function (Chang J.1995). We have seen, in our laboratory, that the binding of p53 to its consensus site in the second promoter of the mdm2 gene, mdm2 P2, changes the footprint of the nearby TATA box, indicating that p53 and TBP interact in vivo (Xiao G., 1998). Because of this evidence, it has been proposed that the transactivation function of p53 is given by its ability to recruit TBP and/or TFIID to the promoter.

The experiments examining protein-protein associations involving p53 have been done with purified proteins and in some cases, with in vitro translated proteins and/or fusion proteins (TBP, p53). These experiments, although highly informative regarding possible protein-protein interactions, do not permit the analysis of such interactions under the presence of other cellular components that might modulate the protein-protein interaction. Additionally, they have not compared a number of the associations in different cell types, different cellular environment or different types of stress such as DNA damage. Another important element missing from these experiments is the contribution of the DNA (p53 binding site) as an active member of the protein complexes. Most of the experiments showing association between p53, TBP, TAFII40 and TAFII60 have been performed by co-immunoprecipitation, which means that they do not address the contribution of the p53 binding site. They give information about protein-

protein interaction but show nothing about the protein-DNA complex. Besides, given that the anti p53 antibodies do not react with all the p53 conformations in the same way, choosing one antibody for the immunoprecipitation imposes an arbitrary bias towards which p53 molecules are being studied and which ones are being excluded.

In order to bridge the gap between the co-immunoprecipitation of in vitro translated proteins type of experiments and more physiologically relevant ones, we decided to study the interaction between p53 and some of its associated proteins by DNA affinity chromatography using extracts derived from different cell types. The p53 cellular level, under normal circumstances, is very low but upon DNA damage it is increased due to decreased degradation of the protein (reviewed in Hall, P. A. 1993). Because of this and in order to have a system with higher amount of p53, we set up the experimental conditions using the temperature-sensitive (ts) mutant p53 Val 135 present in the mouse fibroblast cell line 3-4. The ts mutant p53 Val 135 provides a well-documented system for wild-type p53 dependent growth arrest, when the cells are shifted to 32 °C after being grown at 37 °C (Michalovitz, D. 1990; Martinez, J. 1991).

The DNA affinity chromatography gave us the opportunity of including different p53-binding sites as key elements in the association of p53 and other cellular proteins. The goal of this thesis has been to identify patterns of associated cellular proteins that interact with three different p53-binding sites, Ribosomal Gene Cluster (RGC) The binding site in the second promoter of the mdm2 gene (mdm2 P2) and the Super

consensus Sequence (SCS) with and without p53 bound to these DNA elements. The p53-binding site present in the promoter 2 of the mdm2 gene (mdm2 P2) participates in the p53 dependent activation of transcription of the mdm2 gene (Juven T. 1993). The p53-binding site RGC was the first one identified as cognate for p53 and is located in a non-transcribed region of the cluster, near an origin of replication (Kern SE. 1991). The physiological relevance of this site is not known. The SCS is an ideal p53-binding site that does not exist in the genome (Halazonetis, T.D., 1993). We also analyzed the p53-dependent recruitment of TFIID to the p53 sites described.

We have analyzed the temperature sensitive mutant p53 Val 135 from 3-4 cells as well as wild-type p53 expressed in baculovirus infected Sf21 cells. The p53 present in the 3-4 cells was a mouse p53, while the one expressed in the Sf21 cells corresponded to the human protein. When comparing results from these two proteins, it is important to keep in mind that they had different origins and because of that, these two forms of p53 might not behave identically. Nevertheless, there is evidence suggesting that their behavior is very similar and that physiologically, they are comparable. p53 is a highly conserved protein at the level of amino acid sequence. Knock-in experiments have shown that a chimeric mouse protein having the DNA binding domain of the human p53, behaves as a functional equivalent to the endogenous murine p53. Crystallographic analysis shows that the DNA binding domain of the mouse p53-DNA complex exhibits architectural features very similar to that exhibited by the human p53 core domain-DNA complex. It also has been shown that murine p53 can replace human p53 in competition experiments when analyzing p53-DNA complexes. In addition, hybrid oligomers (human-murine p53) have

been shown to be competent for DNA binding with a binding affinity similar to either homo-oligomer. Also, functional experiments using murine and human p53 proteins have shown the same results (Luo JL. 2001; Zhao K. 2001; Hall AR. 1995;Seto E. 1992). Finally, work done in our laboratory showed that human p53 from TR9-7 cells presented a DNA binding behavior similar to the results from the mouse protein that we present in this work. Together, this evidence suggests that the comparison between the murine and the human p53 protein presented in this work is valid if analyzed with this technical feature in mind.

2. MATERIALS AND METHODS.

2.1. CELL LINES AND EXTRACTS.

The 10-1 is a mouse fibroblast cell line that does not have p53 because the p53 gene is deleted (Martinez J.1991). The 3-4 is a stable p53 expressing cell line derived from the 10-1 cells by co-transfection with the temperature-sensitive (Ts) mutant p53-Val135 plasmid ppLTRp53cGval135 (Michelovitz D. 1990) and a Neomycin resistant one. Spodoptera frugiperda (Sf21) cells were infected with a recombinant baculovirus expressing wild-type human p53.

The 3-4 and 10-1 cells were grown at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) supplemented with 10% heat- inactivated Fetal Bovine Serum (FBS). When the plates reached 80% confluence, the cells were grown at 32 °C for 4 hours. The cells were harvested with 500 micro liters of lyses buffer per plate, 8.8 milliliters of stock buffer (Hepes pH 7.5, 20mM; Glycerol, 20%; NaCl, 10mM; $MgCl_2$, 1.5 mM; EDTA, 0.2 mM; Triton X-100, 0.1%; DTT, 1mM; PMSF, 1mM; Aprotinin, 50 micrograms/ ml.; Leupeptin, 50 micro molar.) and 6.2 milliliters of water. The cell suspension was centrifuged at 2,000 rpm for 15 minutes to pellet the nuclei. The pellet was resuspended in 500 micro liters (per plate) of nuclear lyses buffer (stock buffer 8.8 ml., NaCl 5M 1.47 ml. water 4.7 ml) and rocked for 1 hour at 4 °C. The preparation was then centrifuged in a microfuge for 10 minutes, to pellet the debris. The supernatant was recovered, aliquoted and kept at –80 °C for further use.

Sf21 cells were grown at 27 °C in TC-100 medium (GIBCO), supplemented with 10% heat-inactivated Fetal Bovine Serum. The cells were infected with recombinant baculovirus containing the human p53 gene and harvested 48 hours later by centrifugation at 2,000 rpm for 15 minutes. The pellet was resuspended in 1.6 milliliters of Cowi lyses buffer per plate. The suspension was left on ice for 30 minutes then it was spun at 2,000 rpm for 15 minutes. The supernatant was recovered and spun in a SS34 rotor at 20,000 rpm for 30 minutes, aliquoted and kept at –80 °C.

2.2. DNA AFFINITY CHROMATOGRAPHY.

Three DNA affinity columns were made containing different p53 binding sites. One of them was devised from the p53-binding site present in the Ribosomal Gene Cluster (RGC); another was from the site present in the Promoter two of the mdm2 gene (mdm2 P2) and finally, the synthetic p53-binding site Super Consensus Sequence (SCS) was also used. As a negative, control also we made an affinity column with a mutated version of the RGC site (mtRGC). The general procedure for our work is represented in Fig. 2.2.1.

The synthetic deoxyoligonucleotides used to construct the DNA affinity columns were:

RGC:

5' TCGAGTTGCCT**GGACTTGCCTGGCCTTGCCT**TTTC3'

MDM2-P2:

5'GATCCCT**GGTCAAGTTGGGACACGTCC**GGCGTCGGCTGTCGGAGG**AGCT AAGTCCTGACATGTCT**CCG3'

SCS:

5'TCGAGCC**GGGCATGTCCGGGCATGTCC**GGGCATGTC3'

mtRGC:

5' TCGAGTTTAATGGACTT**TAA**TGGCCTT**TAA**TTTTC3'

The corresponding deoxyoligonucletides were hybridized (65 ug of each ssDNA fragment) (88°C, 2 minutes; 65 °C, 10 minutes; 37°C, 10 minutes and, room temperature, 5 minutes) and phosphorylated with 5uCi of Gama ^{32}P ATP, using T4 PNK (100U) according to the manufacturer's specifications. The total reaction volume for the phosphorylation was 120 ul. The phosphorylated and hybridized DNA was then ethanol precipitated. Before coupling the corresponding DNA to the Sepharose matrix, the deoxyoligonucleotides were ligated to enrich for 10mer fragments. The ligation was carried out using T4 DNA ligase, 30U/ 200-ul total volume. The ligation reaction was performed over night at 15 °C. The extent of ligation was monitored by resolving 0.5 ul of the reaction mixture in a 15% agarose and gel visualizing the DNA with ethidium bromide Fig. 2.2.3. The columns were made by cross-linking 130ug of the respective dsDNA deoxyoligonucletides to 1 ml of CNBr activated Sepharose, according to the method of Kadonaga (Kadonaga, J.T. 1986). We used two variations of the method for the cross linking procedure. We activated the Sepharose beads with CNBr according to the regular protocol (Kadonaga, J.T. 1986). Later on, we used commercially available CNBr activated Sepharose (Pharmacia) (Current protocols in Molecular Biology, alternate protocol). We did not observe any difference in the performance of the DNA affinity columns prepared either way.

The cross linking reaction was carried out over night (12 hrs) at room temperature. After the coupling reaction, the resin was washed as indicated in protocol (Kadonaga, JT., 1986) and the binding of DNA to the Sepharose matrix was monitored by tracking the radioactivity during the washing steps.

The DNA affinity chromatography experiments were normalized for the amount of p53 loaded onto each column. In the case of the 3-4 and Sf21 cells 10 ug of p53 were used. For the 10-1 cells, the amount of total protein corresponding to that of the 3-4 cells was used, this values changed from experiment to experiment but was always between 7 and 12 mg). The Sephacryl excluded nuclear extract prepared as discussed in numeral 2.3 below, was incubated (15 minutes, room temperature) with competitor DNA (Herring sperm DNA; 5ng/ 1 ug of total protein) before loading onto the affinity column. After the incubation, the sample was passed through the corresponding DNA affinity column 10 times, at gravity flow. Then, the column was washed 4 times with 2 milliliters of buffer Z 0.1 molar KCl and eluted with 1 milliliter fractions of buffer Z with increasing salt concentration (KCl, 0.2 to 1.0 molar). The fractions were collected and kept at –80 °C for further use. Buffer Z was 25 mM Hepes (K^+), pH 7.8/ 12.5 mM $MgCl_2$ / 1mM dithiothreitol/ 20% (vol/vol) glycerol/ 0.1% (vol/vol) Nonident P-40. A diagram for the DNA affinity chromatography is presented in Fig. 2.2.2.

The theoretical binding capacity for the DNA affinity columns were calculated based on the formula 7nmoles/1 ml of resin/ 20 bp binding site (Kadonaga, J.T. 1986) and assuming p53 was bound as a tetramer. According to Kadonaga, JT. 1986, the DNA affinity columns yield 30% of the calculated binding capacity.

A. Conversion factor for size of the oligo:

RGC: 35bp/20bp = 1.75

Mdm2 P2: 68bp/20bp = 3.4

SCS: 36bp/20 bp = 1.8

B. Theoretical nmoles of p53/1 ml resin:

RGC: 7nmoles /1.75= 4nmoles

Mdm2 P2: 7nmoles /3.4 = 2nmoles

SCS: 7nmoles / 1.8 = 3.9 nmoles

C. Theoretical binding capacity of the DNA affinity columns or amount of p53/1ml of resin:

0.1nmol of monomeric p53 = 5.3 ug .

0.1 nmol of tetrameric p53 = 21.2 ug.

RGC: 4 nmol x (21.2 ug/ 0.1nmol) = 848 ug.

Mdm2 P2: 2 nmol x (21.2 ug/ 0.1 nmol) = 424 ug.

SCS: 3.9 nmol x (21.2 ug/ 0.1 nmol) = 826.8 ug.

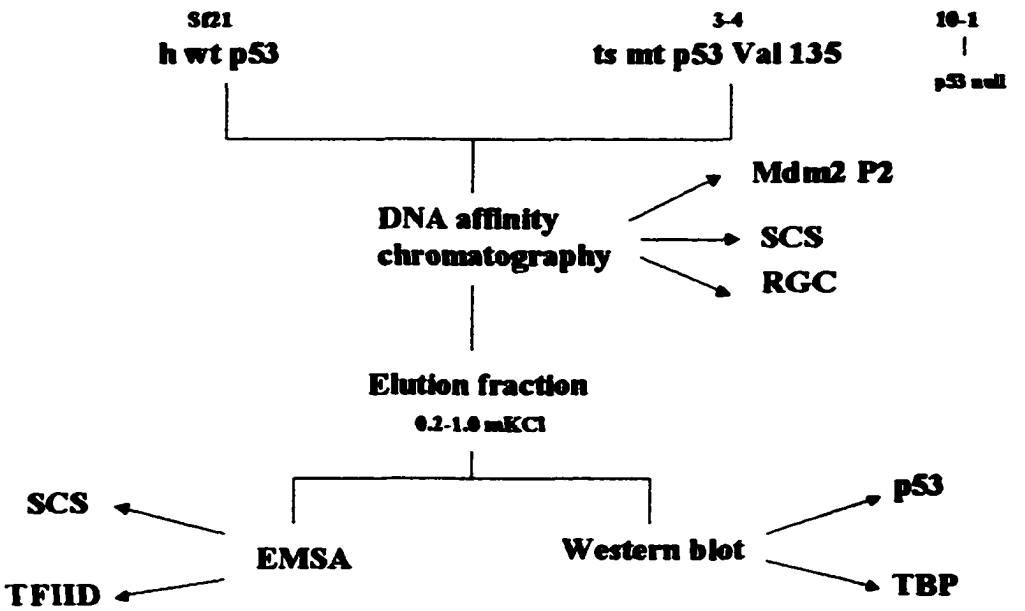
Theoretical maximum amount of p53 that can be eluted from the DNA affinity columns: 30% of C.

RGC: 848 ug x (30/100) = 254.4 ug of p53

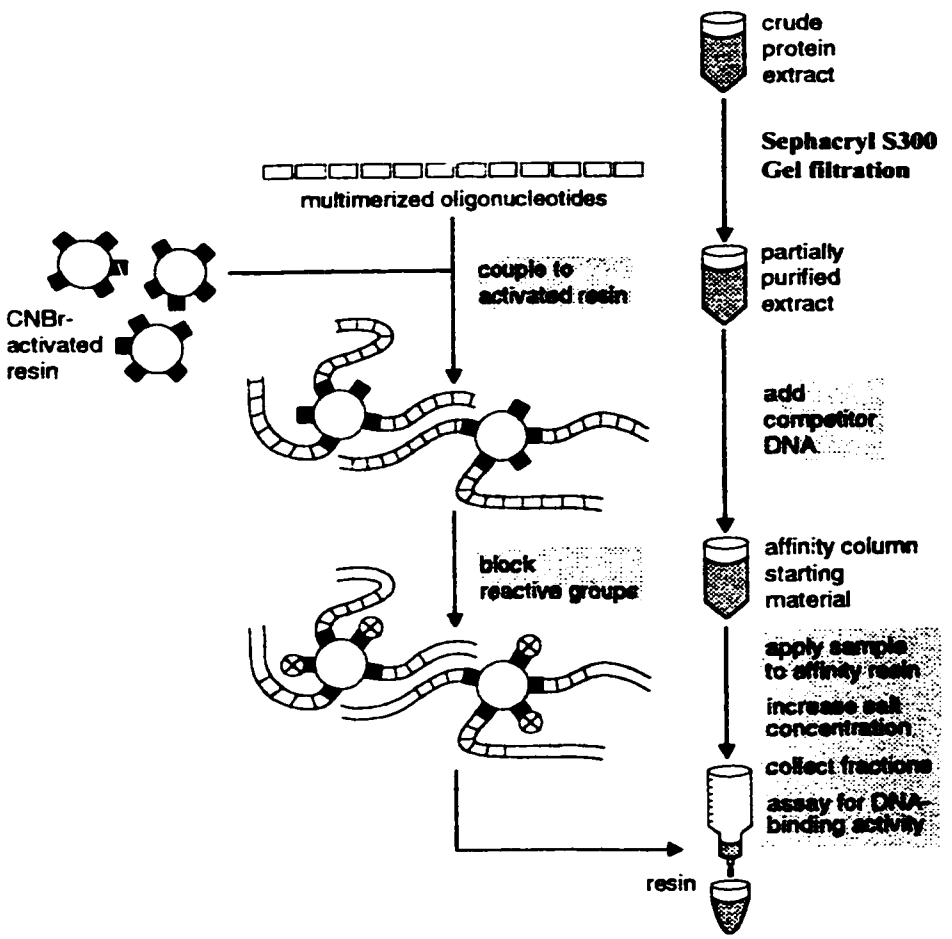
Mdm2 P2: 424 ug x (30/100) = 127.2 ug of p53.

SCS: 826.8 ug x (30/100) = 248 ug of p53.

2.2.1. General Procedure.

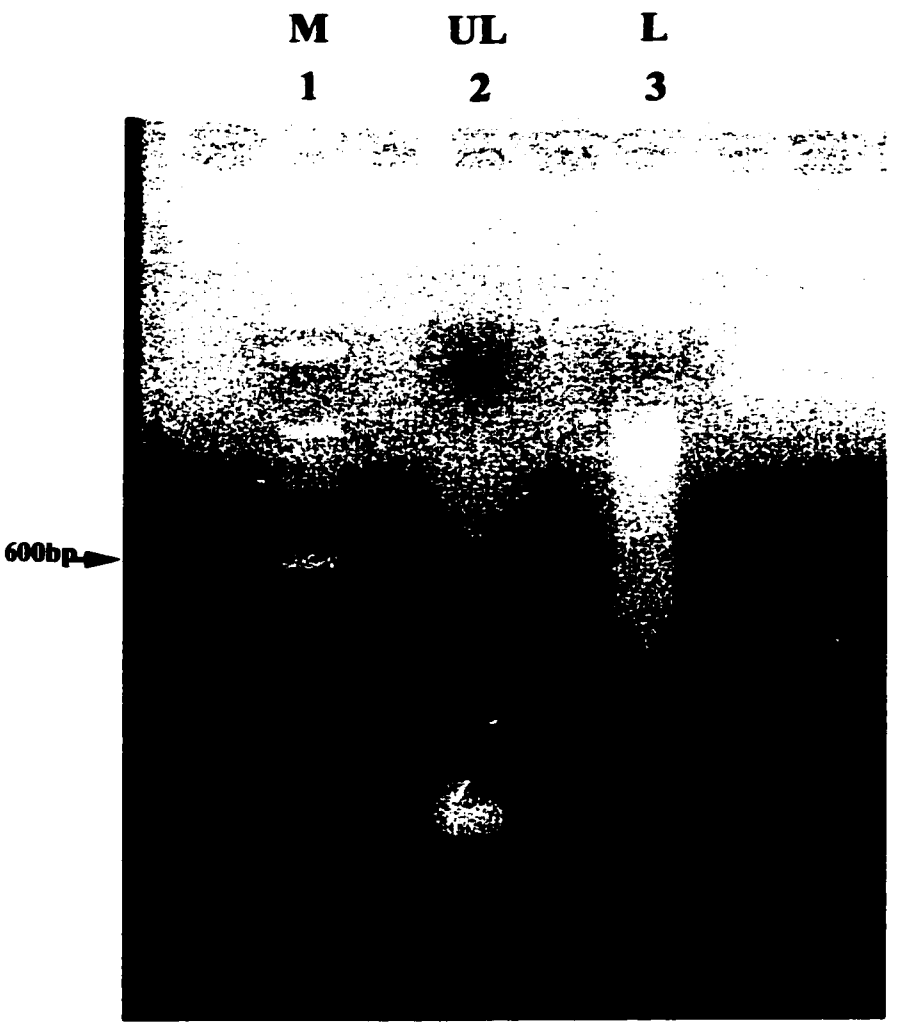


2.2.2. Diagram of DNA affinity chromatography.



Adapted from "Methods in Molecular Biology".

2.2.3. Ligation of the Mdm2 P2 deoxyoligonucleotide.



1 ul of the ligation reaction was resolved in a 4% agarose gel and then visualized by ethidium bromide staining. Lane 1, M, DNA ladder; lane 2, UL, I ul of the reaction mixture before ligation; lane 3, L, 1 ul of the reaction mixture after over night ligation.

2.3. SEPHACRYL S300 GEL FILTRATION.

The crude nuclear extracts were passed through a column made of Sephacryl S300 (Pharmacia) and the gel filtration procedure was performed according to the manufacturer's specifications. The gel filtration column was twenty-five (25) centimeters in length and two (2) centimeters in diameter. 0.1 molar KCl Tris buffer (TM^{+}) was used to run the column. Twenty-five (25) fractions, 1 milliliter each, were collected. The first ten (10) fractions were discarded (void volume) and the remaining fifteen (15) fractions were pooled and stored at – 80°C.

2.4. ELECTROPHORETIC MOBILITY SHIFT ASSAY.

The corresponding deoxyoligonucleotides were radio labeled with ^{32} P using Klenow enzyme. The samples and ^{32}P labeled synthetic deoxyoligonucleotides (Operon) were incubated under DNA binding conditions (Hepes pH 7.8, 20mMolar; KCl, 100mMolar; EDTA, 1mMolar; Glycerol, 10%; DTT, 1mMolar; salmon sperm DNA,1ug per 30 micro liters reaction). The incubation time was 20 minutes. The samples were then resolved on a 4% nondenaturating acrylamide gel and visualized by autoradiography. The deoxyoligonucletides used were Super Consensus Sequence (SCS) (Operon) to detect p53 DNA binding activity and TFIID consensus oligonucleotide (Santa Cruz) to detect TBP DNA binding activity.

2.5. WESTERN BLOT.

The samples were resolved by SDS-PAGE on a 10% acrylamide gel and transferred over night at 0.3 amperes, to a nitrocellulose membrane. The membranes were probed with the corresponding antibodies as indicated. For p53 we used PAb 421, 1801 and 240 produced in hybridoma cells, Dr. Jill Bargonetti laboratory and for TFIID we used anti TBP from Santa Cruz.

2.6. METABOLIC LABELING WITH ^{35}S.

The cells were grown in Dulbeco's Modified Eagle Medium (95 % Methionine free and 5% complete) supplemented with ^{35}S Methionine (20 uCi/ml.) for 5 hours. The proteins were resolved by SDS-PAGE electrophoresis and visualized by autoradiography.

2.7. QUANTIFICATION.

The experiments were normalized either for total protein or for the amount of p53. The Bradford method (Biorad) was used to determine the amount of total protein contained in the nuclear extracts as well as in the Sephacryl fraction pool. The amount of p53 was determined by densitometry and analyzed using Image Quant software.

CHAPTER 3.

SITE SPECIFIC ISOLATION OF P53 BY MDM2 P2 AFFINITY CHROMATOGRAPHY.

3.1. INTRODUCTION.

p53 and MDM2 proteins are two members of a negative feedback loop mechanism in which p53 activates transcription of the mdm2 gene and the mdm2 gene product mediates the inactivation and degradation of p53. The MDM2 protein promotes degradation of p53, in part by binding to the amino terminal domain of p53. In this way, MDM2 targets p53 for degradation by the ubiquitin pathway, given that MDM2 acts as an E3 ligase in the ubiquitin degradation system. Another way in which MDM2 facilitates degradation of p53 is by shuttling p53 from the nucleus to the cytoplasm where the degradation of the protein takes place. Besides promoting its degradation, the binding of MDM2 to the N-terminus of p53 also results in its inactivation because this binding compromises the transactivation domain of p53 (reviewed in El-Deiry WS. 1998; Prives C. 1999; Woods DB. 2001).

The mdm2 gene has two promoter regions. One of these promoters (P1) is located upstream of the first exon and regulates the transcription of the mdm2 gene in a p53 independent manner. The second promoter (P2) on the other hand, is located within the first intron and regulates transcription of the mdm2 gene in a p53 dependent manner (Barak Y. 1994). p53 activates transcription of the mdm2 gene via the binding of p53 to a cognate site present in the second promoter of the mdm2 gene (Xiangwei W. 1993; Juven T. 1993; Zauberman A. 1995).

To make the mdm2 P2 DNA affinity column, we used a synthetic deoxyoligonucleotide that corresponded to the sequence of the p53-binding site present in the second promoter of the mouse mdm2 gene. We analyzed the binding of human wild-type p53 expressed in Sf21 and the binding of the mouse temperature sensitive mutant p53 Val 135 expressed in the 3-4 cells. The p53 expressed in the Sf21 cells was used as a control to evaluate the functionality and binding capacity of the DNA affinity column. The p53 present in the 3-4 is a conditional mutant and, when activated, produces G1 growth arrest via the activation of transcription of p21/waf1, demonstrating that at 32 °C it has a bonafide wild-type p53 activity in this system.

3.2. RESULTS.

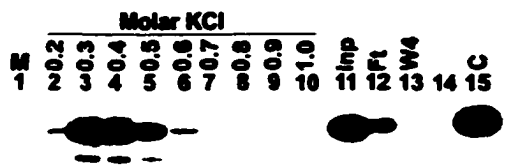
3.2.1. MDM2 P2 ISOLATION OF WILD TYPE P53.

We isolated the baculovirus expressed human wtp53 by mdm2 P2 DNA affinity chromatography (Fig. 3.2.1a and 3.2.1b).

When analyzed the MDM2 P2 elution fractions by Western blot, we observed p53 eluting from the mdm2 P2 site column, mainly in fractions 0.3 to 0.5 molar KCl, (Fig.3.2.1a, lanes 3 to 5). It is important to notice that we did not detect p53 in the last wash, before the salt gradient elution indicating that the p53 observed in the elution fractions was not a remnant of the loaded material (Fig. 3.2.1a, lane 13). This fact indicated therefore that the eluted material was bound to the mdm2 P2 site specifically. This result correlated with the one observed when we analyzed the mdm2 P2 elution fractions by EMSA. For the EMSA analysis we used a deoxyoligonucleotide corresponding to the super consensus sequence as a probe. 5% of each elution fraction was incubated with a ^{32}P labeled p53 Super Consensus Sequence (SCS). PAb 421 anti- p53 antibody was used, where specified, to activate p53 binding to DNA (Hupp TR.1995). The addition of this antibody is a well-established practice although it does not activate the binding of all p53 forms. p53 eluted mainly in fractions 0.3 to 0.5 molar KCl (Fig. 3.2.1b, lanes 11 to 13); although in this case, we detected p53 in all the elution fractions (Fig. 3.2.1b, lanes 10 to 17). The EMSA appeared to be a more sensitive technique compared to Western blot analysis for examining the elution fractions. Noteworthy, we did not detect p53 in wash number 4

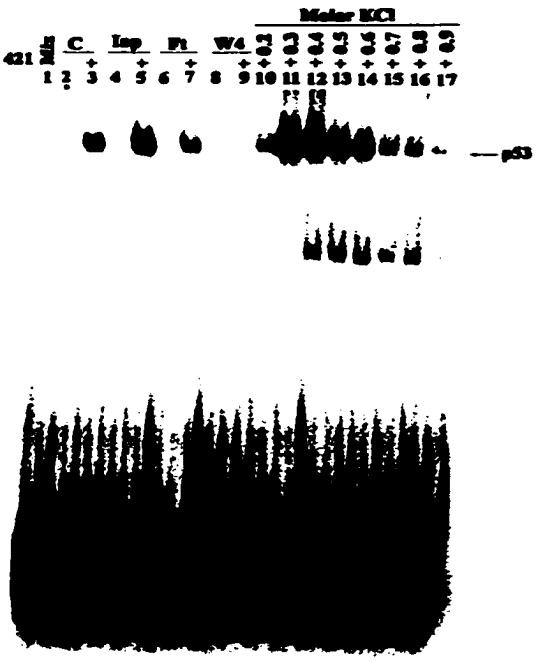
(Fig. 3.2.1b, lanes 8 and 9). The amount of p53 eluted from the mdm2 affinity columns was much less (in the order of nanograms) than the amount loaded (10 micrograms) and much less than the calculated binding capacity of the mdm2 affinity column according to the method described by Kadonaga JT. 1968. These results were reproducible in at least 3 independent experiments. These results showed that wild-type human p53 expressed in Sf21 cells could be successfully isolated by DNA Affinity Chromatography using the p53-binding site present in the promoter 2 of the mdm2 gene (mdm2 P2).

3.2.1a. Western blot analysis of the mdm2 P2 binding -competent wt p53.



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from insect cell extract, was loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by western blot. 80% of each elution fraction (0.2 to 1.0 Molar KCl) from the mdm2 P2 column was resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with a mix of anti- p53 monoclonal antibodies 1801, 421 and 240. Molecular marker, lane 1; mdm2 P2 elution fractions 0.2 to 1.0 molar KCl, lanes 2 to 10; 0.1% input, lane 11; 0.1% flow-through, lane 12; 0.1% of wash four, lane 13;C, 30 nanograms of p53 contained in the Sf21 cells extract as control, lane 15.

3.2.1b. EMSA analysis, on SCS oligo, of the mdm2 P2 competent wt p53.



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from insect cell extract, was loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the p53 binding site SCS; Pab 421 was used, where indicated, to activate p53 binding. Mix, no protein, lane 1; C, control p53 contained in the insect cell extract, lanes 2,3; Inp, 0.1% of input, lanes 4,5; FT, 0.1% of flow-through, lanes 6,7; W4, 0.1% of wash four, lanes 8,9; mdm2 P2 elution fractions 0.2 to 0.9 Molar KCl, lanes 10 to 17.

3.2.2. MDM2 P2 ISOLATION OF THE TS mt P53 VAL 135.

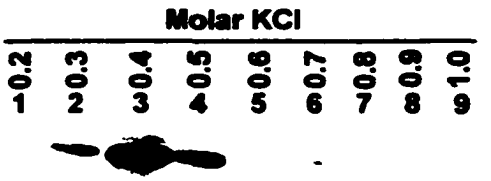
In order to isolate p53 from a mammalian cell line using DNA affinity chromatography, we used 3-4 cells expressing the temperature sensitive (ts) mutant p53 Val 135. The Ts mt p53 Val 135 adopts a wild type conformation when the 3-4 cells are shifted to the permissive temperature (32 °C). We performed the experiment as described previously for the wt p53 and we used Western blot and EMSA to analyze the elution fractions. As a negative control for proteins that bind to the column in a p53 independent manner, we also performed the experiment using equivalent amounts of total protein contained in the Sephacryl excluded nuclear extract from 10-1 cells (which are isogenic to 3-4 cells but do not have p53).

In the case of the 3-4-cell extract, p53 eluted from the mdm2 P2 site column in fractions 0.3 to 0.5 molar KCl as observed by Western blot (Fig 3.2.2a, lanes 2 to 4) and by EMSA (Fig. 3.2.2b, lanes 11 to 13). This p53 was PAb 421 responsive as seen by the induced binding produced by this antibody in the EMSA analysis. The Western blot and the EMSA results presented here (Fig. 3.2.2a and 3.2.2b respectively) correspond to two different experiments. The elution of the mt p53 Val 135 from the mdm2 P2 affinity column was reproducible regarding the elution profile in more than three independent experiments, but the amount of the protein eluted from the column decreased substantially (for reasons unclear) after the first experiment. It is noteworthy that in all cases, only a small fraction (on the order of nanograms) of the total p53 loaded onto the column (10 ug) was tightly bound to it and not lost during the extensive washing. This

reduced amount of p53 bound to the column was not the result of column saturation or degradation, given that the same column was able to bind a greater amount of a control p53 preparation in a subsequent experiment (data not shown). As expected, we did not observe the band corresponding to p53 when we analyzed the mdm2 P2 site elution fractions using 10-1 cells extract (no p53) (compare Fig. 3.2.2c, lanes 10 to 18 to Fig. 3.2.2b, lanes 11 to 13).

These results show that the ts mt p53 Val 135 from the mammalian cell line 3-4 could be successfully isolated by DNA affinity Chromatography using the p53-binding site present in the promoter 2 of the mdm2 gene (mdm2 P2). Our results also showed that the amount of p53 eluted from the column was much less than either the amount loaded or the amount corresponding to the experimental binding capacity of the column.

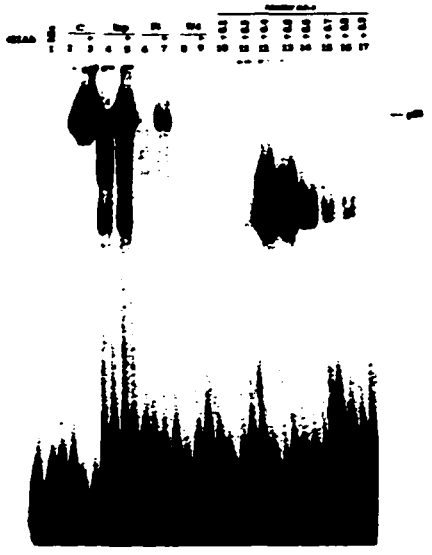
3.2.2a. Western blot analysis of the mdm2 P2 competent Val 135 p53.



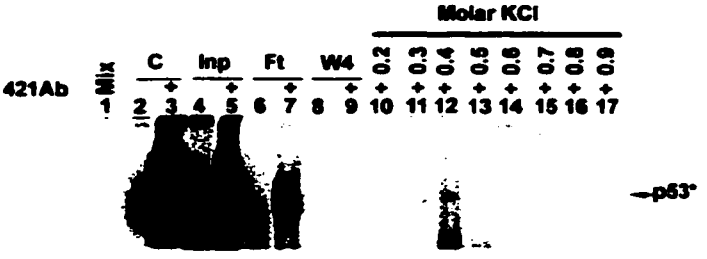
10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells, was loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. 80% of each elution fraction (0.2 to 1.0 molar KCl) was resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with a mix of anti p53 antibodies 1801, 421 and 240. Mdm2 P2 elution fractions, lanes 1 to 9.

3.2.2b. EMSA analysis, on SCS oligo, of the mdm2 P2 competent Val 135.

A. Complete gel.



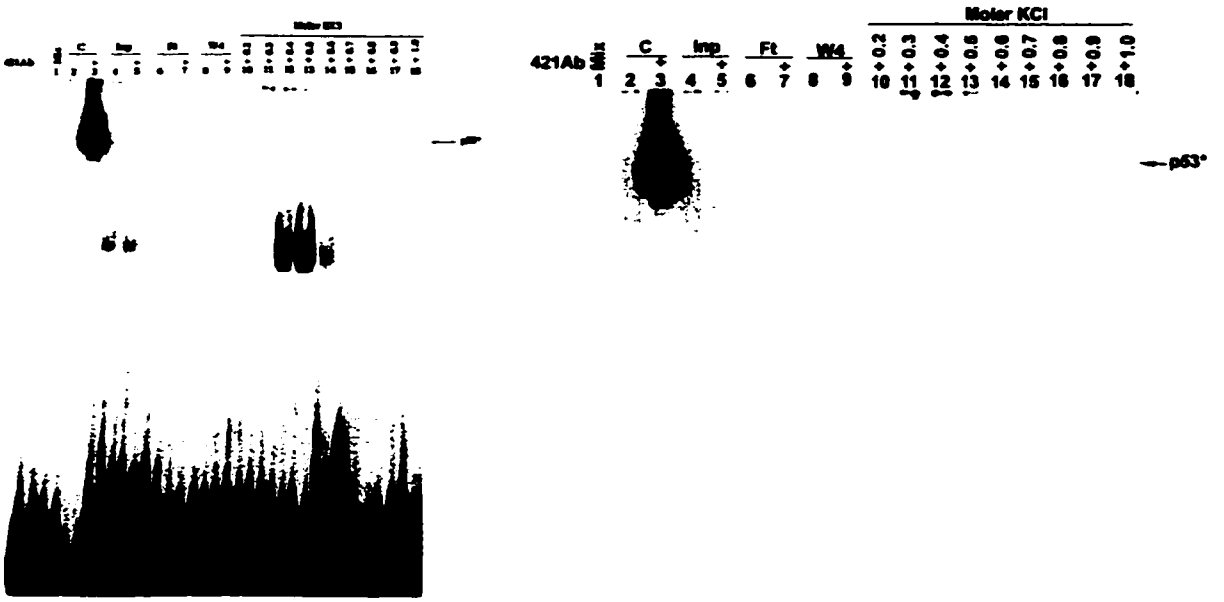
B. Blow-up of Fig. A



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract, was loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the p53 binding site (SCS); 421 antibody was used, where indicated by +, to activate p53 binding. Mix, with no protein, lane 1; C, control, p53 contained in the insect cell extract, lanes 2,3; Inp, 0.1% of input, lanes 4,5; FT, 0.1% of flow-through, lanes 6,7; W4, 0.1% of wash four, lanes 8,9; mdm2 P2 elution fractions 0.2 to 0.9 Molar KCl, lanes 10 to 17.

3.2.2c. EMSA, on SCS oligo, of the mdm2 P2 elution fractions from 10-1 cells.

A. Complete Gel. **B. Blow-up of Fig. A.**



7 mg of total protein (normalized to the 3-4 cells experiment) contained in the Sephacryl S300 fraction pool from 10-1 cells nuclear extract was loaded onto the mdm2 P2 affinity column and the DNA affinity chromatography was performed as specified in methods. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to a p53 binding site (SCS); 421 antibody was used, where indicated, to activate p53 binding. Mix, no protein, lane 1; C, control, p53 contained in insect cells extract, lanes 2,3; Inp, 0.1% of input, lanes 4,5; FT, 0.1% of flow-through, lanes 6,7; W4, 0.1% of wash four, lanes 8,9; mdm2 P2 elution fractions 0.2 to 1.0 Molar KCl, lanes 10 to 18.

3.3. DISCUSSION.

We isolated the wild-type p53 expressed in Sf21 cells and the Ts mt p53 Val 135, expressed in 3-4 cells, using mdm2 P2 DNA affinity chromatography. We detected p53 in the mdm2 P2 elution fractions by Western blot, using antibodies against p53 and also by EMSA. In the EMSA analysis, the identity of the p53* band as being p53 protein is confirmed by its migration pattern, as this band is the result of a super shift induced by the PAb 421, and by its absence in the elution fractions from the 10-1 cells which do not have p53. The fact that no p53 was detected in the last wash (W4) indicated that the p53 detected in the elution fractions was tightly bound to the mdm2 P2 DNA site specifically and was not a remnant from the loaded material.

The amount of wt p53 bound to the column was much less than the amount loaded. This result might be due to the presence of different forms of p53 molecules in the sample, p53 molecules with differential binding characteristics "bindomers" and that the mdm2 P2 site selected for those p53 "bindomers" that were mdm2 P2 competent. Wild-type p53 has been seen to swath between a wild-type and a mutant conformation as a response to cellular conditions such as addition of fresh serum after starvation, and oxidative status (Milner J., 1990; Hainaut P., 1993; Hainaut P., 1995). p53 binds to its cognate site as a tetramer made by a dimmer of dimmers. When only one dimmer, in the tetramer, is in the wild-type conformation, the tetramer binds to DNA in a sequence-specific manner but this binding is short lived, lasting about 15 seconds. On the other hand, when both dimmers are in the wild-type conformation, the binding is stabilized and p53 stays bound to its cognate site for about 20 minutes (Mc Lure KG., 1998; Mc Lure, KG., 1999). The

cellular equilibrium between ATP/ADP also influences the stability of the p53-DNA complex, with the ADP increasing the binding stability (Okorokov AL. 1999).

On the other hand, the amount of wild-type p53 eluted from the mdm2 P2 column might reflect the experimental binding capacity of the column as opposed to the theoretical one. It is also noteworthy that the source of p53 seemed to influence the amount of p53 that was bound to the mdm2 P2 column. Our results consistently showed more p53 eluting from this column when we used the insect cell extract and much less p53 when coming from the 3-4 cells. This fact was not the due to the column saturation or degradation as evidenced by control experiments done with the same column. These results suggest that the mdm2 P2 DNA site selected for a sub-population of the p53 molecules present in the 3-4 extract. The unique characteristic(s) of these p53 molecules remains to be uncovered, although we can speculate that these characteristics might be given, at least in part, by differential post-translational modifications to the p53 protein. The lower amount of p53 from 3-4 cells bound to the mdm2 P2 affinity column compared with the amount bound from the Sf21 cells might also be due to that fact that the p53 expressed in the 3-4 cells is a mutant protein. For this reason, its binding might be less efficient. We are inclined to believe that this difference in binding is due to the presence of the different "bindomers", because the 3-4 cells expressed p53 adopts a wild type conformation under the experimental conditions. Besides, it is physiologically comparable to wild-type p53 given that it produces a p53 dependent G1 growth arrest when activated.

Another result suggesting the differential binding characteristics among the p53 molecules present in the sample was the fact that the amount of Val 135 p53 eluted from the mdm2 P2 column decreased substantially after the first experiment. All the experimental conditions were kept constant during subsequent experiments except for the batch of fetal bovine serum used to grow the 3-4 cells. After this change the amount of p53 eluted from the mdm2 P2 column was hardly detectable by Western blot. This result suggests that the new serum produced a dramatic change in the amount of cellular p53 molecules able to bind to the mdm2 P2 column under our experimental conditions.

Our results suggest that the wild-type p53 and the mt p53 Val 135 were bound to the mdm2 P2 affinity column with similar affinity (0.3 molar main elution). More wild-type p53 was bound to the mdm2 P2 column compared to the Ts Val 135 mt p53 from the 3-4 cells. This fact might be due to a higher stability of binding.

4. SITE SPECIFIC ISOLATION OF WILD-TYPE P53 BY RGC AFFINITY CHROMATOGRAPHY.

4.1. INTRODUCTION.

The Ribosomal Gene Cluster (RGC) is a genomic tandem array of several copies of a series of genes that code for the different ribosomal RNAs. Besides the coding regions, the Ribosomal Gene Cluster has several non-transcribed regions and in one of these non-transcribed regions there is a cognate site for p53. The p53 cognate site in the RGC is located near an origin of replication and was the first site to be identified as bound specifically by p53, but its physiological meaning remains to be elucidated.

To make the RGC DNA affinity column, we used a synthetic deoxyoligonucleotide that corresponds to the sequence of the p53-binding site present in the Ribosomal Gene Cluster (as described in the Materials and Methods. We then performed the experiments as described for the mdm2 P2 affinity column.

4.2. RESULTS.

4.2.1. RGC ISOLATION OF WILD-TYPE P53.

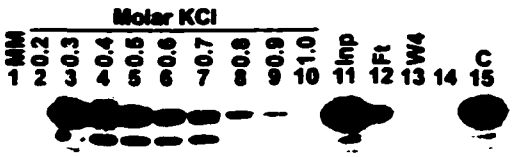
We isolated the RGC DNA binding-site competent p53 by DNA affinity chromatography using baculovirus expressed human wt p53.

When analyzed by Western blot, we observed p53 eluting from the RGC site column, mainly in fractions 0.3 to 0.7 molar KCl, (Fig. 4.2.1a, lanes 3 to 7). Similarly to the mdm2 P2 elution fractions analysis, we did not detect p53 in the last wash (W4) before the salt gradient elution (Fig. 4.2.1a, lane 13) indicating the specific binding of wild-type p53 to the RGC column. The EMSA analysis showed p53 eluting mainly in fractions 0.3 to 0.8 molar KCl (Fig. 4.2.1b, lanes 11 to 16). Again, in this case we detected p53 in all the elution fractions (Fig. 4.2.1b, lanes 10 to 17) indicating that EMSA was more sensitive to detect p53 in the elution fractions compared to Western-blot. The binding specificity of wt p53 to the RGC column was again manifested by the absence of detectable p53 binding activity in the last wash (W4; Fig. 4.2.1b, lanes 8, 9) and its reappearance in the salt elution fractions. These results show that human wt p53 expressed in Sf21 cells, could also be isolated by DNA affinity Chromatography using the p53-binding site present in the Ribosomal Gene Cluster (RGC) and the result of reproducible experiments.

As in the case of the mdm2 P2 affinity column, the amount of wild-type p53 eluted from

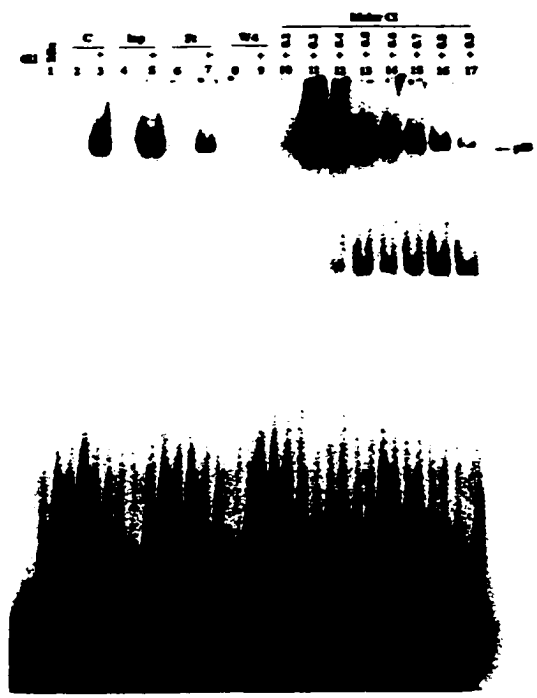
the RGC affinity column was below the calculated binding capacity of the column.

4.2.1a. Western blot analysis of the RGC competent wt p53.



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from insect cell extract were loaded onto the RGC affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by Western blot. 80% of each elution fraction (0.2 to 1.0 Molar KCl) from the RGC column was resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with a mix of anti p53 monoclonal antibodies 1801, 421 and 240. MM, Molecular marker Lane 1; RGC elution fractions 0.2 to 1.0 molar KCl, lanes 2 to 10; Inp, 0.1% input, lane 11; Ft, 0.1% flow-through lane, 12; W4, 0.1% of wash four, lane 13;C, control, 30 ng of p53 contained in the Sf21 cell extract, lane 15.

4.2.1b. EMSA , on SCS oligo, of the RGC competent wt p53.



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from insect cell extract were loaded onto the RGC affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the p53 binding site (SCS); 421 antibody was used, where indicated, to activate p53 binding. Mix, no protein, lane 1; C, control p53 contained in the Sf21 cell extract, lanes 2,3; Inp, 0.1% of input, lanes 4,5; FT, 0.1% of flow-through, lanes 6,7; W4, 0.1% of wash four, lanes 8,9; RGC elution fractions 0.2 to 0.9 Molar KCl, lanes 10 to 17.

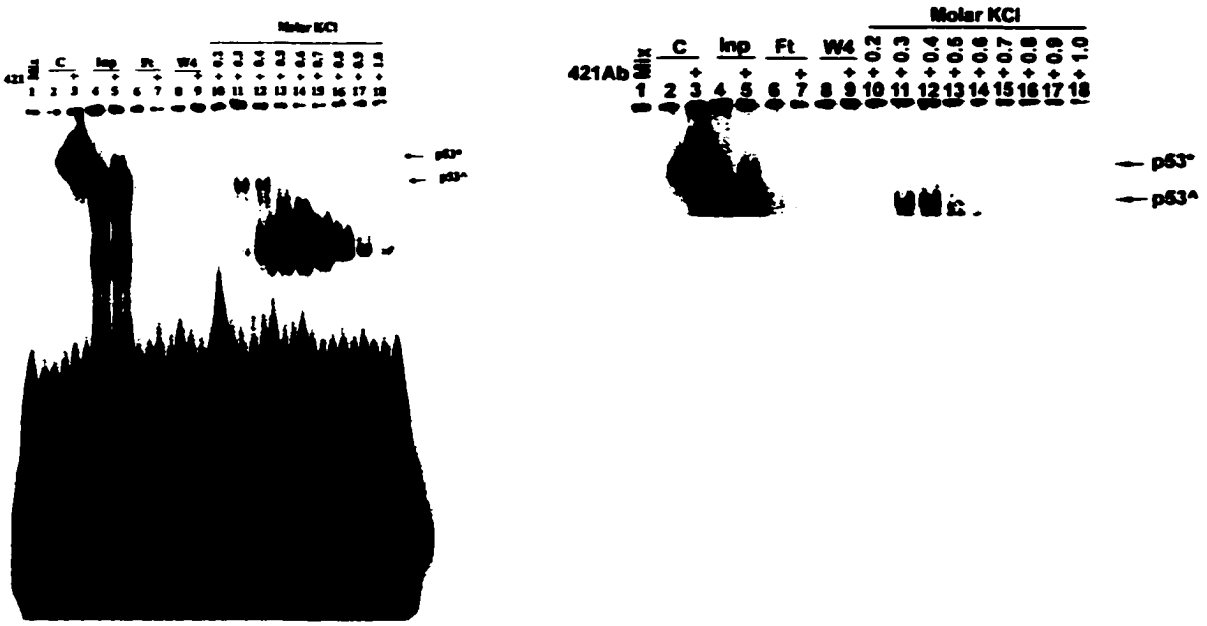
4.2.2. RGC ISOLATION OF THE ts mt P53 VAL 135.

To analyze the binding of a mammalian derived p53 to the RGC site we again used the 3-4 cell line system and performed RGC affinity chromatography as described before. We analyzed the RGC elution fractions by EMSA because of the higher sensitivity exhibited by this technique in the previous experiments, compared to Western blot. We performed the experiments as described for the mdm2 P2 affinity column.

In the case of the 3-4-cell extract, we saw a band (p53^) eluting with fractions 0.3 to 0.5 molar KCl from the RGC site column (Fig 4.2.2a, lanes 11 to 13). This p53^ was not PAb 421 responsive as seen by the lack of super shift produced by this antibody. As a control we performed the experiment with cellular extract from 10-1 cells. In this case we did not observe any DNA binding activity in the elution fractions, Fig. 4.2.2b.

4.2.2a. EMSA analysis, on SCS oligo, of the RGC competent p53 Val 135.

A. Complete gel. **B. Blow-up of Fig. A.**

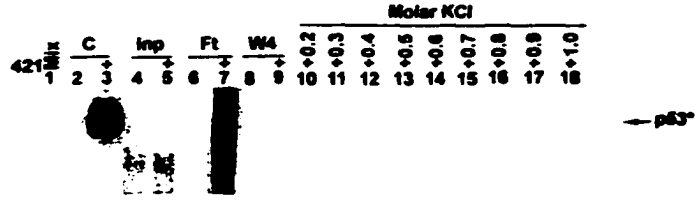


10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract were loaded onto the RGC affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the p53-binding site (SCS); 421 antibody was used, where indicated, to activate p53 binding. Mix, no protein, lane 1; C, control p53 contained in Sf21 cell extract, lanes 2,3; Inp, 0.1% of input, lanes 4,5; FT, 0.1% of flow-through, lanes 6,7; W4, 0.1% of wash four, lanes 8,9; RGC elution fractions 0.2 to 1.0 Molar KCl, lanes 10 to 18.

4.2.2b. EMSA analysis, on SCS oligo, of the RGC elution fractions from 10-1 cells.

A. Complete Gel.

B. Blow-up of Fig. A.



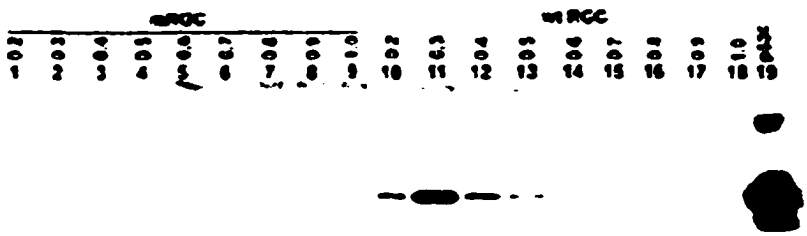
7 mg. of total protein (normalized to experiment 4.2.2a) contained in the Sephacryl S300 fraction pool from 10-1 cells nuclear extract were loaded onto the RGC affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the p53 binding site (SCS); 421 antibody was used, where indicated, to activate p53 binding. Mix, no protein, lane 1; C, control p53 contained in Sf21 cell extract, lanes 2,3; Inp, 0.1% of input, lanes 4,5; FT, 0.1% of flow-through, lanes 6,7; W4, 0.1% of wash four, lanes 8,9; RGC elution fractions 0.2 to 1.0 Molar KCl, lanes 10 to 18.

4.2.3. BINDING OF WT P53 TO THE RGC COLUMN IS P53-COGNATE SITE SPECIFIC.

We observed in the previous experiments that p53 could be isolated using the p53-cognate sites mdm2 P2 and RGC. The fact that we did not observe p53 in the last wash, in either case, mdm2 P2 or RGC, and that the protein reappeared later on with increasing salt concentrations, indicated that the binding of p53 to these DNA affinity columns was specific for DNA. Nevertheless, it did not show if this binding was specific for the p53-cognate site or for DNA in general. In order to determine if p53 could be isolated by DNA affinity chromatography using any DNA fragment or if its isolation was p53-cognate site-specific, we performed DNA affinity chromatography using a nonspecific DNA affinity column. The column was constructed with a mutant version of the RGC site, (mRGC). We run the mRGC column in parallel with a RGC column as described previously (under the same experimental conditions, Fig. 4.2.1). We analyzed the elution fractions by Western blot.

We did not observe p53 in the elution fractions from the mRGC site column (Fig. 4.2.3, lanes 1 to 9). On the other hand, p53 was evident in the elution fractions from the wild-type RGC column (wtRGC) (Fig. 4.2.3, lanes 10 to 13). This result indicated that the binding of p53 to the mdm2 P2 site and to the RGC site columns was specific for these two p53-cognate sites and not a general DNA binding effect.

4.2.3. Western blot analysis of wt RGC and mt RGC elution fractions.



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from insect cell extract was loaded onto the each affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by Western blot. 80% of each elution fraction (0.2 to 1.0 Molar KCl) was resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with a mix of anti p53 monoclonal antibodies 1801, 421 and 240. Mutant p53 site elution fractions 0.2 to 1.0 molar KCl, lanes 1 to 9; wild-type p53 site elution fractions 0.2 to 1.0 molar KCl, lanes 10 to 18; p53c, 30 ng of p53 contained in SF21 cell extract, lane 10.

4.3. DISCUSSION.

We isolated Sf21 cells expressed wild-type p53, using RGC DNA affinity chromatography. We detected p53 in the RGC elution fractions by Western blot and EMSA. In the EMSA analysis, the criteria to confirm the identity of the p53* band as being p53 protein were the same ones used for the mdm2 P2 elution fractions i.e. migration pattern and induction of binding by PAb421. Again, we observed that the binding of wt p53 to the RGC columns was specific given that we did not detect p53 binding activity in the wash four. The amount of p53 bound to the column was much less than the amount loaded and below the theoretical binding capacity of the RGC affinity column. In this regard, our results from the RGC affinity column reflected the same pattern exhibited by the mdm2 P2 affinity column and the same discussion presented in that chapter (3.3) applies here.

Our results from the RGC affinity chromatography of the 3-4 cells showed a band that seems to be p53 but whose identity could not be confirmed (p53^). This species eluted from a p53 specific site affinity column (RGC), it was bound specifically to another p53 cognate sequence in the EMSA analysis (SCS), migrated as p53 in the EMSA and was not present in the RGC elution fractions from the 10-1 cells, which do not have p53. All this evidence suggests that the p53^ band corresponds to wild-type p53. Nevertheless, the fact that we could not super shift this band or induce it to bind more with the PAb 421 raised doubt about its identity.

The sequence-specific binding of p53 to DNA is stimulated by modifications to its c-terminal domain. One of these modifications is the interaction of p53 with the Pab 421 whose epitope is in its carboxyl end (Funk WD., 1992; Mundt M., 1997). The addition of Pab 421 to the reaction mixture in EMSA experiments has been a common practice, to induce p53 binding. Although, it has been shown that not all types of p53 respond to this activation, i.e. p53 molecules phosphorylated at the c-terminus by PKC. Another finding is that the activation of binding by this antibody does not apply to all p53 cognates sites, even more, that Pab 421 can inhibit the binding of p53 (Resnick-Silverman L., 1998). This is another possibility that would explain why this band did not respond to the PAb 421. One way of confirming the presence of the Val 135 p53 in the RGC elution fractions would have been to perform immunodetection by Western blot and EMSA using other anti p53 antibodies. We could not try this approach because we concentrated in characterizing the mdm2 P2 elution fractions.

In this case (RGC affinity chromatography), the source of p53 seemed to influence not only the amount of protein that was bound to the RGC column, but also the kind of peptide. Our results consistently showed a substantial and identifiable amount of p53 eluting from the RGC affinity column when we used the insect cell extract. In contrast, when coming from the 3-4 cells, our results showed an RGC binding protein that either was not p53 or was a p53 protein with a different migration profile when analyzed by EMSA. This protein bound much less to the RGC site compared with the wild-type p53 expressed in Sf21 cells.

CHAPTER 5.

ISOLATION OF p53 ASSOCIATED PROTEINS BY DNA AFFINITY CHROMATOGRAPHY.

5.1. INTRODUCTION.

The association of p53 with other proteins is an important factor to consider when analyzing p53 function. It has been reported that p53 can associate with several proteins such as SP1, BP1, BP2, MDM2, TFIIH and others (reviewed in Hupp TR., 2000; Prives C., 1999 and Levine A., 1997). Of special importance because of their role in transcription is the association between p53 and TBP, TAFII40 and TAFII60. Most of the reported associations between p53 and the mentioned proteins have been studied in reconstituted solutions using in vitro translated or purified p53 and TFIID proteins, and these associations have been detected by co-immunoprecipitation experiments. These types of experiments do not account for all of the post-translational modifications of p53 under different cellular conditions, for the participation of other proteins in this process or for the presence of the DNA element in determining the association. Besides, the immunoprecipitation approach carries an intrinsic bias because not all the p53 forms react with all the anti-p53 antibodies.

In order to study p53 and associated proteins in a different (and perhaps more physiological environment) we looked at the proteins that co-eluted with p53 from each

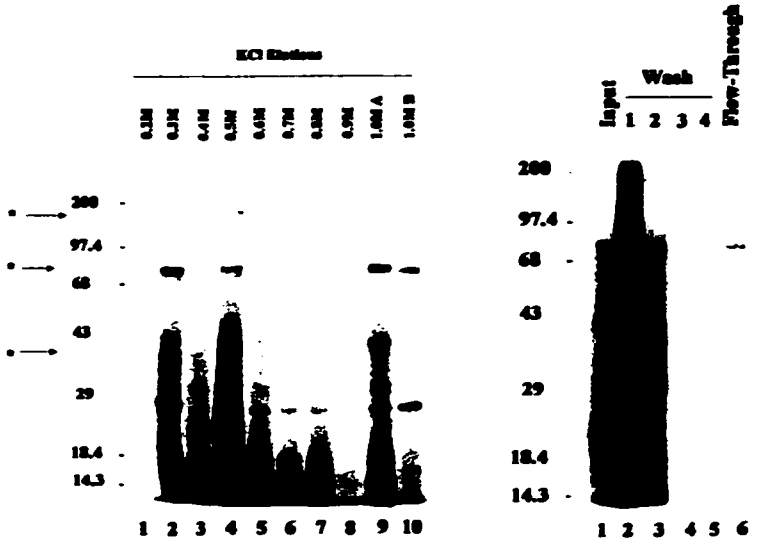
one of the DNA affinity columns (mdm2 P2 and RGC) using nuclear extract from the 3-4 cells.

5.2. RESULTS.

5.2.1. ISOLATION OF P53 ASSOCIATED PROTEINS BY MDM2 P2 AND RGC AFFINITY CHROMATOGRAPHY.

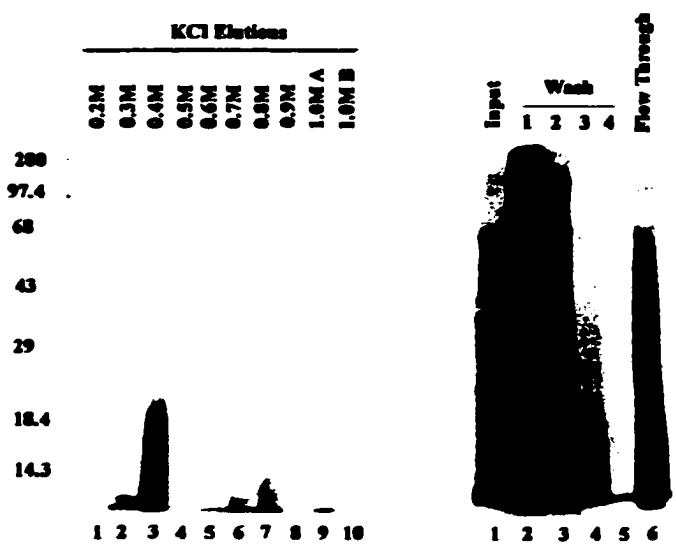
In order to study the p53 co-eluting proteins, we performed mdm2 P2 affinity chromatography. We analyzed the proteins by SDS-PAGE after biotynilation. We observed a group of proteins that co-purified with p53 from the mdm2 P2 affinity column (Fig. 5.2.1a, left panel, lanes 2 to 10). These co-purifying proteins seemed to bind either to the mdm2 P2 column or to p53 specifically because they did not appear in the last wash (Fig. 5.2.1a, right panel, lane 5). Noteworthy, we did not observe these p53 co-eluting proteins in the mdm2 P2 elution fractions from the 10-1 cells, which did not have p53 (Fig. 5.2.1b, lanes 1 to 10). We could see p53 by immunodetection of these mdm2 P2 elution fractions of the 3-4 cells; nevertheless we could not see it by the biotinylation technique.

5.2.1a. Biotinylated mdm2 P2 elution fractions from 3-4 cells.



10ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cell extract, were loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as described in methods. The elution fractions were biotinylated, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized by chemoluminescence. Left panel: Elution fractions 0.2 to 1.0 molar KCl, lane 1 to 1 Right panel: Input, 1% of the input, lane 1; Wash, washes 1 to 4, lanes 2 to 5; Flow through, 1% of flow-through, lane 6.

5.2.1b. Biotinylated mdm2 P2 elution fractions from 10-1 cells.



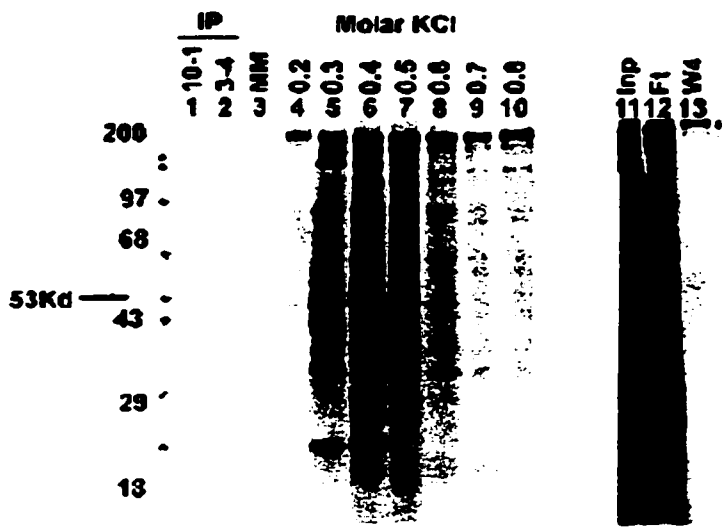
5 mg. of total protein from the 10-1 cells were loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as described in methods. The elution fractions were biotinylated, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized by chemoluminescence. Left panel: Elution fractions 0.2 to 1.0 molar KCl, lane 1 to 10. Right panel: Input, 1% of the input, lane 1; Wash, washes 1 to 4, lanes 2 to 5; Flow through, 1% of flow-through, lane 6.

In order to identify p53-associated proteins, it is important to detect all the proteins that co-eluted with p53 from the mdm2 affinity column. We had chosen the biotinylation of such proteins to accomplish this goal but this technique was not appropriate because not all the proteins present in the sample seemed to incorporate the biotin moiety to the same extent. The differential biotinylation was evident because we did not detect p53 in the biotinylated mdm2 P2 elution fractions of the 3-4 cells (Fig. 5.2.1a, lanes 1-10). Nevertheless we detected p53 in elution fractions 0.3, 0.4 and 0.5 Molar KCl when we analyzed these elution fractions by Western blot. In addition, we chose to label the cellular proteins with ^{35}S. We grew the 3-4 cells in DMEM containing ^{35}S Methionine, prepared the nuclear extract and Sephacryl S300 fraction pool, as described in methods. Then, we used this Sephacryl S300 fraction pool preparation to perform mdm2 P2 and RGC affinity chromatography experiments. We included an immunoprecipitation from 10-1 (no p53) and 3-4 (ts. Val 135 p53) cells as a control for those proteins that coimmunoprecipitate with p53 in the absence of the DNA binding site.

Our results showed that a series of proteins co-eluted with p53 from the mdm2 P2 affinity column (Fig. 5.2.1c) as well as from the RGC affinity column (Fig. 5.2.1d). The co-eluting proteins were not a remnant of the loaded material because they were not present in the last wash of the mdm2 P2 affinity column (Fig. 5.2.1c, lane 13) or in the last wash of the RGC affinity column (Fig.5.2.1d, lane 15). These co-eluting proteins reappeared in the elution gradient, mainly with fraction 0.3 molar KCl and after (Fig. 5.2.1c, lanes 5 to 10 for the mdm2 P2 site and Fig. 5.2.1d, lanes, 5 to 12 for the RGC site). As a control for the position of p53 in the gel, we immunoprecipitated p53 from ^{35}S labeled 3-4 and 10-1

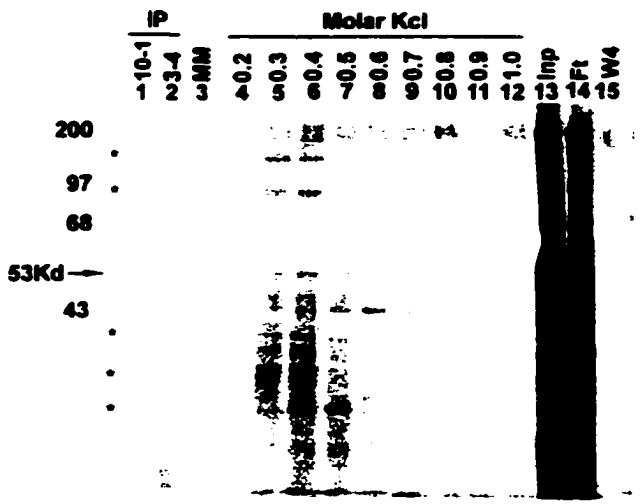
cells nuclear extract, using the anti p53 PAb 421. The arrow marked as 53 Kd, points to the band corresponding to p53 in the control lanes (Fig.5.2.1c lane 2 and Fig. 5.2.1d, lane 2). The stars show the most prominent bands in each case. The fact that many proteins co-eluted with p53 from both DNA columns made it difficult to determine the identity of each band at this point. To identify some of the co-eluting proteins we then analyzed the elution fractions using EMSA. This experiment was performed once.

5.2.1c. Auto radiography of Val 135 p53 associated proteins from the mdm2 P2 elution fractions.



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from nuclear extract of 3-4 cells that had been metabolically labeled with ^{35}S Methionine, were loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were resolved by SDS-PAGE and visualized by auto radiography. 10-1 IP, 10-1 cells anti p53 immunoprecipitation, lane 1; 3-4 IP, 3-4 cells anti p53 immunopecipitation lane 2; MM, molecular marker, lane 3; MDM2 P2 elution fractions 0.2 to 0.8 molar KCl, lane 4 to 10; Inp, 0.1% of input, lane 11; Ft, 0.1% of flow-through, lane 12; W4, 0.1% of wash four, lane 13.

5.2.1d. Auto radiography of p53 associated proteins from the RGC affinity column.



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from nuclear extract of 3-4 cells that had been metabolically labeled with ^{35}S Methionine, were loaded onto the RGC affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were resolved by SDS-PAGE and visualized by auto radiography. 10-1 IP, 10-1 cells anti p53 immunoprecipitation, lane 1; 3-4 IP, 3-4 cells anti p53 immunopecipitation lane 2; MM, molecular marker, lane 3; RGC elution fractions 0.2 to 1.0 molar KCl, lane 4 to12; Inp, 0.1% of input, lane 13; Ft, 0.1% of flow-through, lane 42;W4, 0.1% of wash four, lane 15.

5.2.2. P53 DEPENDENT SUPERSHIFT OF A TBP COMPLEX

p53 and the TATA Binding Protein, TBP, have been reported to co-immunoprecipitate. It has also been reported that when p53 dependent protection of the mdm2 P2 promoter occurred, a concomitant increase in protection of the nearby TATA box was seen, (Xiao, et al, 1998). These results suggest that p53 plays a role in recruiting TBP to the DNA. We wanted to see if TBP co-eluted with p53 from the DNA affinity columns. We performed mdm2 P2 and RGC affinity chromatography as described in the Materials and Methods chapter using 3-4 cells nuclear extract. We analyzed the elution fractions by EMSA using a radio labeled TATA box as a probe. 5% of each elution fraction was incubated with a TATA Box (TBP consensus oligo, Santa Cruz).

The mdm2 P2 elution fractions from 3-4 cells showed a band that eluted mainly with fractions 0.4 to 0.8 molar KCl. (Fig.5.2.2a lanes, 8 to 12). We think this band corresponded to TBP plus other proteins and because of that we called it TBP complex. Surprisingly, this TBP complex was also present in the mdm2 P2 elution fractions of the 10-1 cells (no p53) (fig. 5.2.2b, lanes 8 to11). This result indicated that the binding of this TBP complex to the p53-binding site present in the mdm2 P2 was not p53 dependent.

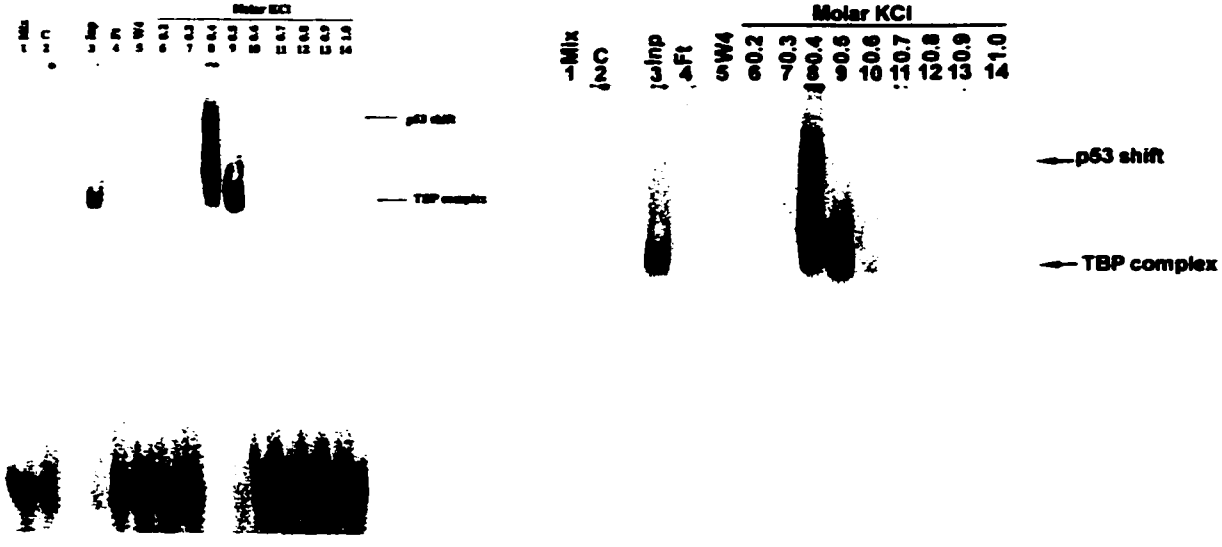
Although the binding of the observed TBP complex to the mdm2 P2 affinity column occurred without p53, we observed a p53 dependent super shift of this TBP complex band in the mdm2 P2 fractions (Fig. 5.2.2a, lane 8). This super shift correlated with the presence of p53 in this elution fraction (Fig. 3.2.2b, lane 12). The observed super shift

was p53 dependent because it did not occur in the mdm2 P2 elution fractions of the 10-1 cells (no p53) (Fig. 5.2.2b, lanes 7 to 14). This result suggests that the mdm2 P2 binding p53 associated with this TBP complex in the presence of the mdm2 P2 DNA.

The presence of p53 in the sample did not seem to influence the affinity or stability of binding of the TBP complex to the mdm2 P2 affinity column. We observed it eluting at the same salt concentrations, 0.4 molar KCl, either with or without p53 (Fig5.2.2a, lane 8, and Fig. 5.2.2b, lane 8). This result was observed in two independent experiments.

5.2.2a. EMSA on TATA Box, of the mdm2 P2 elution fractions from 3-4 cells.

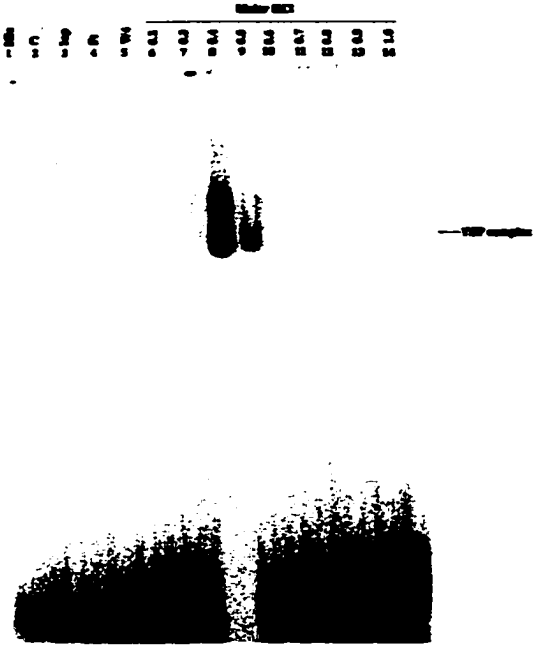
A. Complete gel. **B. Blow-up of Fig. A.**



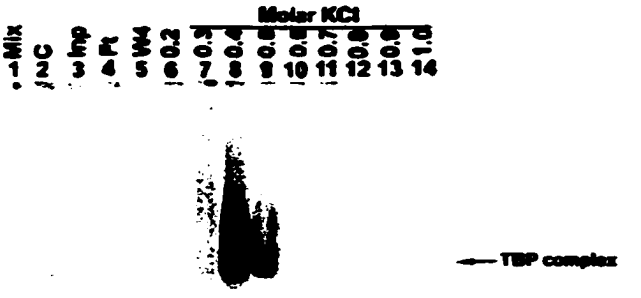
10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract, were loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. The mdm2 P2 elution fractions were then analyzed by EMSA, as described in methods. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to a TATA box (TFIID consensus oligo, Santa Cruz). Mix, no protein, lane 1; C, control, p53 contained in Sf21 cells extract, lane 2; Inp, 0.1% of input, lane 3;Ft, 0.1% of flow-though, lane 4; W4, 0.1 % of wash four, lane 5; mdm2 P2 elution fractions 0.2 to 1.0 molar KCl, lanes 6 to 14.

5.2.2b. EMSA, on TATA Box, of the mdm2 P2 elution fractions from 10-1 cells.

A. Complete gel.



B. Blow-up of Fig. A.



7 mg. of total protein (normalized to experiment 5.2.2a) contained in the Sephacryl S300 fraction pool from 10-1 cells nuclear extract, were loaded onto the mdm2 P2 affinity column and the experiment was conducted as for 5.2.2a. Mix, no protein, lane 1; C, control, p53 contained in Sf21 cells extract, lane 2; Inp, 0.1% of input, lane 3;Ft, 0.1% of flow-through, lane 4; W4, 0.1 % of wash four, lane 5; mdm2 P2 elution fractions 0.2 to 1.0 molar KCl, lanes 6 to 14.

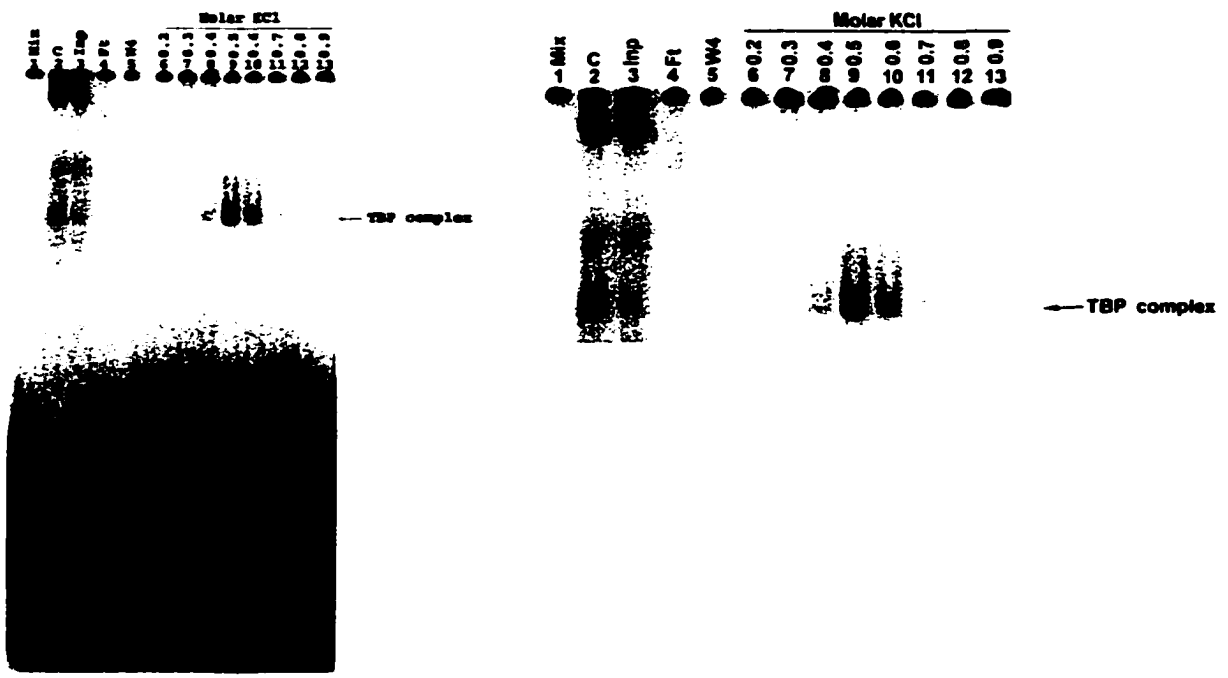
In the case of the RGC elution fractions of the 3-4 cells, we detected the TBP complex eluting mainly, with fractions 0.5 and 0.6 molar KCl (Fig. 5.2.2c, lanes 9 and 10). It is noteworthy that in the case of the RGC elution fractions, we did not detect the p53 dependent super shift of this TBP complex seen in the elution fractions of the mdmP2 column (compare Fig. 5.2.2c lanes 9 and 10 with Fig. 5.2.2a, lane 8).

As in the case of the mdm2 P2 elution fractions, we also observed this TBP complex in the RGC elution fractions of the 10-1 cells (no p53) (Fig, 5.2.2d, lanes 9 and 10). This result shows that the binding of this TBP complex to the RGC column was not specific for the p53-binding site present in the mdm2 P2 and further suggested that this binding was not p53 dependent.

Again, the presence of p53 in the sample did not seem to influence the affinity of this TBP complex for the RGC site. In both cases, with and without p53 (3-4 and 10-1 cells) the elution profiles were similar: 0.5, 0.6 molar KCl (Fig. 5.2.2c, lanes 9,10 and Fig. 5.2.2d, lanes 9,10).

5.2.2c. EMSA, on TATA Box, of the RGC elution fractions from 3-4 cells.

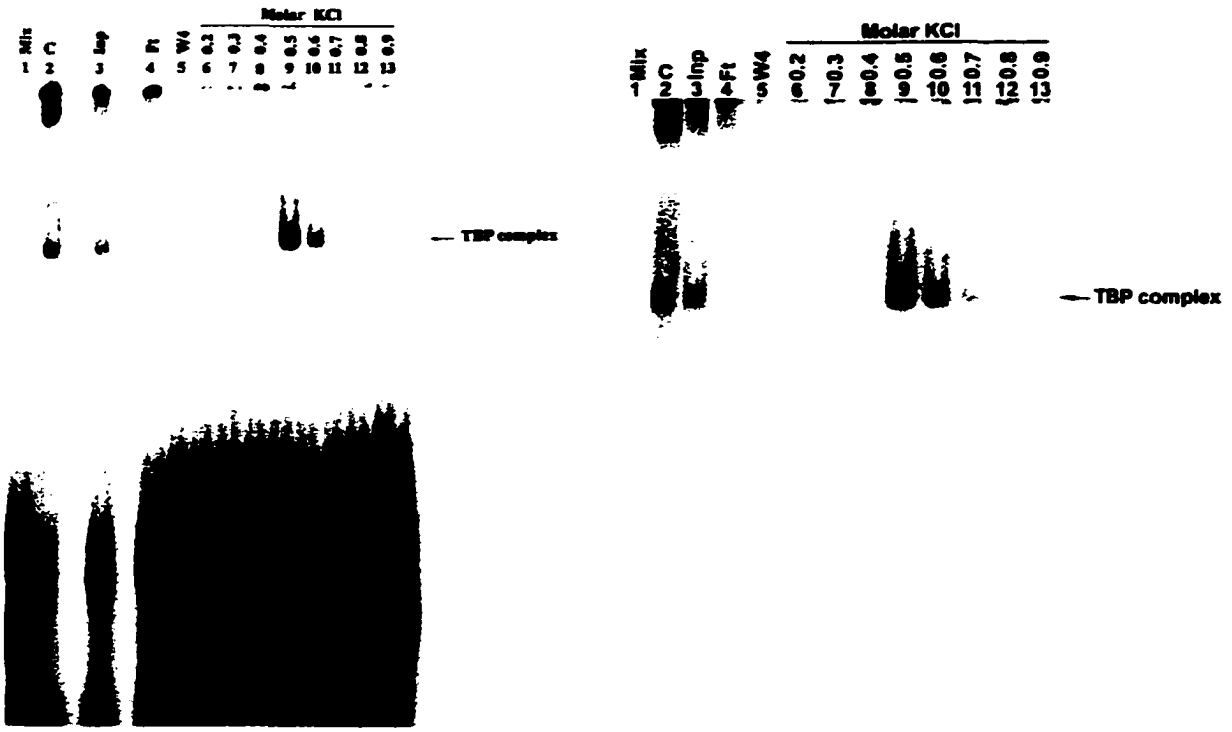
A. Complete gel. **B. Blow-up of Fig. A.**



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract, were loaded onto the RGC affinity column and DNA affinity chromatography was performed as specified in methods. The RGC elution fractions were then analyzed by EMSA, as described in methods. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to a TATA box (TFIID consensus oligo, Santa Cruz). Mix, no protein, lane 1; C, control p53 contained in Sf21 cell extract, lane 2; Inp, 0.1% of input, lane 3; Ft, 0.1% of flow-though, lane 4;W4, 0.1 % of wash four, lane 5; RGC elution fractions 0.2 to 0.9 molar KCl, lanes 6 to 13.

5.2.2d. EMSA, on TATA Box, of the RGC elution fractions from 10-1 cells.

A. Complete gel. **B. Blow-up of Fig. A.**



7 mg. of total protein (normalized to experiment 5.2.2c) contained in the Sephacryl S 300 fraction pool from 10-1 cells nuclear extract, were loaded onto a RGC affinity column and the experiment was conducted as described. The RGC elution fractions were then analyzed by EMSA. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to a TATA box (TFIID consensus oligo, Santa Cruz). Mix, no protein, lane 1; C, control p53 contained in Sf21 cell extract, lane 2; Inp, 0.1% of input, lane 3; Ft, 0.1% of flow-through, lane 4;W4, 0.1% of wash four, lane 5; RGC elution fractions 0.2 to 0.9 molar KCl, lanes 6 to 13.

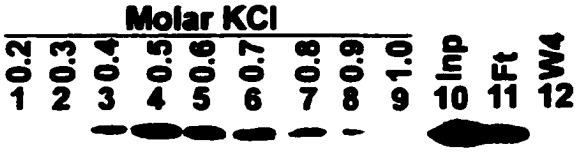
5.2.3. TBP COMPLEX FROM 3-4 CELLS CONTAINS TBP, TAFII40 AND TAFII60.

In order to identify some of the proteins present in what we called TBP complex, we analyzed the mdm2 P2 elution fractions of the 3-4 cells by Western blot and immunodetection. The nitrocellulose membrane was probed with anti-TBP antibody (Santa Cruz). Our results showed the presence TBP eluting with fractions 0.4 to 0.9 molar KCl (Fig. 5.2.3a, lanes 3 to 8). This result correlated with the presence of the TBP complex in these fractions (Fig. 5.2.2a, lanes 8 to 12) confirming that TBP was a member of this TBP complex.

In order to corroborate the presence of TBP in the TBP complex band, we performed EMSA analysis with anti TBP antibody to produce a super shift and using a TBP consensus oligonucleotide as a probe. 5% of elution fraction 0.5 molar KCl from the mdm2 P2 column was incubated with TBP consensus sequence (Santa Cruz). This fraction contained the TBP complex alone and did not exhibit the p53 dependent super shift. The anti-TBP antibody (generous gift from Dr. Roeder Laboratory) was included in the reaction mixture, as indicated, to produce an Ab dependent super shift of the TBP complex (5.2.3b lanes 2,3). As expected, 2ul of the anti TBP preparation produced a slower migrating species compared with the TBP complex band (Fig.5.2.3b, lane 2). This slower migrating species was not observed in the control lane, which had no antibody (Fig.5.2.3.b, lane 4). When the amount of anti TBP antibody was increased to 6 ul, there was a corresponding increase in the amount of the new species observed (Fig. 5. 2.3b, lane 3). This result further suggested that TBP was present in this TBP complex.

Given that TAFII40 and TAFII60 have been seen to associate with p53 in co-immunoprecipitation experiments using purified proteins, we wanted to know if these other 2 members of the TFIID were present in the TBP protein complex. We repeated the experiment presented in fig. 5.2.3b but this time, we included anti TAFII40 and anti TAFII60 antibodies (generous gift from Dr. Roeder Laboratory) in the reaction mixtures as indicated (Fig. 5.2.3c). Our results suggested the presence of TAFII40 and TAFII60 as evidenced by the induction of a slower migrating species compared with the TBP protein complex (Fig. 5.2.3c, lane 3 for TAFII40 and lane 4 for TAFII60). This antibody-induced species was not observed when a non-specific antibody (Sp1) was included in the reaction mixture (fig. 5.2.3c lane 2) or when no antibody was included (fig. 5.2.3c, lane 1). These experiments were performed only once.

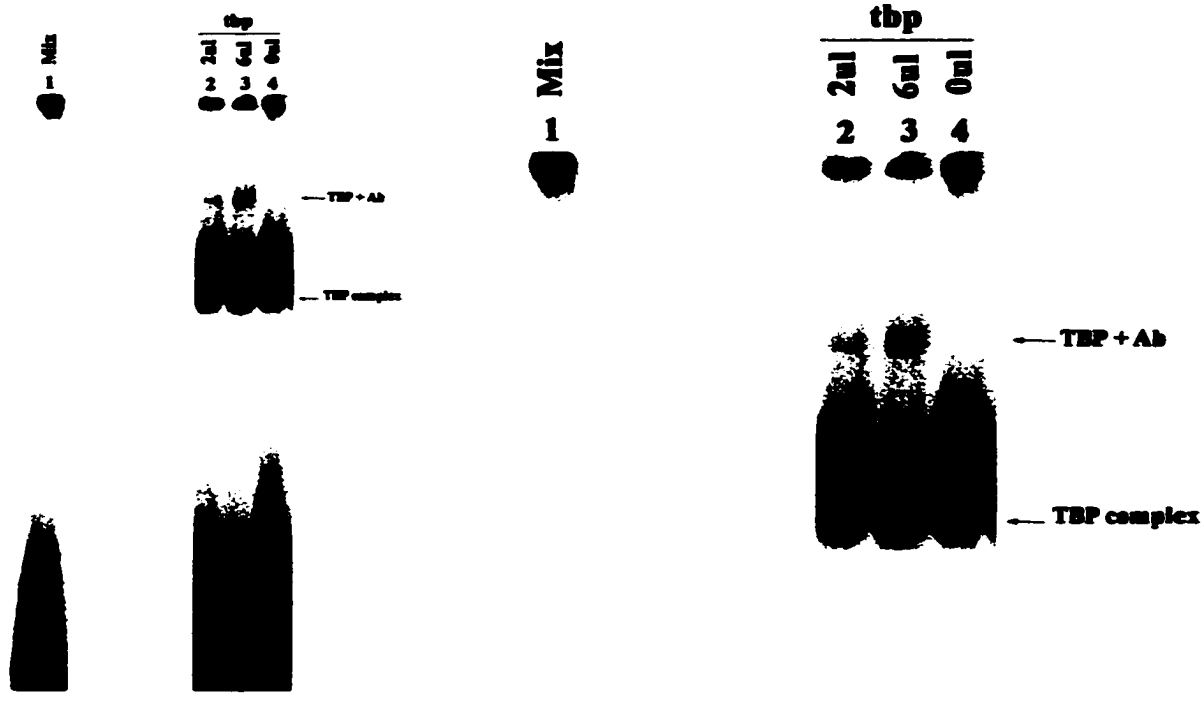
5.2.3a. <u>TBP complex from 3-4 cells contains TBP; Western blot Analysis.</u>



10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract were loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as described before. 80% of each elution fraction was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti TBP antibody (Santa Cruz). Mdm2 P2 elution fractions 0.2 to 1.0 molar KCl, lanes 1 to 9; Inp, 0.1 % of input, lane 10; Ft, 0.1% of flow-through, lane 11; W4, 0.1% of wash four, lane 12.

5.2.3b. TBP complex from 3-4 cells contains TBP; EMSA on TATA Box.

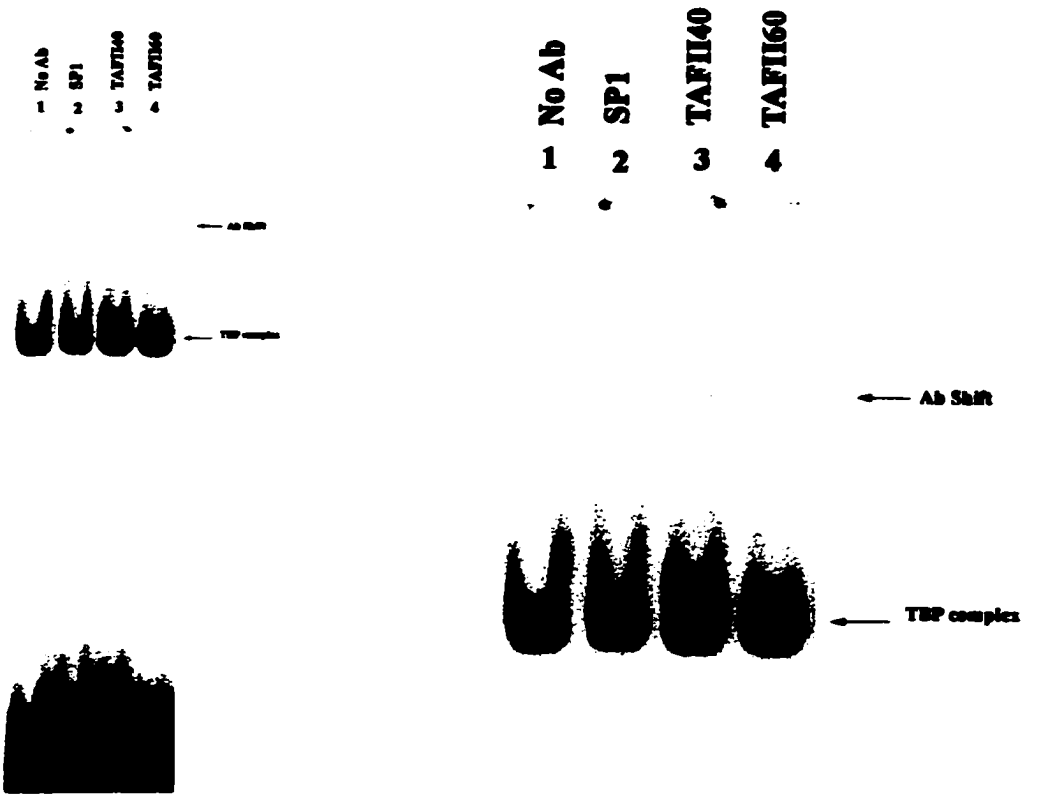
A. Complete gel. **B. Blow-up of Fig. A.**



5% of 3-4 cells mdm2 P2 elution fraction 0.5 molar KCl was incubated with ^{32}P labeled TATA Box (Santa Cruz). Different amounts of a polyclonal anti-TBP antibody preparation (generous gift from Dr. Roeder laboratory) were included in some of the reaction mixtures, as indicated. Mix, no protein, lane 1; 2ul, 2ul of anti TBP, lane 2; 6 ul, 6 ul of anti TBP, lane 3; 0 ul, no antibody, lane 4.

5.2.3c. TBP complex from 3-4 cells contains TAFII40 and TAFII60; EMSA on TATA Box.

A. Complete gel. **B. Blow-up of Fig. A.**



5% of 3-4 cells mdm2 P2 elution fraction 0.5 molar KCl was incubated with ^{32}P labeled TATA Box (Santa Cruz). 6 ul of polyclonal anti TAFII40 and anti TAFII60 antibody preparations (generous gift from Dr. Roeder laboratory) were included in the reaction mixtures, as indicated. No Ab, no antibody, lane 1; SP1, anti SP1 antibody, lane 2;TAFII40, anti TAFII40 antibody, lane 3; TAF II60, anti TAFII6 antibody, lane 4.

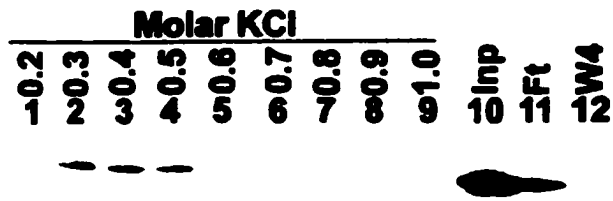
5.2.4. TBP COMPLEX FROM 10-1 CELLS CONTAINS TBP, TAFII40, AND TAFII60.

Previously when we analyzed the mdm2 P2 elution fractions of 10-1 cells, we observed the TBP complex (Fig.5.2.2b). In order to investigate if the TBP complex from the 10-1 cells also contained TBP, TAFII40, and TAFII60 we analyzed the mdm2 P2 elution fractions of the 10-1 cells by Western blot and immunodetection as we did with the elution fractions of the 3-4 cells

Our results showed the presence of TBP eluting with fractions 0.3 to 0.7 molar KCl (Fig. 5.2.4a, lanes 2 to 6). This result correlated with the presence of the TBP complex in these fractions (Fig. 5.2.2b, lanes 7 to 11) suggesting that TBP is a member of the TBP complex of 10-1 cells.

In order to corroborate the presence of TBP, TAFII40 and TAFII60 in this TBP complex band, we also performed EMSA in the same way we did for the 3-4 cells elution fractions (Fig 5.2.4b) using 6ul of the anti-TBP, and TAFII60 and anti-TAFII40 preparation respectively. Each antibody produced a slower migrating species compared to the TBP complex band (Fig.5.2.4.b, lane 3, 4 and 5). We did not observe these species in the control, which had no antibody (Fig.5.2.4b, lane 2) or when a non-relevant antibody was included (fig. 5.2.4b, lane 6). This result corroborated the presence of TBP, TAFII60, and TAFII40 as a member of this TBP complex.

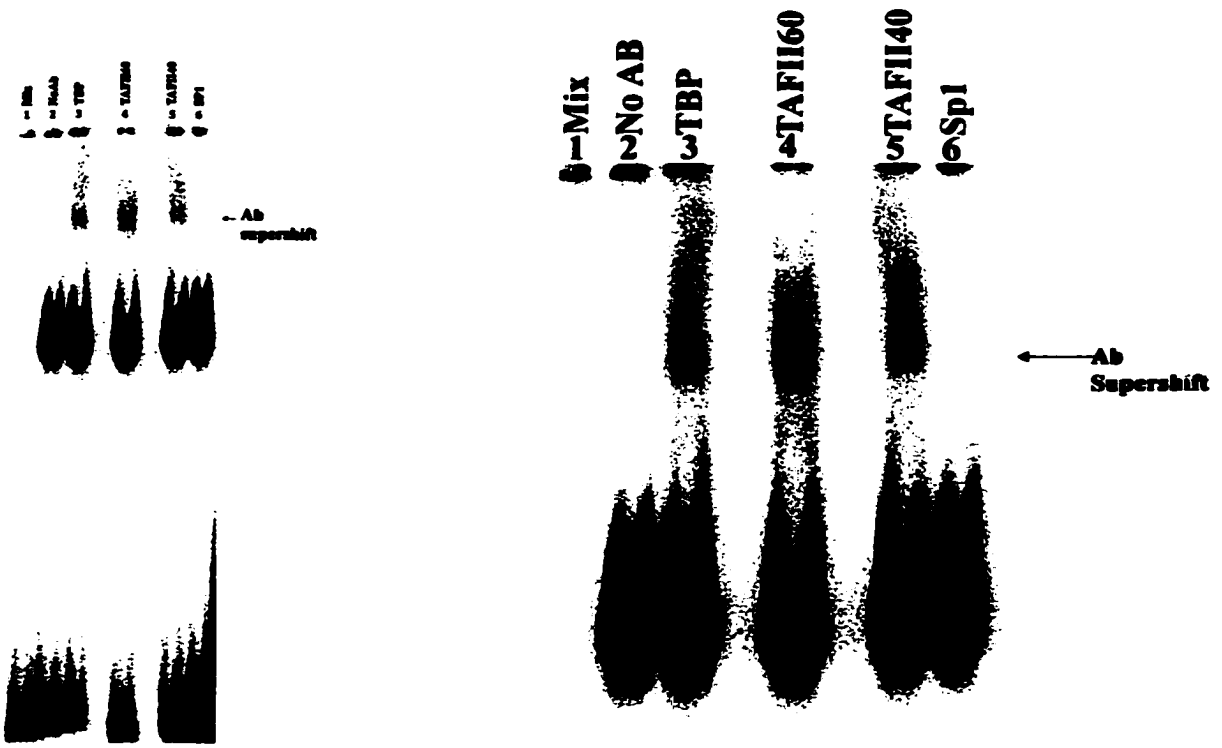
5.2.4a. <u>TBP complex from 10-1 cells contains TBP; Western blot analysis</u>.



7 mg. of total protein contained in the Sephacryl S300 fraction pool prepared from 10-1 cells nuclear extract were loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. 80% of each elution fraction was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-TBP antibody (Santa Cruz). Mdm2 P2 elution fractions 0.2 to 1.0 molar KCl, lanes 1 to 9; Inp, 0.1 % of input, lane 10; Ft, 0.1% of flow-through, lane 11; W4, 0.1% of wash four, lane 12.

5.2.4b. TBP complex from 10-1 cells contains TBP, TAFII40 and TAFII60; EMSA on TATA Box.

A. Complete gel. **B. Blow-up of Fig. A.**



5% of 10-1 cells mdm2 P2 elution fraction 0.5 molar KCl was incubated with ^{32}P labeled TATA Box (Santa Cruz). 6ul of a polyclonal anti TBP, anti TAFII40 and anti TAFII60 antibody preparation (generous gift from Dr. Roeder laboratory) were included in some of the reaction mixtures, as indicated. Mix, no protein, lane 1; No Ab, no antibody, lane, 2.; TBP, anti TBP, lane 3; TAFII60, lane 4; TAFII40, lane 5; SP1, lane 6.

5.3. DISCUSSION.

We found that a group of proteins co-eluted with p53 from the mdm2 P2 and the RGC, affinity columns as evidenced by auto radiography. Some of these proteins formed a complex that we named TBP complex because it shifted a TATA box oligonucleotide in EMSA experiments. Our results suggested that this TBP complex was formed, at least in part, by TBP and two of its associated factors TAFII40 and TAFII60. The autoradiographic profile of the mdm2 P2 and RGC elution fractions indicated the presence of other proteins in this complex but their identity remains to be investigated. We detected the TAFs in the TBP complex by EMSA and using antibodies to produce a super shift. The slower migrating species in the presence of the Ab is promising indication of the presence of these proteins. However, further quantitative experiments are needed to verify that this was indeed a super shift.

The TBP complex that eluted from the mdm2 P2 site was bound to the affinity column in a p53 independent manner; although p53 from 3-4 cells, when eluted from the mdm2 P2 site changed the migration pattern of this TBP complex suggesting an interaction between these four proteins. The p53 independent binding of this TBP complex to the mdm2 P2 column might be due to its recruitment by a p53 analog such as p63 or p73. It could also be due to the ability of TBP to bind DNA in both a specific and non-specific way, which has been reported, or to the presence of other protein(s) in the complex that may be able to bind DNA non-specifically. Interactions between TBP-p53 and TAFII60, TAFII40 - p53 have been reported before, but with purified and/or in vitro translated proteins and by

co-immunoprecipitation experiments. To the best of our knowledge, this is the first report of an interaction between these 4 proteins as a complex with the p53 DNA binding site and using proteins from cellular extracts.

Initially, we used biotinylation to detect the proteins eluting from the columns. We found that it was not an appropriate technique for this purpose because not all the proteins get biotinylated at the same extent.

CHAPTER 6.

SITE SPECIFIC ISOLATION OF P53 BY SCS AFFINITY CHROMATOGRAPHY.

6.1. INTRODUCTION.

The Super Consensus Sequence (SCS) corresponds to an ideal p53 cognate site. This sequence has been developed as a consensus of the p53 binding sites identified in the genome by sequential point mutations and analysis of the binding affinity of p53 to each substitution until the ideal sequence (highest affinity) was determined (Halazonettis TD., 1993). This sequence of nucleotides has not been seen in the genome.

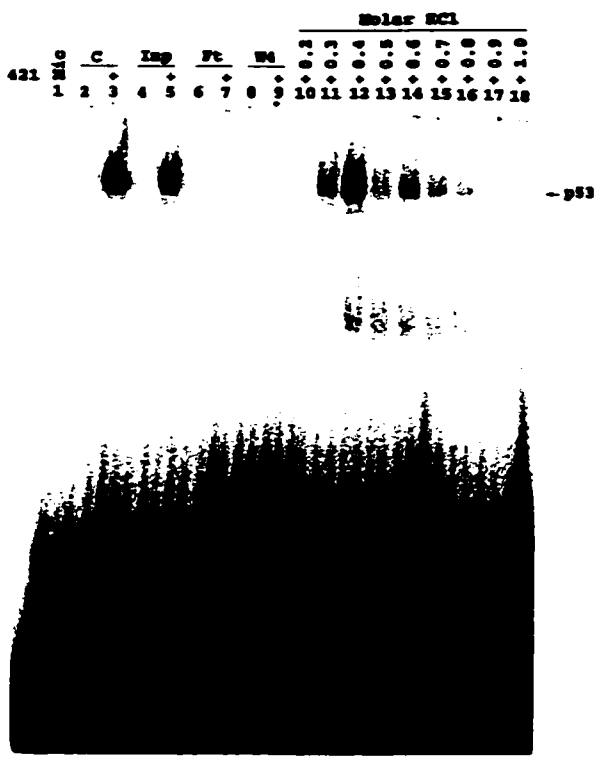
Given that SCS is the highest affinity but non-natural occurring p53 cognate site known, we wanted to compare p53 binding to this site with its binding to the two natural occurring p53 binding sites previously studied (mdm2 P2 and RGC). We performed SCS affinity chromatography in the same way we did for the mdm2- P2 and RGC sites.

6.2. RESULTS.

6.2.1. SCS ISOLATION OF WILD-TYPE P53.

We studied, by DNA affinity chromatography, the binding of p53 from 3-4 cells to the synthetic ideal p53-binding site called Super Consensus Sequence (SCS). In order to determine the capacity of the SCS affinity column to bind p53, we used cell extract from Insect Cells expressing wt p53 and passed the equivalent to 10 micrograms of p53 through the SCS affinity column. We determined the presence of p53 in the elution fractions by EMSA (as we did before) and using an SCS deoxyoligonucleotide as probe. We detected p53 in the elution fractions 0.3 to 0.8 molar KCl (Fig. 6.2.1a, lanes 11 to 16). This result showed that wt p53 could be isolated by SCS affinity chromatography. This is the result of one experiment.

6.2.1a. EMSA, on SCS oligo, of the SCS competent wt p53.



10 ug of p53, contained in the Sephacryl S300 fraction pool from Insect Cells extract, were loaded onto the SCS affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA as described in methods. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the p53 binding site SCS. Mix, no protein, lane 1;C, control, p53 contained is Sf21 cellular extract, lanes 2 and 3; Inp, 0.1% of input, lanes 4 and 5; Ft, 0.1% of flow-through, lanes 6 and 7; W4, 0.1% of wash four, lanes 8 and 9; SCS elution fractions 0.2 to 1.0 molar KCl, lanes 10 to 18.

6.2.2. SCS ISOLATION OF THE ts mt p53 VAL 135.

In order to analyze the binding of p53 from the mammalian system, we repeated the experiment presented above using 3-4 nuclear extract (Fig. 6.2.2a). We observed ts mt p53 Val 135 eluting with fractions 0.3 and 0.4 molar KCl (Fig 6.2.b, lanes 11 and 12). Again, only a small fraction of the total p53 loaded onto the column (10 ug) was bound to it. The p53 DNA binding activity was barely detectable although the experimental binding capacity of the column was higher as observed before (Fig. 6.2.1a, lanes 11-16). The ts. Val 135 mt p53 eluted from the SCS column at a lower salt concentration (0.3 molar KCl) compared to the wt p53, which eluted mostly at 0.4 molar KCl (Fig. 6.2.2a, lane 11 and Fig. 6.2.1a, lane 12). It is important to notice that although the SCS site is a perfect one, the amount of Val 135 p53 bound to the SCS column was very similar to the amount of p53 bound to the mdm2 P2 column as determined by densitometry and presented ahead (Fig.7.2.1a).

6.2.2a. EMSA, on SCS oligo, of the SCS competent ts mt p53 Val 135.

10 ug of p53, contained in the Sephacryl S300 fraction pool 3-4 Cells extract, were loaded onto the SCS affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA as described in methods. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the p53 binding site SCS. Mix, no protein, lane 1; C, controlp53 contained in Sf21 cells extract, lanes 2 and 3;Inp, 0.1% of input, lanes 4 and 5;Ft, 0.1% of flow-through, lanes 6 and 7;W4, 0.1% of wash four, lanes 8 and 9; SCS elution fractions 0.2 to 0.9 molar KCl, lanes 10 to 17.

6.2.3. TBP COMPLEX BINDS TO THE SCS SITE.

In order to determine if the TBP complex co-eluted with p53 from the SCS column, we analyzed the SCS elution fractions, from 3-4 cells, by EMSA using a TATA box as a probe and as described for previous experiments.

We detected TBP complex eluting with fractions 0. 4 to 0.8 molar KCl (Fig. 6.2.3a, lanes 8 to 12). Again, as in the case of the RGC elution fractions, we did not observe the p53 dependent super shift of this TBP complex that we saw with the mdm2 P2 elution fractions. This may be because the p53 subtype that was bound to the SCS column could not induce the super shift or because it was present in a very low amount and we could not detect the super shift. Although in this case, we identified p53 in the elution fractions (Fig. 6.2.3a, lanes 9 to 12).

Regarding the affinity of TBP complex for the SCS site, we observed this TBP complex eluting at higher salt concentration (beginning at 0.5 molar KCl) compared with its elution profile from the mdm2 P2 column (0.4 molar KCl) (Fig. 6.2.3a, lane 9 compared to Fig. 5.2.2a, lane 8).

6.2.3a. TBP complex in SCS elution fractions of 3-4 Cells extract. EMSA on TATA Box.

10 ug of p53, contained in the Sephacryl S300 fraction pool of 3-4 cells extract, were loaded onto the SCS affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA as described in methods. 5% of each elution fraction was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to a TATA box (Santa Cruz). M, no protein, lane 1; C, control, 3-4 cells nuclear extract, lane 2; Inp, 0.1% of input, lane 3; Ft, 0.1% of flow-through, lane 4; W4, 0.1% of wash four, lane 5; SCS elution fractions 0.2 to 0.9 molar KCl, lanes 6 to 13.

6.3. DISCUSSION.

We isolated the wild-type p53 expressed in insect cells and the ts mtp53 Val 135 from 3-4 cells, using SCS DNA affinity chromatography. We detected and identified p53 in the SCS elution fractions in the same way we did for the mdm2 P2 and RGC experiments.

In the case of the SCS affinity chromatography as in the case of the mdm2 P2 and RGC, the source of p53 seemed to influence the amount of p53 that was bound to the SCS affinity column. Our results showed a substantial amount of p53 eluting from the SCS affinity column when we used the insect cell extract. In contrast, when coming from the 3-4 cells, our results showed that much less p53 was bound to the column.

We also observed the TBP complex eluting from the SCS affinity column, as was the case with the mdm2 P2 and RGC affinity columns. p53 eluting from the SCS affinity column did not induce the super shift (slower migrating species) of this TBP complex observed in the mdm2 P2 elution fractions. This result seemed to indicate that either the p53 species that was bound to the SCS column did not associate with this TBP complex. However, given that very little p53 was detected in the elution fractions, it might also indicate that the amount of p53 bound to the column was not enough to produce such association. Another possibility is that the association took place but was below the detection limit.

The fact that the much less ts mt p53 Val 135 was bound to the SCS column compared to the mdm2 P2 one seemed to confirm the idea of the different p53 bindomers present in the sample.

CHAPTER 7.

QUANTITATIVE ANALYSIS.

7.1. INTRODUCTION.

In order to further our understanding of the binding of p53 to the different p53-binding sites used in this work, we performed a quantitative analysis of the p53 bound to the two DNA affinity columns mdm2 P2 and SCS. As a reminder, we have presented results using 2 types of p53 as follows: human p53 derived from Sf21 cells and a ts mt p53 Val 135 derived from 3-4 cells. The Sf21 cells do not produce human wt p53 unless infected with the specific baculovirus. In the Sf21 cells, wt p53 is not activated in the same way as in the mammalian cells. On the other hand, because of the infection procedure their stress related kinases might be activated and this might help to produce a highly active DNA binding form of p53. The mammalian 3-4 cells, on the other hand, produce the mouse ts. mt p53 Val 135 in a background suitable for physiologically relevant post-translational modifications as well as protein-protein associations and the protein adopts a wild type conformation at the permissive temperature (see description in materials and methods).

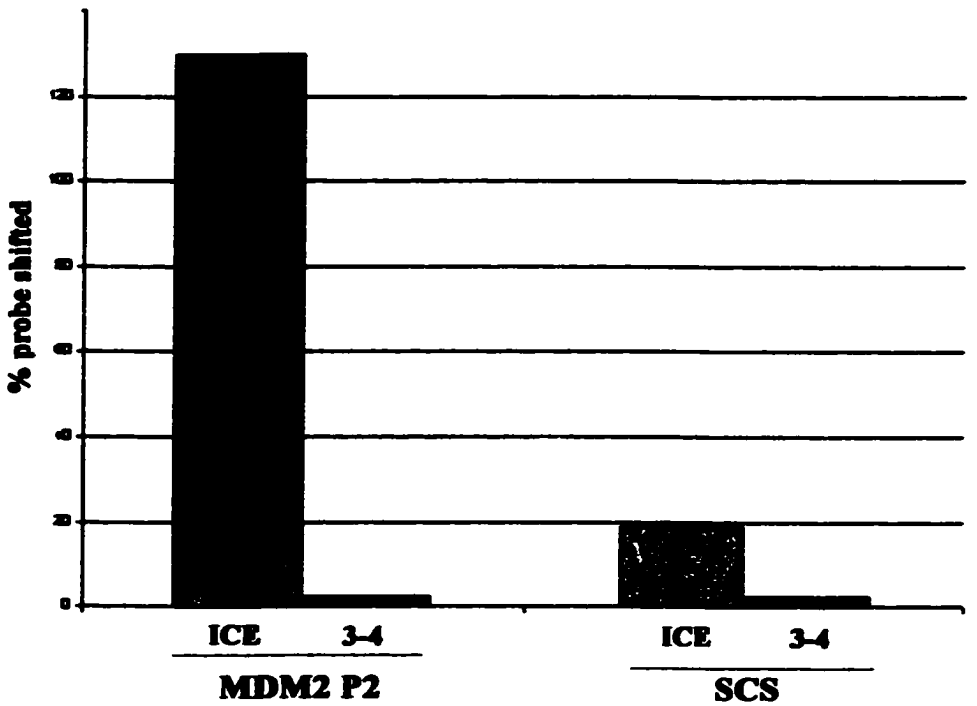
7.2. RESULTS

7.2.1. THE SAME AMOUNT OF P53 FROM 3-4 CELLS DNA BINDING ACTIVITY IS PRESENT IN THE ELUTION FRACTIONS FROM THE IDEAL SCS AND THE GENOMIC MDM2 P2 SITES.

Given that the SCS is an ideal consensus sequence, it would be expected to bind more p53 than the mdm2 P2 site, which shows a somewhat degenerate sequence. Surprisingly, we detected the same amount of p53 eluting from both DNA affinity columns mdm2 P2 and SCS (Fig. 7.2.1a; 3-4/mdm2 Vs 3-4/SCS). The values plotted in Fig. 7.2.1a correspond to the experiments shown in Fig. 3.2.2b for 3-4/mdm2 and Fig. 6.2.2a for 3-4/SCS. In each case, we measured the band corresponding to p53 and added this value for all the elution fractions were a p53 dependent DNA shift was observed. The amount of p53 from 3-4 cells bound to each column was not limited by the binding capacity of the columns. The mdm2 P2 and SCS columns had higher experimental binding capacity as observed in the results from the experiments with the wt p53 (Fig. 7.2.1a, ICE/ mdm2 and ICE/SCS). These values correspond to the experiments shown in Fig. 3.2.1b for ICE/mdm2 and 6.2.1a for ICE/SCS. The ts mt p53 Val 135 from the 3-4 cells bound much less to the mdm2 P2 and SCS columns compared to the amount of the wtp53 from insect cells (ICE) bound to them (Fig. 7.2.1a; 3-4/ mdm2 Vs ICE/mdm2 and 3-4/ SCS Vs ICE/SCS). Fig 7.2.1a shows the amount of probe shifted, detected in all the elution fractions for each affinity column (0.2 to 1.0 molar KCl) expressed as a percentage of the

probe loaded in one reaction mixture, that is why, this value is more than 100% in the case of the wild type p53 ICE/ mdm2 P2 binding site.

7.2.1a. Amount of p53 eluted from the mdm2 P2 and SCS columns.



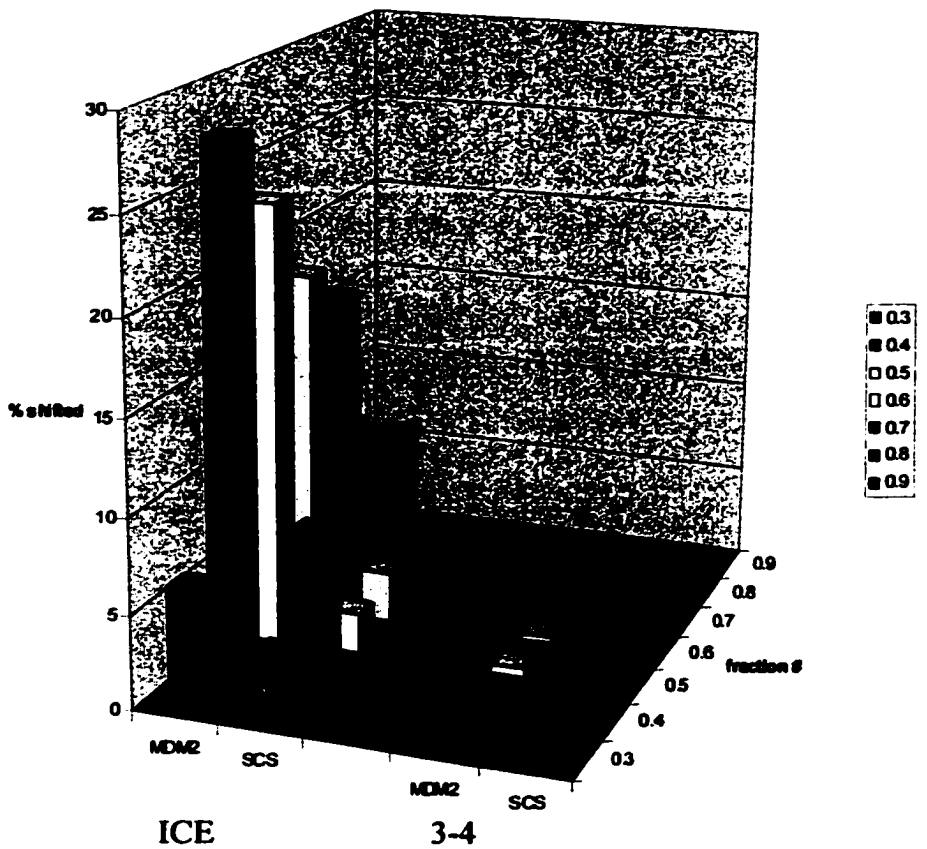
We analyzed by densitometry the EMSA films for each one of the DNA affinity columns (Mdm2 P2 and SCS) and for each one of the sources of p53 [3-4 and ICE]. We then plotted the amount of total probe shifted by p53, in each case (elution fractions 0.2 to 1.0 molar KCl) expressed as a percentage of the amount of probe present in one reaction mixture.

7.2.2. BINDING AFFINITIES OF P53 FROM 3-4 CELLS vs. P53 FROM Sf21 CELLS.

Although the amount of the ts mt p53 Val 135 from 3-4 cells bound to the two p53 cognate sites was very similar, the elution profiles from the two columns were quite different from each other. From the SCS column, p53 eluted at fraction 0.3 molar KCl. On the other hand, from the mdm2 P2 column, we observed p53 eluting with fractions 0.3 up to 0.6 molar KCl [Fig. 7.2.2a, 3-4: mdm2 Vs SCS]. These results are the average value of 2 independent experiments for the 3-4_mdm2 P2 and for the ICE-mdm2 P2 systems. This pattern of binding was observed in more than 5 independent experiments. For the ICE- SCS and 3-4-SCS systems this is the result of one experiment.

Our results showed that the source of p53 as well as the DNA site determined the characteristics of the binding of p53 to its cognate site at least, in terms of the amount of the p53 bound and in terms of the affinity/stability of such binding.

7.2.2a. Elution profiles of wt and Val 135 p53.



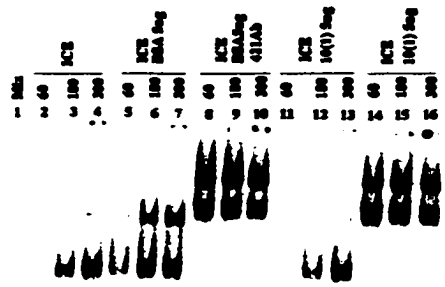
The elution fractions from each column and each cell type were analyzed by EMSA, as described in methods and using SCS deoxyoligonucleotide as a probe. The binding was then quantitated by densitometry and expressed as the percentage of probe shifted per each elution fraction. X-axis, percentage of probe shifted; Y-axis, cell/ affinity column type; Z-axis, elution fractions 0.3 to 0.9 molar KCl.

7.2.3. 3-4 NUCLEAR EXTRACT DOES NOT REDUCE THE BINDING OF WT P53 TO THE MDM2 P2 SITE.

We observed more wt p53 (expressed in Sf21 cells) eluting from the mdm2 P2 affinity column, compared with the amount of ts mt p53 Val 135 from 3-4 cells obtained from the same column. In fact, p53 from 3-4 cells was bound less to both sites (mdm2 P2 and SCS) compared to p53 from insect cells. We wanted to know if the decreased binding of the Val 135 p53 was due to and inhibitory effect exerted by a factor(s) present in the nuclear extract of the 3-4 cells. In order to answer this question, we analyzed by EMSA the binding of the wtp53 expressed in the insect cells mixed with 10-1 cells nuclear extract, isogenic to 3-4 cells but without p53, to see if there was a factor(s) in the 3-4 nuclear extract that inactivated the binding of p53 to its cognate site. p53 from the insect cells extract (ICE) was bound the same to the mdm2 P2 deoxyoligonucleotide with or without 10-1 cells nuclear extract (Fig. 7.2.3a, lanes 2 &11; 3 & 12 and 4 &13). In both cases, with and without 10-1 nuclear extract, this p53 was responsive to the PAb 421 as evidenced by its super shift (Fig. 7.2.3a, lanes 8,9,10 and 14,15,16). As a control for the effect of non-specific proteins present in the reaction mixture, we added Bovine Serum Albumin (BSA) normalized to the amount of protein present in the 10-1 cells extract, to some reactions, as specified. Our results showed that the presence of non-specific proteins did not affect the binding of p53 to the mdm2 P2 probe in a significant way (Fig. 7.2.3a, lanes 5,6,7).

These results indicated that the lower binding of p53 from the 3-4 cells to the mdm2 P2 affinity column was not due to the presence of inhibitors in the nuclear extract of the 3-4 cells, but to a characteristic of the p53 molecules present in that extract.

7.2.3a. Wild-type p53 mixed with 10-1 cell extract. EMSA on mdm2 P2 oligo.



Different amounts of p53 (60, 180 and 300 nanograms) contained in the Sephacryl S300 fraction pool of insect cells extract, were added to each reactions mixture as specified. EMSA was performed as described in methods and using mdm2 P2 deoxyoligonucleotide as a probe. 5 micrograms of protein from 10-1 cells extract or BSA were added to some reaction mixtures as indicated. PAb 421 was included in some reaction mixtures to produce a super shift, as indicated. Mix, no protein, lane 1; ICE, 60, 180 and 300 ng of p53 contained in insect cell extract, lane 2,3 and 4; ICE p53, 60 , 180 and 300 ng. plus 5ug of BSA, lanes 5, 6 and 7; ICE p53, 60 , 180 and 300 ng. plus 5ug of BSA plus PAb421, lanes 8,9 and 10; ICE p53, 60 , 180 and 300 ng. plus 5ug of 10-1 cells extract lanes 11, 12 and 13; ICE p53, 60 , 180 and 300 ng. plus 5ug of 10-1 cells extract plus PAb421, lanes 14,15 and 16.

7.3. DISCUSSION.

Our quantitative analysis showed that p53 from 3-4 cells bound less to the mdm2 P2 and SCS affinity columns compared with p53 from the insect cells. According to our data, this lower binding seemed to be due to a characteristic(s) of the p53 molecules themselves and not to the presence of inhibitors of binding in the 3-4 nuclear extract. Among those characteristics may be the fact that the p53 from the 3-4 cells is a temperature sensitive mutant protein and that because of that, its ability to bind to the p53 cognate sites is reduced compared to the human wild-type protein. This idea, although possible, is opposed by the reported data showing that the ts Val 135 p53 adopts the wild-type conformation at the permissive temperature (32 °C). It has also been reported that at 32 °C, the ts Val 135 p53 produces a response in the 3-4 cells characteristic of the wild-type protein. Another piece of evidence that seems to oppose this idea is the fact that, in our experiments, we observed both p53 proteins having a similar elution profile when eluting from the mdm2 P2 column (salt concentration 0.3 molar KCl and up). This result suggests that the ts Val 135 p53 and the wt-p53 proteins have a similar potential affinity for binding. Even more, other experiments done in our laboratory with mammalian wild-type p53 have shown the same pattern of binding as the ts Val 135 p53 to the mdm2 P2 site. All these evidence seems to point at physiologically relevant modifications to the ts Val 135 p53 as determinants of the binding profile that we observed.

Contrary to what we expected, we did not observe a significant difference in the amount of Val 135 p53 eluting from the SCS column, compared to the amount eluting from the mdm2 P2 column. One would expect that more p53 would bind to the SCS site because

of its reported higher affinity given that it is a perfect consensus sequence. The amount of Val 135 p53 eluted from these affinity columns might correspond to the total p53 molecules competent for binding in the sample. In this case our results would indicate that the DNA binding site did not make a difference regarding the p53-DNA interaction determined. Another possibility is that, although the amount of p53 bound to both sites was similar, the molecules of p53 that were bound to each site were not identical. This idea seems to be supported by the differential elution profile that we observed for the Val 135 p53 when coming from the mdm2 P2 column vs. the SCS one. The p53 eluted from the mdm2 P2 column exhibited a range of populations of p53 molecules regarding their affinity for that site (0.3 to 0.6 molar KCl). On the other hand, the p53 eluting from the SCS column seemed to be more homogeneous in its affinity (0.3 molar KCl). These other p53 populations were bound to the mdm2 P2 site with higher affinity and/or produced more stable p53-DNA complexes, which required higher salt concentrations to elute (0.4 to 0.6 molar KCl). Significantly, we find that the stronger binding p53 molecules were detected in the elution fractions of the physiological DNA p53-binding site (mdm2 P2) and not in the non-physiological one (SCS).

CHAPTER 8.

DNA AFFINITY CHROMATOGRAPHY Vs ELECTROPHORETIC MOBILITY SHIFT ASSAY.

8.1. INTRODUCTION.

In this study, we have used two techniques: EMSA and DNA affinity chromatography. EMSA has been the technique of choice to analyze protein-DNA interactions and in the case of p53 has been used traditionally to detect and analyze p53 DNA binding activity. DNA affinity chromatography has been used primarily to identify and purify sequence specific DNA binding proteins. In this study, we used DNA affinity chromatography in a non-traditional way. We used it to study if the p53-binding site would influence the association of p53 with other cellular factors.

Our findings contradicted the initial assumption that the p53-binding site would influence a differential association of p53 with other proteins. Instead, we observed the same group of proteins (TBP, TAFII40, and TAFII 60) co-eluting with p53 from the different DNA affinity columns that we studied (mdm2 P2, RGC and SCS). In the process of analyzing the differential protein- protein association, we gathered information about the interaction between p53 and some of these p53-binding sites.

Given that DNA Affinity chromatography is more expensive and time consuming that EMSA, we wanted to see if we could have obtained the information that we gathered from the DNA affinity experiments, using EMSA. We limited this comparative analysis to two topics. First we compared the requirement for PAb421 induction of sequence-specific p53 binding between the two techniques. Second, we looked for the p53-TAFII40-TAFII60 interaction using cellular extracts and EMSA.

8.2. RESULTS.

8.2.1. MDM2 P2 AFFINITY CHROMATOGRAPHY DID NOT REQUIRE PAb 421 TO INDUCE P53 BINDING.

In EMSA experiments for p53 binding, PAb 421 is usually required to induce the binding of p53 to the deoxyoligonucleotide. In fact, without this anti- p53 antibody it is not always possible to detect p53 in these experiments. In order to compare the results we obtained by DNA affinity chromatography to those obtained by EMSA, we analyzed the wt p53 mdm2 P2 elution fractions from the experiment shown in fig. 3.2.1b as described ahead. 5% of mdm2 P2 elution fractions 0.5 and 0.6 molar KCl, where we knew there was p53, was incubated with radiolabeled SCS deoxyoligonucleotide and we performed EMSA as described in methods. We included PAb421 were specified, to activate p53 binding. By contrast, PAb 421 addition was not necessary for the mdm2 P2 affinity chromatography. EMSA did not show p53 dependent shift of the probe in the absence of PAb 421 (Fig. 8.2.1a, lanes 4 y 6). Although p53 was present in the sample as evidenced by the shift observed when we added PAb 421 to the reaction mixture (Fig. 8.2.1a, lanes 5 and 7). It is important to remember that the p53 present in these reaction mixtures was coming from the mdm2 P2 site affinity column; that means that it already had been able to bind to the mdm2 P2 site without the induction by the PAb 421. Nevertheless, the EMSA failed to detect the p53 present in these fractions.

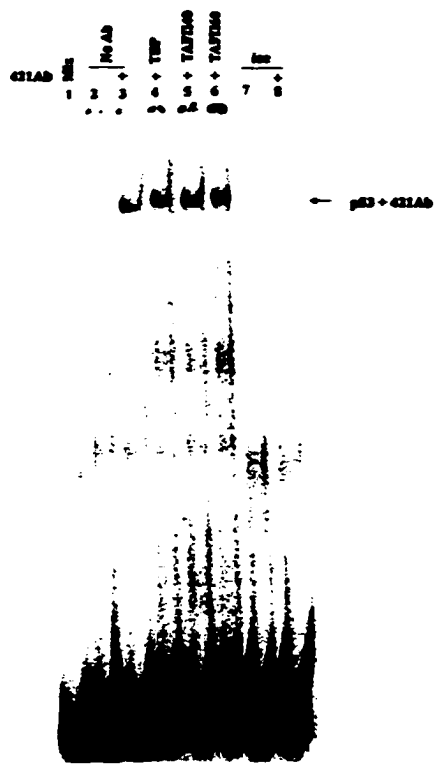
8.2.1a. Mdm2 P2 affinity fractions with and without PAb 421. EMSA on SCS oligo.

10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from insect cell extract was loaded onto the mdm2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA. 5% of elution fractions 0.5 and 0.6 molar KCl was incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the p53 binding site (SCS); PAb 421 was used, were indicated, to activate p53 binding. Mix, no protein, lane 1; ice, insect cell extract control, lanes 2,3; 0.5, 5% of elution fraction 0.5 molar KCl, lanes 4, 5; 0.6, 5% of elution fraction 0.6 molar KCl, lanes 6,7.

8.2.2. EMSA DID NOT SHOW TBP COMPLEX-P53 INTERACTION.

When we analyzed 3-4 nuclear extract, by EMSA directly, prior to mdm2 P2 affinity chromatography, we could not detect any interaction (or super shift) between p53 and any of the members of the TBP complex identified before (Fig. 8.2.2a). We included specific antibodies against TBP (Fig. 8.2.2a, lane 4) against TFII40 (Fig. 8.2.2a, lane 5) and against TAFII60 (Fig.8.2.2a, lane 6) as indicated to induce an antibody dependent super shift. Neither one of these antibodies produced the slower migrating species seen before in the elution fractions from the mdm2 P2 column (Fig.5.2.2a, lane 8; Fig. 5.2.3b, lanes 2,3 and Fig. 5.2.3c, lanes 3,4). These results suggest that DNA affinity Chromatography showed, and perhaps enriched for, some DNA-protein interactions that the EMSA failed to detect.

8.2.2a. EMSA, on mdm2 P2 oligo, of 3-4 cells nuclear extract.



2 ug of total protein contained in the nuclear extract of 3-4 cells were added to each reaction mixture and EMSA was performed as specified in methods. The samples were incubated with a ^{32}P labeled deoxyoligonucleotide corresponding to the mdm2 P2 p53-binding site. PAb 421 was included, were specified, to activate p53 binding. Mix, no protein, lane1; No Ab, 3-4 nuclear extract, lanes 2 and; TBP, 3-4 nuclear extract plus 6 ul of anti TBP, lane 4; TAFII40, 3-4 nuclear extract plus 6ul of anti TAFII40, lane 5; TAFII60, 3-4 nuclear extract plus 6 ul of anti TAFII60, lane 6; ice, insect cell extract control, lanes 7 and 8.

8.3. DISCUSSION.

Our results showed that without the addition of PAb 421, EMSA failed to detect p53 in samples where we knew there was p53. On the other hand, we were able to isolate p53 by the DNA affinity chromatography approach without the help of the PAb 421. This result, again, reinforced the idea of the p53-binding site selecting for a specific p53 species "bindomer". The lack of detection by EMSA might be because the amount of the mdm2 P2 competent p53 bindomer present in the sample was very low and below the detection limit. Another possibility is that this type of p53 required a three dimensional conformation of the DNA and the length of the probe in the EMSA was not enough to support its binding.

With the DNA affinity chromatography we also detected an interaction between p53 and TBP, TAFII40 and TAFII60 when we examined the elution fractions using the TATA box oligonucleotide for the EMSA. We could not see this interaction when we analyzed the nuclear extract directly by EMSA using the mdm2 P2 site oligonucleotide. The fact that we did not detect this interaction in the EMSA of the nuclear extract suggests that the interaction did not take place. It might also be due to a very low amount of the specific p53 "bindomer" that is competent for the binding to the p53-binding site analyzed and, the association. Another possible explanation is that, in order to induce binding in the EMSA, we had to add PAb421 to the reaction mixtures. The presence of this antibody might have disrupted the mentioned interaction.

CHAPTER 9.

DISCUSSION.

In this study we examined p53-DNA interactions within different cellular contexts. The first cell line, Sf21, is an insect cell line that does not harbor a native p53 gene. It was infected with a baculovirus containing a human wild-type p53 gene. This wild-type p53 initially served as a control preparation to evaluate the experimental binding capacity of the columns. The second cell line, 3-4, once transfected with ts mutant p53 Val 135 (see materials and methods) exhibits the properties of wild-typ3 p53 cell line. Three p53 binding sites were examined in this study, two naturally occurring ones (mdm2 P2 and RGC) and an idealized binding site (SCS) that does not occur naturally in the genome.

We isolated wild-type p53 expressed in Sf21 cells using mdm2 P2, RGC and SCS DNA affinity chromatography. We detected wild-type p53 in the different elution fractions by Western blot, using antibodies against p53 and also by Electrophoretic Mobility Shift Assay (EMSA). In the EMSA analysis, the identity of the p53* band as being p53 protein was confirmed by its migration pattern, by its specific binding to a p53 cognate site and by the responsiveness of this band to the p53 specific PAb 421. The fact that no p53 was detected in the last wash (W4) indicated that the p53 detected in the elution fractions was bound to the mdm2 P2, RGC or SCS DNA sites, specifically and was not a remnant from the loaded material. The specificity of binding was also confirmed by the absence of p53

in the elution fractions of the mutant RGC (mt RGC) column, made with a mutated version of the p53 cognate site present in the Ribosomal gene cluster.

Our results showed that within each p53 preparation there were p53 molecules with different binding strengths. This was obvious from the different salt concentrations needed to elute the p53 subtypes. The combination that exhibited the greatest variety of p53 binding subtypes was the wt p53 expressed in the Sf21 cells eluted from the mdm2 P2 affinity column (Fig. 7.2.2a, 0.3 –0.9 Molar KCl). Within the same series of experiments, the SCS elution profile showed a smaller range of p53 subtypes, compared to mdm2 P2, i.e. 0.3- 0.7 Molar KCl. In both cases, mdm2 P2 and SCS, the amount of wt p53 eluted from the columns was much less than the amount loaded. This difference might reflect DNA binding site selection of p53 subtypes or be due to experimental saturation of the columns.

We also isolated the Ts mt p53 Val 135 from the 3-4 cells using mdm2 P2 and SCS DNA affinity chromatography. We analyzed the elution fractions by EMSA because it gave as a more sensitive way of detecting the presence of p53 in the elution fractions. The identification criteria were the same as for the wt p53 from the Sf21 cells plus the fact that the band corresponding to p53 was absent from the elution fractions of the 10-1 cells, which do not have p53. When we analyzed the mammalian p53 cell extract, 3-4, we only saw different p53 subtypes (0.3-0.6 Molar KCl) at the physiological binding site, mdm2 P2. The SCS elution profile exhibited only one p53 subtype (0.3 Molar KCl). Although, the amount of each subtype was much less than observed for the wt p53 preparation when

analyzed on the same columns, mdm2 P2 and SCS. In this case, we knew that the columns were not saturated because of the results from the wt-p53 extract. Then, the column binding capacities were not the limiting factor but instead a quality of the mammalian nuclear extract or a condition(s) impinged upon the p53 molecules present in 3-4 cell extract. We did not observe much difference between the amounts of Val 135 p53 eluting from the SCS column compared with the amount of Val 135 p53 eluting from the mdm2 P2 site. This result suggests that the closeness of the cognate site to the consensus sequence is not the only determinant for the affinity and stability of the binding of p53. Although it could also mean that such amount corresponded to the total amount of p53 competent for binding present in the nuclear extract of the 3- 4 cells. We think that the different p53 subtypes may reflect differences in binding affinities and that the differences in the amounts of each p53 subtype eluted from each site (0.3 – 0.9 molar) may be due to differences in stability of binding.

Our results from the RGC affinity chromatography of the 3-4 cells showed a band that seemed to be p53 but whose identity could not be confirmed (p53^). This species eluted from a p53 specific site affinity column (RGC), was bound specifically to another p53 cognate sequence in the EMSA, migrated as p53 in the EMSA and was not present in the RGC elution fractions from the 10-1 cells, which do not have p53. All this evidence suggests that the p53^ band corresponded to p53. Nevertheless, the fact that we could not super shift this band or induce it to bind more with the PAb 421 raised doubt about its identity. It is a known fact that some post-translational modifications of p53, such as phosphorylation by Protein Kinase C (PKC), disturb the binding of the PAb421 to its

epitope on the p53 molecule. This is another possibility that would explain why this band did not respond to the PAb 421. At this point, we concentrated on the results from the mdm2 P2 affinity column and because of that we did not pursue the identification of this species.

Together these data seems to indicate that in each cellular extract there is a variety of p53 molecules with different binding affinity and stability. We refer to these p53 subtypes with different binding characteristics as p53 "bindomers". We think that each p53-binding site selected for some of these p53 "bindomers". We do not know what determines the characteristics of each "bindomer" but our data suggests that those features are impinged upon the p53 molecules themselves and that but the cellular background used as a source of p53 played a role in determining such characteristics. We think this is the case because when we took a preparation of each type of p53 (wt or Val 135) and mixed it with a mock preparation of the other cell extract, the p53-binding pattern to the mdm2 P2 was not changed. The modifications to the p53 protein are determined not only by the cell type but also by the cell status, cell cycle stage, the presence or absence of stress and the type of stress (as described in chapter 1). These factors combined can induce the cell to produce a variety of p53 molecules by means of post-translational modifications and differential splicing, among others. Based on our data, we propose a model in which each post-translational modification of p53 would produce a change (either in conformation or in its ability to associate with other factors) that would make that particular molecule of p53 more or less avid for one specific p53-binding site. In this way, the deviation of each binding site from the consensus would not

be a degeneracy of the site, but another step of control for specific pathways. This model would explain, in part, the versatility observed in p53 function.

This hypothesis is supported by several independent findings. Firstly, p53 can be post-translationally modified in several different ways. Secondly, it has been shown that the p53 dimmers that tetramerize to bind DNA in a sequence-specific manner can exist in a wild-type and in a mutant conformation and that the equilibrium between these two conformations may affect the stability of binding (McLure. KG., 1999). The binding stability can also be modified by the cellular ATP/ADP ratio (Okorokov, AL., 1999). Thirdly, a new p53-binding site has been identified which behaves atypically with respect to its responsiveness to the PAb 421 (Resnick-Silverman L.,1998).

The amount of p53 from 3-4 cells bound to the columns was very low. We think that by changing some of the experimental conditions would be feasible to increase the amount of p53 recovered to the levels that we obtained with the p53 from the insect cell extract. The changes in the experimental condition could be achieved by activating p53 by treating the cells with DNA damaging agents. Another important consideration is the p53-binding site selected. Based on this evidence, we think that with the appropriate matching of the cells conditions (drug treatment, metabolite deprivation, etc) and the p53-binding site, DNA affinity chromatography is a good technique to isolate and enrich for p53 molecules with specific binding characteristics (p53 bindomers). Another application could be to detect complex and physiological relevant protein-protein interactions and enrich for the factors involved in them.

We found that a group of proteins co-eluted with p53 from the mdm2 P2 and the RGC, affinity columns as evidenced by autoradiography. The fact that we observed so many bands in the autoradiography of the elution fractions made it very difficult to identify the co-eluting proteins. For the same reason, it also was not possible to determine conclusively, if there was a clear difference between the co-eluting proteins from the mdm2 P2 column compared with the ones from the RGC column. Because TBP and its associated factors TAFII40 and TAFII60 have been seen co-immunoprecipitate with p53 and because some of the autoradiographic bands fell in the size range of these proteins, we looked for their presence in the elution fractions.

When we analyzed, by EMSA, the mdm2 P2, RGC and SCS elution fractions from the 3-4 cells in order to identify some of the p53 co-eluting proteins, we saw a TATA box binding activity. Because we detected this activity with a TATA box as a probe and because we observed many bands in the autoradiography, we thought that the observed TATA box binding activity might correspond to a complex formed by TBP and other proteins. Because of this, we called this activity TBP complex. The binding of this TBP complex to the p53 cognate DNA sites studied, was not p53-dependent as evidenced by its presence in the elution fractions of the 10-1 cells. Nevertheless, it is noteworthy that only the mdm2 P2 competent p53 was able to induce a slower-migrating species/super shift of this TBP complex. The modification to the TBP complex that we observed correlated with the presence of p53 in the elution fractions. We observed them both in the mdm2 P2 elution fraction 0.4 molar KCl. This modification suggested an association

between p53 and this TBP complex. The fact that only the mdm2 P2 competent p53 induced super shift of this TBP complex further suggested the idea of the p53 cognate sites selecting for specific types of p53 molecules among all the p53 present in the sample.

The fact that this TBP complex was able to bind to the mdm2 P2 and RGC columns in a p53 independent manner argues against p53 as a recruiter of TBP and /or TFIID to the promoter. We observed that p53 did not influence the stability of binding of this TBP complex to the DNA as evidence by the same elution profile comparing 3-4 to 10-1 cells, for each site studied. This argues against a role for p53 as a stabilizer of the interaction between TFIID and the DNA. We did not rule out the presence of a p53 analog(s) in the extracts, which might be the recruiter in the case of the 10-1 cells. The presence of this TBP complex in the mdm2 P2 elution fractions in absence of p53 (10-1 cells) could also be due to the ability of TBP to bind DNA in both specific and non-specific way. Finally, it could also be due to the presence of other protein(s) in this complex, which may be able to bind DNA non-specifically

Our results showed that the TATA Binding Protein (TBP) and two of its associated factors TAFII40 and TAFII60 were members of this TBP complex. The presence of other proteins in this complex remains to be investigated. Interactions between TBP-p53 and TAFII60, TAFII40 - p53 have been reported before but, with purified and/or in vitro translated proteins and by co-immunoprecipitation experiments. To the best of our knowledge, this is the first report of an interaction between these 4 proteins as a complex

with the DNA p53 binding site and using endogenous proteins from cellular extracts. The fact that only the mdm2 P2 competent p53 modified the migration patterns of this TBP complex, further supports the idea of the p53 bindomers and suggests that not all the p53 bindomers associate in the same way with this TBP complex.

Our results showed that EMSA failed to detect p53 in samples where we knew there was p53. The EMSA could detect this p53 only after induction of binding by the PAb 421. On the other hand, we were able to obtain p53 by the DNA affinity chromatography approach without the help of the PAb 421. This result, again, reinforces the idea of the p53-binding site selecting for a specific p53 bindomer. The lack of detection by EMSA might be because the amount of the mdm2 P2 competent p53 species present in the sample is very low, below the detection limit. Another possibility is that this p53 species requires a specific conformation of the DNA element and that the length of the probe used in the EMSA was not enough to support its binding. The DNA element in the affinity chromatography procedure in longer than in the EMSA and may adopt a three-dimensional conformation more favorable for this association.

With the DNA affinity chromatography we also detected an interaction between p53 and TBP, TAFII40 and TAFII60. We could not see this interaction using the EMSA. This might also be due to the low amount of the specific species that is competent for the binding to the p53-binding site analyzed and the association with this TBP complex. Another possible explanation is that, in order to induce p53 binding in the EMSA, we had

to add PAb421 to the reaction mixtures. The presence of this antibody might have disrupted the mentioned interaction.

CHAPTER 10.

FUTURE DIRECTIONS.

The next step would be to confirm the hypothesis of the different p53 bindomers. **In a given physiological sample containing p53, there are a variety of p53 molecules, which exhibit different binding affinities and stability. We called these differentially binding p53 molecules "p53 bindomers". Each genomic p53-binding site selects for specific p53 bindomers as another control mechanism in the p53 pathway.**

To test this hypothesis the experimental conditions should be adjusted in order to increase the amount of p53 bound to the DNA affinity column e.g. activating p53 in the cells with DNA damaging agents. It is important to match the p53-binding site chosen to make the affinity column with the specific cellular conditions to maximize the amount of p53 bound to it. Different p53 bindomers can then be isolated under conditions where the limiting factor becomes the amount of p53 competent for binding in the sample and not the binding capacity of the column itself. The isolated p53 bindomers can then be characterized with respect to their relative amounts in each experimental conditions and their biochemical features. It would be most interesting to see, if indeed, each p53 binding site is selecting for p53 molecules with different post-translational modifications.

Our results also showed the presence of a protein resembling p53 in the fractions from the RGC column, but further study is required for unequivocal identification. This is of special interest because the p53-binding site found in the ribosomal gene cluster does not

seen to play a role in p53-dependent activation of transcription. The physiological function of this p53-binding site is not known but is has been proposed to participate in DNA replication given its proximity to an origin of replication.

Finally, it would be interesting to investigate the presence of a p53 analog in the TBP complex observed in our results, given that the recruitment of this TBP complex to the p53-binding sites studied seemed to be p53- independent.

CHAPTER 11.

REFERENCES.

Abarzua P., LoSardo J.E., Gubler M.L., Neri A. 1995. Microinjection of monoclonal antibody PAb421 into human SW480 colorectal carcinoma cells restores the transcription activation function to mutant p53. Cancer Res., **55**: 3490-3494.

Addison C., Jenkins J.R., Sturzbecher H.W. 1990. The p53 nuclear localization signal is structurally linked to a p34 cdc kinase motif. Oncogene, **5**: 423-426.

Agami R., Bernards R. 2000. Distinc initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell **102**: 55-66.

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

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

Anderson M.E., Woelker B., Reed M., Wang P., Tegtmeyer P. 1997. Reciprocal interference between the sequence-specific core and nonspecific C-terminal DNA-

binding domains of p53: implications for regulation. Mol. Cell. Biol., **17**: 6255-6264.

Ashcroft M., Vousden K.H. 1999. Regulation of p3 stability. Oncogene, **18** :7637-7643.

Ashcroft M., Taya Y., Vousden K.H. 2000. Stress signals utilize multiple pathways to stabilize p53. Mol. Cell. Biol., **20:** 3224-3233.

Avantaggiati M.L., Ogryyzko V., Gardner K., Giordano A., Levien A.S., Kelly K. 1997. Recruitment of p300/CBP in p53-dependent signal pathway. Cell, **89**:1175-1184.

Bakalkin G., Yakovleva T., Selivanova G., Magnusson K.P., Szekely L., Kiseleva E., Klein G., Terenius L., Wiman K.G. 1994. p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. Proc. Natl. Acad. Sci. USA. **91**:413-417.

Bakalking G., Selivanova G., Yakivleva T., Kiseleva E., Kashuba E., Magnusson K.P., Szekely L., Klein G., Terenius L., Wiman K.G. 1995. P53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. Nucleic Acids Res., **23**: 362-369.

Baker S.J., Markowitz E.R., Willson J.K., Vogelstein B. 1990. Suppression of human colorectal cell growth by wild-type p53. Science **249**, 912-915.

Barak Y., Gotlieb E., Juven-Gershon T., Oren M. 1994. Regulation of mdm2 expression

by p53: alternative promoters produce transcripts with non-identical translation potential. Genes Dev. **8(15):** 1739-49.

Bar-Or L., Segel MR., Alon U., Levine AJ. Oren M. 2000. Generation of oscillations by the p53-mdm2 feedback loop: A theoretical and experimental study. Proc. Natl. Acad. Sci. USA. **97**: 11250-11255.

Bargonetti J., Friedman P.N., Kern S.E., Vogelstein B., Prives C. 1991. Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. Cell **63**:1083-1091.

Bargonetti J., Manfredi J.J., Chen X., Marshak D.R., Prives C. 1993. A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. Genes Develop.. 7 **(12B):** 2565-2574.

Bargonetti J., Chicas A., White D., Prives C. 1997. p53 represses Sp1 DNA binding and HIV-LTR directed transcription. Cell Mol Biol. **43(7):** 935-49.

Bayle J.H., Elenbaas B., Levine A.J. 1995. The carboxyl-terminal domain of p53 protein regulates sequence-specific DNA binding through its nonspecific nucleic acid-binding activity. Proc. Natl. Acad. Sci. USA, **92**: 5729-5733.

Beneke R., Geisen C. 2000. DNA excision repair and DNA damage-induced apoptosis are linked to poly(ADP-ribosyl)ation but have different requirements for p53. Mol. Cell. Biol. **20**: 6695-6703.

Bennett M., Macdonald K., Chan S.W., Luzio P.J., Simari R., Weissberg P. 1998. Cell surface trafficking of fas: A rapid mechanism of p53 mediated apoptosis. Science **282**: 290-293.

Beroud, C., Soussi T. 1998. p53 gene mutations: software and database. Nucleic Acids Res. **26**: 200-204.

Bill C.A., Yu Y., Miselis N.R., Little J.B., Nickoloff J.A. 1997. A role for p53 in DNA end rejoining by human cell extracts. Mutat. Res., **385**: 21-29.

Blagosklonny M.V., An W.G., Romanova L.Y., Trepel J., Fojo T., Neckers L. 1998. p53 inhibits hypoxia- inducible factor-stimulated transcription. J. Biol. Chem., **273**:11995-11998.

Blaydes J.P. Wynford-Thomas D. 1998. The proliferation of human fibroblasts is dependent upon negative regulation of p53 function by MDM2. Oncogene, **16:** 3317-3322.

Blaydes J.P., Gire V., Rowson J.M., Wynford-Thomas D. 1997. Tolerance of high levels of wild-type p53 in transformed epithelial cells dependent on autoregulation by mdm-2.

Oncogene, **14**: 1859-1868.

Bond J.A., Wyllie F.S., Wynford-Thomas D. 1994. Escape from senescence in human diploid fibroblasts induced directly by mutant p53. Oncogene, **9**: 1885-1889.

Borellini F., Glazer R. 1993. Induction of SP1-p53 DNA-binding heterocomplexes during granulocyte/macrophage colony-stimulating factor-dependent proliferation in human erythroluekemia cell line TF-1. J. Biol. Chem., 268:7923-7928.

Bottger A., Bottger V., Sparks A., Liu W.L., Howard S.F., Lane D.P. 1997. Design of a synthetic Mdm2-binding mini protein that activates the p53 response *in vivo*. Curr. Biol., 7: 860-869.

Boyd S.D., Tsai K.Y., Jacks T. 2000. An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. Nat. Cell Biol., **2**: 563-568.

Brugarolas J., Chandrasekaran C., Gordon J.I., Beach D., Jacks T., Hannon G.J. 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. Natures, **377**: 552-557.

Bucholzt T.A., Weil M.M. Story M.D., Strom E.A., McNeese M.D. 1999. Tumor suppressor genes and breast cancer. Radiat Oncol Investg. **77(2):** 55-65.

Buschmann T., Fuchs S.Y., Lee C.G., Pan Z.Q., Ronai Z. 2000. SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases MDM2 ability to ubiquitinate p53. Cell **101**: 753-762.

Caelles C., Helmberg A., Karin M. 1994. p53-dependent apoptosis in the absence of trancriptional activation of p53-target genes. Nature, **370 (6486)**: 220-223.

Cain C., Miller S., Ahn J., Prives C. 2000. The N-terminus of p53 regulates its dissociation from DNA. J. Biol. Chem. **275**: 39944-39953.

Cairn C.A., White R.J. 1998.p53 is a general repressor of RNA polymerase III transcription. EMBO J., **17 (11)**: 3112-3123.

Cardinali M., Kratochvil F.J., Ensley J.F., Robbins K.C., Yeudall W.A. 1997. Functional characterization in vivo of mutant p53 molecules derived from squamous cells carcinomas of head and neck. Mol. Carcinog., **18**: 78-88.

Caron de Fromental C., Gruel N., Venot C.. Debusshe L., Conseiller E., Dureuill C., Teillaud J.L., Tocque B., Bracco L. 1999. Restoration of transcriptional activity of p53 mutants in human tumor cells by intracellular expression of anti-p53 single chain Fv fragments. Oncogenne, **18**: 551-557.

Caspari TR. 2000. How to activate p53. Curr Biol. 10(8):R315-7.

Chan J., Kim DM., Lee SW., Cho KY., Sung YC. 1995. Transactivation ability of p53 transcriptional activity domain is directly related to the binding affinity to TATA-binding protein. J Biol Chem. 270(42): 25014-9.

Chan T.A., Hermeking H., Lengauer C., Kinzler K.W., Vogelstein B. 1999. 14-3-3 sigma is required to prevent mitotic catastrophe after DNA damage. Nature (London) **401**: 616-620.

Chehab N.H., Malikzay A., Appel M., Halazonetis T.D., 2000. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev. **14**: 278-288.

Chen X., Ko L.J., Jayaraman L., Prives C. 1996. P53 levels, functional domain, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev. **10**: 2438-2451.

Chen Y., Chen P.L., Lee W-H. 1994. Hot-spot mutants interact specifically with two cellular proteins during progression on the cell cycle. Mol. Cell Biol., **14**: 6764-6772.

Chernov M.V., Adler W., Stark G.R. 1998. Stabilization and activation of p53 are regulated independently by different phosphorylation events. Proc Natl Acad Sci USA. **95**:2284-2289.

Cho,Y.,Gorina, S. Jeffrey, P.C., Pavletich, N.P. 1994. Crystal structure of a p53 tumor suppressor-DNA complex. Understanding tumerigenic mutations. Science **265**: 346-355.

Chumacov, PM. 2000. Function of the p53 gene: choice between life and death. Biochemistry (mosc.). **65(1);** 28-40.

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

Clore G.M., Ernst J., Clubb R., Omichinski J.G., Kennedy W.M., Sakaguchi K., Apella E., Gronenborn A.M. 1995. Refined solution structure of the oligomerization domain of the tumor suppressor p53. Nat. Struct. Biol., 2: 321-333.

Colman MS., Afshari CA., Barret JC. 2000. Regulation of p53 stability and activity in response to genotoxic stress. Mut Res. 462(2-3): 179-88

Craig A.L., Bruch L., Vojtesek B., Mikutowska J., Thompson A., Hupp T.R. 1999. Novel phosphorylation sites of human tumor suppressor protein p53 at Ser20 and Thr18 that disrupt the binding of mdm2 (mouse double minute 2) protein are modified in human cancers. Biochem. J. **342**: 133-141.

Craig A.L., Balydes J.P., Bruch L.R., Thompson, Hupp T.R. 1999. Dephosphorylation of p53 at Ser 20 after cellular exposure to low levels of non-ionizing radiation. Oncogene, **18**:6305-6312.

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

Dameron KM., Volpert OV., Tainsky MA., Bouck N. 1994. Control of angiogenesis in fibroblasts by p53 regulation of Thrombospondin 1. Science **265**: 1582-1584.

Davison T.S., Yin P., Nie E., Kay C., Arrowsmith C.H. 1998. Characterization of the oligomerization defects of two p53 mutants found in families with Li-Fraumeni and Li-Fraumeni-like syndrome. Oncogene, **17**: 651-656.

De Rozieres S., Maya R., Oren M. Lozano G. 2000. The loss of mdm2 induces p53 mediated apoptosis. Oncogene, **19**: 1691-1697.

Deb S., Jackson C.T., Subler M.A., Martin D.W. 1992. Modulation of cellular and viral promoters by mutant p53 proteins found in tumor cells. J. Virol., **66**: 6164-6170.

Deng C., Zhong P., Harper J.W., Elledge S.J.. Leder P. 1995. Mice lacking p21/CIP1/Waf1 undergo normal development, but are defective in G1 checkpoint control.

Cell, **82 (4)**: 675-684.

Descombes P., Chojkier M., Lichtsteiner S., Falvey E., Schibler U. LAP a novel member of the C/EBP gene family encodes a liver-enriched transcriptional activator protein. 1990. Genes Dev., **4**: 1541-1551.

Diller L., Kassel J., Nelson CE., Gryka MA., Litwak G., Gebhardt M., Bressac B., Ozturk M., Baker SJ., Vogelstein B . et al. 1990. p53 functions as a cell cycle control protein in osteosarcomas. Mol Cell Biol. 10(11): 5772-81.

Ding H.F., Lin Y.L., McGill G., Zhu H., Blenis J., Yuan J., Fisher D.E. 2000. Essential role for Caspase-8 in transcription independent apoptosis triggered by p53. J Biol Chem. 275(49): 38905-11.

Dittmer, D., Pati, S. Zambetti G., Chu, S. Tersky, A.K. Moore, M. Finlay C. and Levine AJ.,1993. Gain of function mutations in p53. Nature Genet. 4:42-45.

Donehower L.A., Harvey M., Slagle B.L., Mcarthur M.J., Montgomery C.A., Butel J.S., Bradley A. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature **356** (6366): 215-221.

Dudenhoffer C., Rohaly G., Will K., Depper W., Wiesmuller L. 1998. Specific mismatch recognition in heteroduplex intermediates by p53 suggests a role in fidelity control of homologous recombination. Mol. Cell. Biol. **18**: 5332-5342.

El-Deiry, W.S., Kern S. E., Pietenpol J. A., Kinzler K. W., Vogelstein. B. 1992. Human genomic DNA sequences define a consensus-binding site for p53. Nature Genet. **1(1):** 44-49.

El-Deiry, WS., Kern,S.E., Pietenpol,J.A., Levy,D.B., Parsons,R., Trent,J.M., Mercer,W.E., Kinzler,K.W., and Vogelstein,B. 1993. Waf1, a potential mediator of p53 tumor suppression. Cell, **75**:817-825.

El-Deiry W.S., Tokino T., Waldman T., Oliner J.D., Velculescu V.E. Burrel M., Hill D.E., Healy E., Rees J.L. Hamilton S.R., Kinzler K.W., Vogelstein B. 1995. Topological control of p21 (Waf1/Cip1) expression in normal and neoplastic tissues. Cancer Res **55**: 2910-2919.

El-Deiry, W.S. 1998. Regulation of p53 downstream genes. Seminars in Cancer Biology. **8**: 345-357.

El-Deiry WS[2]. 1998. p21/p53, cellular growth control and genomic integrity. Current topics in Microbiology and Immunology 227: 121-137.

Eliyahu D., Michalovitz D., Eliyahu S., Pinhasi-Kimhs O., Oren M. 1989. Wild-type p53 can inhibit oncogene mediated focus formation. Proc. Natl. Acad. Sci. USA. **86**: 8763-8767.

Eller M.S., Maeda T., Magnoni C., Atwal D., Gillchrest B.A. 1997. Enhancement of DNA repair in human skin cells by thymidine dinucleotides: evidence for a p53-mediated mammalian SOS response. Proc. Natl, Acad. Sci. U.S.A. **94**: 12627-12632.

Fang S., Jensen J.P., Luddwig R.L., Vousden K.H., Weissman A.M. 2000. Ubiquitin protein ligase activity of Mdm2: Differential RING finger requirements for ubiquitination and proteosomal targeting of Mdm2 and p53. J. Biol. Chem., **275**: 8945-8951.

Farmer,G.,Bargonetti,J.,Zhu,H.,Friedman,P.,Prywes,R.and Prives,C. 1992. Wild-type p53 activates transcription in vitro. Nature, **358**:83-86.

Fields S., Jang S.K. 1990. Presence of potent transcription activation sequence in the p53 protein. Science, **249**: 1046-1049.

Fiendley H.W., Gu L., Yeager A.M., Zhou M. 1997. Expression and regulation of Bcl-2, Bcl-xl, and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. Blood **89**: 2986-2993.

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

Fiscella M., Ullrich S.J., Zambrano M., Shields M.T., Lin D., Lees-Miller S.P., Anderson C.W., Mercer W.E., Apella E. 1993. Mutation in the Serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. Oncogene, **8**:

1519-1528.

Ford J.M., Baron E.L., Hanawalt P.V. 1998. Human fibroblasts expressing the human papillomavirus E6 gene are deficient in global genomic nucleotide excision repair and sensitive to ultraviolet irradiation. Cancer Res. **58**: 599-603.

Frazier M.W., He X., Wang J. Gu A., Clevelan J.L., Zambetti G.P. 1998. Activation of c-myc gene expression by tumor- derived p53 mutants requires a discrete c-terminal domain. Mol. Cell. Biol., **18**: 3735-3743.

Freedman D.A., Wu L., Levin A.J. 1999. Functions of the MDM2 oncoprotein. Cell Mol. Life Sci. **55**: 96-107.

Fu L., Minden MD., Benchimol A. 1996. Transcriptional regulation of human p53 gene expression. EMBO J. **15**: 4392-4401.

Fu L., Ma W.L., Benchimol S. 1999. A translation repressor element resides in the 3' untranslated region of human p53 mRNA. Oncogene, **18**: 6419-6424.

Fuchs S.Y., Adler V., Buschmann T., W.u X., Ronai Z. 1998. Mdm2 Association with p53 targets its ubiquitination. Oncogene, **17**, 2543-7.

Fuchs S.Y., Adler V., Pincus M., Ronai Z. 1998. MEKK1/JNK signaling stabilizes and

activates p53. Proc. Natl. Acad. Sci. USA, **95**: 10541-10546.

Funk WD., Pak DT., Kara RH., Wright WE., Shay JW. 1992. A transcriptionally active DNA-binding site for human p53 protein complexes. Mol Cell Biol. **12**:2866-2871.

Gao Y., Ferguson D.O., Xie W., Manis J.P., Sekiguchi J., Frank K.M., Chaudhuri J., Horner J., dePinho R.A., Alt F.W. 2000. Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability, and development. Nature, **404**: 897-900.

Gaitonde S.V., Riley J.R., Qiao D., Martinez J.D. 2000. Conformational phenotype of p53 is linked to nuclear translocation. Oncogene **19**: 4042-4049.

Geyer R.K., Yu Z.K., Makiu C.G. 2000. The MDM2 RING-finger domain is required to promote p53 nuclear export. Nat. Cell. Biol., **2**: 569-573.

Giaccia A.J., Kastan M.B. 1998. The complexity of p53 modulation: emerging patterns from diverging signals. Genes Dev. **12 (19)**: 2973-83.

Gingsberg D., Mechta F., Yaniv M., Oren M. 1991. Wild-type p53 can down-regulate the activity of various promoters. Proc Natl Acad Sci. USA. **88:** 9979-9983.

Gorina S., Pavletich N.P. 1996. Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. Science, **274**: 1001-1005.

Gotissa M., Hengstermann A., Fogal V., Sandy P., Scheffner M., DelSal G. 1999. Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. EMBO J. **18**: 6462-6471.

Gottlieb E., Haffner R., von Uden T., Wagner E.F., Oren M. 1994. Down-regulation of wild-type p3 activity interferes with apoptosis of IL-3-dependent hematopoietic cells following IL-3 withdrawal. EMBO J., **13:**1368-1374.

Gottlieb, TM. Oren, M. 1998. p53 and apoptosis. Seminars in Cancer Biology. **8**, 359-368.

Gryaznov SM., Schultz R., Cahturvedi S.K., Letsinger RL. 1994. Enhancement of selectivity in recognition of nucleic acids via chemical autoligation. Nucleic Acids Res., **22**:2366-2369.

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

Gu W., Shi X.L., Roeder R.G. 1997. Synergistic activation of transcription by CBP and p53. Nature (London) **387**: 819-823.

Haapajarvi T., Pitkanen K., Tsubaris M., Laiho M. 1997. p53 Transactivation and

protein accumulation are independently regulated by UV light in different phases of the cell cycle. Mol Cell Biol, **17**: 3074-3080.

Hainaut P., Milner J. 1993. Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. Cancer Res. 53(19): 4469-73.

Hainaut P., Rolley N., Davies M., Milner J. 1995. Modulation by copper of p53 conformation and sequence-specific DNA binding : role for Cu(II)/Cu(I) redox mechanism. Oncogene, **10:** 27-32.

Haldar S., Negrini M., Monne M., Sabbioni S., Croce C.M. 1994. Down-regulation of bcl-2 by p53 in breast cancer cells. Cancer Res. **54**: 2095-2097.

Halazonettis TD., Davis LJ., Kandil AN. 1993. Wilde-type p53 adopts a "mutant"- like conformation when bound to DNA

Hall A.R., Milner J. 1995. Structural and kinetic analysis of p53-DNA complexes and comparison of human and murine p53. Oncogene, **10(3):** 561-7.

Hall PA., McKee PH., Menage HD., Dover R., Lane DP. 1993. High levels of p53 protein in UV-irradiated normal human skin. Oncogene. 8(1): 203-7.

Hall PA. Meek D. and Lane D.P 1996. p53-integrating the complexity. J Pathol. 180(1):

1-5.

Hansen R., Oren M., 1999. P53: from inductive signal to cellular effect. Curr Opin Genet Devel 7:46-51.

Hao M., Lowy A.M., Kapoor M., Deffie A., Liu G., Lozano G. 1996. Mutation of phosphoserine 389 affects p53 function *in vivo*. J. Biol. Chem., **271**: 299380-29385.

Harper J.W., Adami G.R., Wei M., Keyomarsi K., Elledge S.J. 1993. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin dependent kinases. Cell, **75:** 805-816.

Harris CC., Hollstein M. 1993. Clinical implications of the p53 tumor-suppressor gene. N. Engl. Med. **(18):** 1318-1327.

Hartwell, L. 1992. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell, **71**:543-546.

Haupt R., fears TR., Heise H., Loiacono G., De Terlizzi M., Tucker MA. 1997. Risk of secondary leukemia after treatment with etoposide (VP-16) for Langerhan's cell Histiocytosis in Italian and Austrian-German populations. Int J Cancer. 71(1): 9-13.

Haupt Y., Rowan S., Shaulin E., Vousden K.H., Oren M. 1995. Induction of apoptosis in HeLa cells by trans-activation- deficient p53. Genes Dev. **9**: 2170-2183.

Haupt Y., Maya R., Kazar A., Oren M. 1997. Mdm2 promotes the rapid degradation of p53. Nature, **387**, 296-9.

Hermeking H., Lengaver C., Polyak K., He T.C., Zhang L., Thiagalingam S., Kinzler K.W., Vogelstein B. 1997. 14-3-3 Sigma is a p53 regulated inhibitor of G2/M progression. Mol. Cell, **1**:3-11.

Hiebert S.W., Packham G. Strom D.K., Haffner R., Oren M., Zambetti G., Cleveland J.C. 1995. E2F-1: DP-1 induces p53 and overrides survival factors to trigger apoptosis. Mol. Cell. Biol. **15**: 6864-6874.

Hinaut P., Milner J. 1993. Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. Cancer Res., **53**:4469-4473.

Hirao A., Kong Y.Y.. Matsuoka S., Wakeham A., Ruland J., Yoshida H.D.L., Elledge S.J., Mak T.W. 2000. DNA damage-inducible activation of p53 by the checkpoint kinase Chk2. Science, **287**: 1824-1827.

Hoffman R., Craik D.J., Pierens G., Bolger R.E.. Otvos Jr. L. 1998. Phosphorylation of the C-terminal sites of human p53 reduces non-sequence-specific DNA-binding as modeled with synthetic peptides. Biochemistry, **37**: 13755-13764.

Hollstein, M.; Sidrowsky, D.; Vogelstein, B.; Harris. C.C.1991. p53 mutations in human cancers.. Science **253**:49-53.

Hollstein, M., Rice, K., Greenblatt, MS., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., Harris, CC.1994. Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res., **22**, 3551-5.

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

Honda R., Yasuda H.1999. Association of p19 (ARF) with MDM2 inhibits ubiquitin ligase activity of MDM2 for tumor suppressor p52. EMBO J. **18**: 22—2227.

Howley PM., 1991. Role of the human papillomavirus in human cancer. Cancer Res., **51**: 5019s-5022s.

Hsiao M., Low J., Dorn E., Ku D., Pattengale P., Yeargin J., Hass M. 1994. Gain-of-function mutations of the p53 gene induce lympho-haematopoietic metastatic potential and tissue invasiveness. Am. J. Pathol., **145**: 702-714.

Huang L.C., Claikin K.C., Wahl G.M. 1996. Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. Proc. Natl. Acad. Sci. USA. **93**: 4827-4832.

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

Hupp T.R., Lane D.P. 1994. Allosteric activation of latent p53 tetramers. Curr. Biol. **4**: 865-875.

Hupp T.R., Lane D.P. 1994. Regulation of the cryptic sequence-specific DNA-binding function of p53 by protein kinases. Cold Spring Harbor Symposia Quantit Biol., **59:** 195-206.

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

Hupp Tr., Lane DP., Ball KL. 2000. Strategies for manipulating the p53 pathway in the treatment of human cancer. Biochem J. 352 pt. 1: 1-17.

Jacks T., Remington L., Williams BO., Schmitt EM., Halachmi S., Bronson RT. Winnberg RA. 1994. Tumor spectrum analysis in p53- mutant mice. Curr. Biol. **4(1):** 1-7.

Janus F., Albrechtsen A., Dornreiter J., Wiesmuller L., Grosse F., Deppert W. 1999. The dual role model for p53 in maintaining genomic integrity. CMLS, **55**:12-27.

Janus F., Albreechtsen N., Knippschild U., Wiesmuller L., Grosse F., Deppert W. 1999a. Different regulation of the p53 core domain activities 3' to5' exonuclease and sequence-specific DNA binding. Mol. Cell. Biol., **19**: 2155-2168.

Janus F., Albrechtsen N., Dornreiter I., Wiesmuller L., Grosse F., Deppert W. 1999b. Cell. Mol. Life Sci., **55**: 12-27.

Jayaraman J., Prives C. 1995. Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. Cell. **81**:1021-10029.

Jayaraman L., Murthy K.G.K., Zhu C., Curran T., Xanthoudakis S., Prives C. 1997. Identification of redox/repair protein Ref-1 as a potent activator of p53. Genes Dev. **11**:558-570.

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

Jayaraman L., Prives C. 1999. Covalent and non-covalent modifiers of the p53 protein. Cell. Mol. Life Sci., **55**: 76-87.

Jeffrey P.D., Gorina S., Pavletich N.P. 1995. Crystal structure of the tetramerization domain of p53 tumor suppressor at 1.7 angstroms. Science **267**:1498-1502.

Jones S.N., Roe A.E., Donehower L.A., Bradley A. 1995. Rescue of embryonic lethality in MDM2-deficient mice by absence of p53. Nature, **378**: 206-208.

Juven T., Barak Y., Zauberman A., George DL., Oren M. 1993. Wild-type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene. Oncogene. 8(12): 3411-6.

Juan L.J., Shia W.J., Chen M.H., Yang W.M. Seto E., Lin Y.S., Wu C.W. 2000. Histone deacetylases specifically down-regulate p53-dependent gene activation. J. Biol. Chem. **275**: 20436-20443.

Kachnic LA., Wu B., Wunsch H., Mekeel KL., DeFrank JS., Tgang W., Powel SN. 1999. The ability of p53 to activate downstream genes p21 (Waf 1/Cip 1) and mdm2, and cell cycle arrest following DNA damage is delayed and attenuated in scid cells deficient in the DNA-dependent protein kinase. J Biol Chem. **274(19):** 13111-7.

Kadonaga, JT. Purification of sequence- specific binding proteins by DNA affinity chromatography. Methods Enzymoly. **208**: 10-23.

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

Kastan MB., Zhan Q., El-Deiry WS., Carrier F., Jaks T., Walsh WV., Plunkett BS., Vogelstein B. and Fornace AJ.1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in Ataxia-Telangiectasia. Cell, **71**:587-597.

Kawamura M., Yamashita T., Segawa K., Kaneuchi M., Shindoh M., Fujinaga K. 1996. The 273rd codon mutant of p53 shows growth modulation activities not correlated with p53-specific transactivation activity. Oncogene, **12**: 2361-2367.

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

Khosravi R., Maya R., Gottlieb T., Oren M., Shiloh Y., Shkedy D. 1999. Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. Proc. Natl. Acad. Sci. USA, **96**: 14973-14977.

Kieser A., Weich H.A., Brandner G., Marme D., Kolch W. 1994. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. Oncogene, **9**: 963-969.

Kim, I.S., Kim D.H., Han S.M., Chin M.U., Nam H.J., Cho H.P., Choi S.Y., Son E.R., Bae Y.S., Moon Y.H.. 2000. Truncated forms of importin alpha identified in breast cancers inhibit nuclear import of p53. J. Biol. Chem. **275**: 23139-23145.

Kirch H.C., Falswinkel S., Rumpf H., Brockmann D., Esche H.. 1999. Expression of human p53 requires synergistic activation of transcription from the p53 promoter by AP-

1. NF-kappaB and Myc/Max. Oncogene, **18**: 2728-2738.

Ko, LJ., Prives C., 1996. P53: Puzzle and paradigm. Genes Dev 10: 1054-1072.

Ko LJ., Chen X., Shiehh S., Jayaraman L., Tamaik K., Taya Y. 1997 p53 is phosphorylated by CDK7/cyclin H in a 36/MAT 1 dependent manner. Mol. Cell Biol. **17**:7220-7229.

Kobet E., Zheng X., Zhu Y., Keller D., Lu H. 2000. MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with two proteins. Proc. Natl. Acad. Sci. USA **97**: 112547-12552.

Komarova E.A., Diatchenko L., Rokhlin O.W., Hill J. E., Wang Z.J., Krivokrysenko V.I., Feinstein E., Gudkov A.V. Stress- induced secretion of growth inhibitors: a novel tumor suppressor function of p53.1998. Oncogene, **17**: 1089-1096.

Krause K., Haugwitz U., Wasner M., Wiedmann M., Mossner J., Engeland K. 2001. Expression of the cell cycle phosphatase cdc25C is down-regulated by the tumor suppressor p53 but not by p73. Biochem Biophys Res Commun. **284(3):** 743-50.

Kubbutat M.H.G., Jones S.N., Vousden K.H. 1997. Regulation of p53 stability by Mdm2. Nature, **387**: 299-303.

Kubbutat M.H.G., Vousden K.H., 1997. Proteolytic cleavage of human p53 by calpain: A potential regulator of protein stability. Mol. Cell Biol., **17**: 460-468.

Kuerbitz S.J., Plunkett B.S., Walsh W.V.; Kastan M.B. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA, **89**:7491-7495.

Kulez-Martin M.F., Lisafeld B., Husang H., Kisiel N.D., Lee L. 1994. Endogenous p53 protein generated from wild-type alternatively spliced p53 RNA in mouse epidermal Cells. Mol. Cell. Biol., **14**: 1698-1708.

Kumari S.R., Mendoza-Alvarez H., Alvarez-Gonzalez R. 1998. Functional interaction of p53 with poly (ADP-ribose) polymerase (PARP) during apoptosis following DNA damage: covalent poly(ADP-ribosyl)ation of p53 by exogenous PARP and covalent binding of p53 to the 85,000 proteolytic fragment. Cancer Res. **58**: 5075-5078.

Kussie P.H., Gorina S., Marecha V., Elenbaas B., Moreasu J.. Levine A.J., Pavletich N.P. 1996. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science, **274**: 948-953.

Lakin N.D., Jackson S.P. 1999. Regulation of p53 in response to DNA damage. Oncogene, **18**: 7644-7655.

Lambert P.F., Kashanchi F., Radonovicch M.F., Shiekhattar R., Braddy J.N. 1998. Phosphorylation of p53 Serine 15 increases interaction with CBP. J. Biol. Chem. **273**: 33048-33053.

Lane D.P. 1992. P53: guardian of the genome. Nature, **358**: 15-16.

Lanyi A., Deb D., Seymur RC., Ludes-Meyer J.H., Subler M.A., Deb S. 1998. "Gain of function" phenotype of tumor- derived mutant p53 requires oligomerization/non sequence- specific nucleic acid-binding domain. Oncogene, **16**: 3169-3176.

Laronga C., Yang H.Y., Neal C., Lee M.H. Yang HY., 2000. Association of the cyclin-dependent kinases and 145-3-3 sigma negatively regulates cell cycle progression. J. Biol. Chem. **275**: 23106-23112.

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

Lee S., Cavallo L., Griffith J. 1997. Human p53 binds Holliday junctions strongly and facilitates their cleavage. J. Biol. Chem., **272**: 7532-7539.

Lee W., Harvey t.S., Yin Y., Yau P., Litchfield D. Arrowsmith C.H. 1994. Solution structure of the tetrameric minimum-transforming domain of p53. Nast. Struct. Biol., **1**:

877-890.

Levine AJ., 1991. Rare diseases do occur. Arch Emerg Med. 8(4):296.

Levine, AJ. 1997. p53, the cellular gatekeeper for growth and division. Cell. **88,**323-31.

Li B., Lee MY. 2001. transcriptional regulation of the human DNA polymerase delta catalytic subunit gene POLD1 by p53 tumor suppressor and SP1. J Biol Chem. **276(32):** 29729-39.

Li Y., Jenkins C.W., Nichols M.A., Xiong Y. 1994. Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. Oncogene, **9**: 2261-2268.

Lill N.L., Grossman S.R., Ginsberg D., DeCaprio J., Livingston D.M. 1997. Binding and modulation of p53 by p300/CBP coactivators. Nature, **387**: 823-837.

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

Lin Y., Ma W., Bhenchimol S. Pidd, a new death- domain-containing protein, is induced by p53 and promotes apoptosis. 2000. Nature Genet. **26**: 122-127.

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

Liu X., Miller C., Koeffler P.H., Berl A.J. 1993. The p53 activation domain binds the TATA -binding protein polypeptide in Holo-TFIID and a neighboring p53 domain inhibits transcription.. Mol. Cell. Biol., **13 (6)**: 3291-3300.

Liu Z.G., Baskaran R., Lea-Chou E.T., Wood L.D., Chen J., Karin M., Wanng J.Y. 1996. Three distinct signaling responses by murine fibroblasts to genotoxic stress. Nature, **384**: 273-276.

Ljungman M., 2000. Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. Neoplasia. May-Jun **2(3):** 208-25.

Lomax M.E., Barnes D.M., Hupp T.R., Pickksley S.M., Cammplejohn R.S. 1998. Characterization of p53 oligomerization domain mutations isolated from Li-Fraumeni and Li-Fraumeni-like family members. Oncogene, **17**: 643—649.

Lowe S., Schmitt E.M., Smith S.W. Osborne B.A., Jacks T.. 1993. p53 is required for radiation induced apoptosis in mouse thymocytes. Nature, **362**: 847-849.

Lowe S.W. 1999. Activation of p53 by oncogenes. Endcr. Realt. Cancer, **6**:45-48.

Lozano G., Montes de Oca Luna R. 1998. MDM2 function. Biochim. Biophhys. Acta. **1377** M55-59.

Ludes-Meyer J.H., Subler M.A., Schiivakumar C.V., Munoz R.M., Jiang P., Bigger J.E., Brown D.R. Deb S.P, Deb P.1996. Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol. Cell. Biol., **16**: 6009-6019.

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

Luo J.L., Yang Q., tong W.M., Hergenhahn M., Weang Z.Q., Hollstein M. 2001. Knock-in mice with a chimeric human/murine p53 gene develop normally and show wild-type p53 response to DNA damaging agents: a new biomedical research tool. Oncogene, **20(3):** 320-8.

Ma Y., Yuan R., Meng A., Goldberg I.D., Rosen I.M., Fan S. 2000. P53-independent down-regulation of Mdm2 in human cancer cells treated with Adriamycin. Mol. Cell. Biol. Res. Commmun. **3**: 122-128.

Macleod MC. 1993. Identification of a DNA structural motif that includes the binding sites for Sp1, p53 and GA-binding protein. Nuc Acids Res. 21(6):1439-47.

Mailand N., Falcck J.. Likas C., Syljuasen R.G., Welcker M., Barteck J., Likas J. Rapid destruction of human Cdc 25A in response to DNA damage. 2000. Science. **288 (5470):** 1425-1429.

Malkin D., Li, F.P., Strong L.C., Fraumeni J.F Jr., Nelson C.E., Kim D.H., Kassel J., Gryka M.A., Bishoff F.Z., Tainsky M.A. 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science, 250:1233-1238.

Maltzman W., Czyzyk L. 1984. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell Biol., **4**: 16898-1694.

Martin D.W. Munoz RM.. Subler MA., Deb S. 1993. P53 binds to the TATA binding protein TATA complex. J. Biol. Chem. **268**:13062-13067.

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

Matlashewiski G. 1999. P53: twenty years on, Meeting Review. Oncogenne. **18**: 7618-7620.

Mayo L.D., Turchi J.J., Berberiich S.J. 1997. Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. Cancer Res. **57**: 5013-5016.

McLure KG., Lee PWK. 1998. How p53 binds DNA as a tetramer. EMBO J., **17**: 3342-3350.

McLure KG., Lee PWK. 1999. p53 DNA binding can be modulated by factors that alter conformational equilibrium. EMBO J. **18**: 763-770.

Meek D.W. 1997. Post-translational modification of p53 and the integration of stress signals. Pathol Biol., **45**: 804-814.

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

Meek D.W. 1999. Mechanisms of switching on p53: A role for covalent modifications. Oncogene, **18**: 7666-7675.

Mekeel K.L., Tang W., Kchnic L. A., Luo C.M., DeFrank J.S., Powell S.N. 1997. Inactivation of p53 results in high rates of homologous recombination. Oncogene, **14**: 1847-1857.

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

Midgley C.A., Owens B., Briscoe C.V., Thomas D.B., Lane D.P., Hall P.A. 1995. Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type in vivo. J. Cell Sci. **108**: 1843-1848.

Midgley C.A., Desterro J.M., Saville M.K. Howard S., Sparks A., Hay R.T., Lane D.P. 2000. An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. Oncogene, **19**: 2312-2323.

Milner J., Watson JV. 1990. Addition of fresh medium induces cell cycle and conformation changes in p53, a tumor suppressor protein. Oncogene, **5:** 1683-1690.

Milner J. 1995. DNA damage, p53 and anticancer therapies. Nat Med. 1(9): 879-80.

Miller C.W., Imai Y., Aslo A., Li L., Koeffler H.P. 1992. Proc. Am. Assdoc. Cancer Res.**33,** 386.

Miller CW., Simon K., Aslo A., Kok K., Yokota J., Buys CH., Terada M., Koeffler HP. 1992. p53 mutations in human lung tumors. Cancer Res. **52(7):** 1695-8.

Miller S.D., Moses K., Jayaraman L., Prives C. 1997. Complex formation between p53 and replication protein A inhibits the sequence-specific DNA binding of p53 and is regulated by single-stranded DNA. Mol. Cell. Biol. **17:** 2194-2201.

Miu X., Miller CW., Koeffler PH., Berk AJ. 1993. The p53 activation domain binds the TATA box-binding polypeptide in a holo TFIID and a neighboring p53 domain inhibits transcription. Mol. Cell Biol. **13**: 3291-3300.

Miyashita T., Krajewski S., Krajewski M., Wang H.G., Lin H.K., Liebermman D.A., Hoffman B., Reed J.C. 1994. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene **9**: 1799-1805.

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

Moberg KH., Tyndall WA., Hall DJ. 1992. Wild-type murine p53 represses transcription from the murine c-myc promoter in a human glial-cell line. J. Cell Biochem. **49**:208-215.

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

Momand J., Zambetti GP., George DL., Levine AJ. 1992. The mdm2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell, **69**: 1237-1245.

Monique G., van Oijen, Slotweg PJ. 2000. Gain-of-function mutations in the tumor suppressor gene p53. Clinical Cancer Research, **6**:2138-2145.

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

Mosner J., Mummenbrauer T., Bauer C., Sczakiel G., Gross F., Deppert W. 1995. Negative feedback regulation of wild-type p53 synthesis. EMBO J., **14**: 4442-4449.

Muller B., Paulsen D., Deppert W. 1996. Specific binding of MAR/SAR DNA- elements by mutant p53. Oncogene, **12**: 1941-1952.

Muller M., Strand S., Hug H., Heinemann E.M., Walczak H., Hofmann W.J., Stremmel W., Krammer P.H., Galle P.R. 1997. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1Fas) receptor/ligand system and involves activation of wild-type p53. J. Clin. Invest. **99**:403-413.

Muller S., Berger M., Lehembre F., Seeler J.S., Haupt Y., Dejean A. 2000. C-Jun and p53 activity modulated by SUMO-1 modification. J. Biol. Chem. **275**: 13321-13329.

Mummenbrauer T., Janus F., Muller B., Wiesmuller L., Deppert W., Grosse F. 1996. p53 protein exhibits 3'-to 5' exonuclease activity. Cell, **85**: 1089-1099.

Mundt M., Hupp T., Fritsche M., Merkle C., Hansen S., Lane D., Groner B. 1997. Protein interactions at the carboxyl terminus of p53 result in the induction of its in vitro transactivation potential. Oncogene, **15**:237-244.

Nie Y., Li H.H., Bula C.M., Liu X. 2000. Stimulation of p53 DNA binding by c-Abl requires the p53 C-terminus and tetramerization. Mol. Cell. Biol. **20**: 741-748.

Nishimori H., Shiratsuchi T., Urano T., Kimura Y., Kiyono K., Tatsumi K., Yoshida S., Ono M., Kuwano M., Nakamura Y., Tokino T. 1997. A novel brain- specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. Oncogene, **15**: 2145-2150.

O'Connell M.J. Walworth N.C., Carr A.M. 2000. The G2-phase DNA- damage checkpoint. Trends Cell Biol, Jul **10(7):** 296-303.

Oda E., Ohki R., Murasawa H., Nemoto J., Shibue T., Yamashita T., Tokino T., Taniguchi T., Tanaka. 2000. Noxa a BH3-only member of the Bcl2 family and candidate mediator of p53-induced apoptosis. Science, **288**: 1053-1058.

Oda K., Arakawa H., Tanaka T., Matsuda K., Tanikawa C. Mori. 2000. p53 AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell,**102**: 849-862,

Offer H., Wolkkowicz R., Matas D., Blumenstein S., Livneh A., Rotter V. 1999. Direct involvement of p53 in the base excision repair pathway of DNA repair machinery. FEBS Lett. **450**: 197-204.

Ohki R., Nemoto J., Murasawa H., Oda E., Inazawa J., Tanaka N., Taniguchi T. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. 2000. J. Biol. Chem. **275**: 22627-22630.

Okorokov AL., Milner J. 1999. An ATP/ADP dependent molecular switch regulates the stability of p53-DNA complexes. MCB. **19**:7501-7510.

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

Oliner JD., Pietenpol JA., Thiagalingam S., Gvuris J., Kinzler KW., Vogelstein B. 1993. Oncoprotein MDM2 conceals the activation domain of tumor suppressor-p53. Nature **362**:857-860.

Oltvai Z.N., Korsmeyer S.J., 1994. Cheeckpoints of dueling dimmers foil death wishes. Cell, **79**: 189-192.

Owen-Schuab L.B., Zhang W., Cusack J.C., Angelo L.S., Santee S.M., Fujiwara T., Roth J.A., Deisseroth A.B., Zhang W.W., Kruzel E., Radinsky R. 1995. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Molec. Cell Biol. **15:** 3032-3040.

Pariat M., Carillo S., Molinari M., Salvat C., Debussche L., Bracco L., Milner J. Piechaczyk M. 1997. Proteolysis by calpain: A possible contribution to degradation of p53. Mol. Cell Biol., **17**: 2806-2815.

Pavlovich, A.G., Tocczyski DP. Hartwell LH. 1997. When checkpoints fail. Cell, vol. **88**, 315-321.

Pavletich N.P., Chambers K.A., Pabo C.O. 1993. The DNA-binding domain of p53 contains four conserved regions and the major mutation hot spots. Genes Dev., **7 (12B)**: 2556-2564.

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

Pietenpol J.A., Tokino T., Thiagalingam S., el-Deiry W.S., Kinzler K.W., Vogelstein B. 1994. Sequence-specific transcriptional activation is essential for growth suppression by p53. Proc. Natl. Acad. Sci. USA, **91**: 1998-2002.

Piette J., Neel H., Marechal V. 1997. MDM2: keeping p53 under control. Oncogene **15:** 1001-1010.

Pise-Masison C.A., Radonovich M., Sakaguchi K., Apella E., Brady J.N. 1998. Phosphorylation of p53: a novel pathway for p53 inactivation in human T-cell lymphotropic virus type 1-transformed cells. J. Virol., **72**: 6348-6355.

Pochampally R., Fodera B., Chen L., Shao W., Levine A., Chen J. 1999. A 60Kd MDM2 isoform is produced by caspase cleavage in non-apoptotic tumor cells. Oncogene, **17**:2629-2636.

Pochampally R., Fodera B., Chen L., Lu W., Chen J. 1999. Activation of an MDM2-specific caspase by p53 in absence of apoptosis.

Polyak K., Xio Y., Zweier J.L., Kinzler K.W., Vogelstein B. 1997. A model for p53-induced apoptosis. Nature **289**: 300-305.

Pomerantz J., Scheiber-Agus N., Ligeois N.J., Silvermann A., Alland L., Chin L., Potes J., Chen K., Orlow I., Lee H.W., Codon-Carddo C., Depinho R.A. 1998. The Ink4A tumor suppressor gene product, p19ARF, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell, **92**: 713-23.

Prives C. 1994. How loops, beta sheets and alpha helices help to understand p53. Cell,

78, 543-6.

Prives C. 1998. Signaling to p53: breaking the Mdm2-p53 circuit. Cell. **95 (1):** 5-8.

Prives C. Hall P. 1999. The p53 Pathway. J. Pathol, **187**: 112-126.

Prokocimer M., Harris N., Brill E., Rotter V. 1987. Expression of p53 in human leukemia lymphoma. Recent Advances in Leukemia Lymphoma. 243-264. Alan R. Liss, Inc.

Purdie CA., Harrison DJ., Peter A., Dobbie L., White S., Howiee SEM., Salter DM., Bird CC., Wyllie AH., Hooper ML., Clarke AR. 1994. Tumor incidence spectrum and ploidy in mice with large deletion in the p53 gene. Oncogene **9(2):** 603-609.

Raman V., Martensen SA., Reisman D., Evron E., Odenwald WF., Jafee E., Marks J., Sukumar S.. 2000. Compromised HOXA5 function can limit p53 expression in human breast tumors, Nature, **405**: 974-978.

Raycroft L., Wu H., Lozano G. transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene.1990. Science. **249**: 1049-1051.

Reed M., Woelker B., Wang P., Wang Y., Anderson M.E., Tegtmeyer P. 1995. The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. Proc. Natl.

Acad. Scie. USA, **92**: 9455-9559.

Reisman D., Elkind N.B., Roy B., Beamon J., Rotter V. C-myc trans-activates the p53 promoter through a required downstream CACGTG motif. 1993. Cell. Growth Differ. **4**: 57-65.

Reisman D., Loging T. 1998. Transcriptional regulation of the p53 tumor suppressor gene. Cancer Biology, **8**, 371-324.

Resnick-Silverman L., St. Clair S., Mauretg M., zhao K., Manfredi J.J. 1998. Identification of a novel class of genomic DNA-binding sites suggests a mechanism for selectivity in target gene activation by tumor suppressor protein p53. Genes Dev., **12**-2102-2107.

Rodriguez N.R., Rowan A., Smith M.E... Kerr IB., Bodmer W.F., Gannon J.V., Lane D.P. 1990. p53 mutations in colorectal cancer. Proc. Natl,.Acad. Sci.. U.S.A. **87**: 7555-7559.

Rodriguez M.S., Desterro J.M.P. Lain S., Midgley C.A., Lane D.P.. Hay R.T. 1999. SUMO-1 modification activates the transcriptional response of p53. EMBO J. **18**:6455-6461.

Rogan E.M., Bryan T.M., Hukku B. 1995. Alterations in p53 and p16 Ink4 expression

and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. Mol Cell Biol; **15**:4745-4753.

Rosse T., Olivier R., Monney L., Rager M., Conus S., Fellay I., Jansen B., Bornerm C. Bcl2 prolongs cell survival after bax-induced release of cytochrome c. 1998. Nature, **391**: 496-499.

Roth J., Dobbelstein M., Freedman D.A., Shenk T., Levine A.J. 1998. Nucleo-cytoplasmic shuttling of hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. EMBO J. **17**:554-564.

Salles-Passador I., Fotedar A., Fotedar R. 1999. Cellular response to DNA damage. Link between p53 and DNA-PK. C R Acad Sci III, **322(2-3):** 113-20.

Sakaguchi K., Herrera J.E., Saito S., Miki T., Bustin M., Vassilev A., Anderson CW., Apella E. 1998. DNA damage activates p53 through a phosphorylation- acetylation cascade. Genes Dev. **12**:2831-2841.

Sakamuro D., Sabbatini P., White E., Prendergast G.C. The polyproline region of p53 is required to activate apoptosis but not growth arrest. 1997. Oncogene, **15**: 887-898.

Santhanam U., Ray A., Sehgal PB. 1991. Repression of the interleukin-6 gene promoter by p53 and the retinoblastoma susceptibility gene-product. Proc. Natl. Acad. Sci. USA.

88:7605-7609.

Schackelford, RE., Kaufmann WK., Paules RS. 1999. Cell Cycle Control, Checkpoint Mechanisms, and Genotoxic Stress. Environ Health Perspect, **107 Suppl 1**:5-24.

Scharer E., Iggo R. 1992. Mammalian p53 can function as a transcription factor in yeast. Nucl. Acids Res. **20**:1539-1545.

Schwartz D., Rotter, V. 1998. P53-dependent cell cycle control: Response to genotoxic stress. Seminars in Cancer Biology, **8**: 325-336.

Scolnick D.M., Chehab N.H., Stavridi. E.S., Lien M.C., Caruso L., Moran E., Berger S.L., Halazonetis T.D. 1997. CREB-binding protein and P300/CBP-assocaited factor are transcriptional coactivators of the p53 tumor suppressor protein. Cancer Res., **57**: 3693-3696.

Selivanova G., Iotsova V., Kiseleva E., Strom M., Bakalkin G., Grafstrom R.C., Wiman K.G. 1996. The single-stranded DNA end-binding site of p53 coincides with the C-terminal regulatory region. Nucleic Acids Res., **24**: 3560-3567.

Selivanova, G., Kawasaki, T., Ryabchenko,L., Wiman, K.G. 1998. Reactivation of mutant p53: a new strategy for cancer therapy. Semin Cancer Biol., **8**, 367-78.

Selvakumaran M., Lin H.K., Miyashita T., Wang H.G., Krajewki S., Reed J.C., Hoffman B., Liebermman D. 1994. Immediate early up-regulation of bax expression by p53 but not TGF beta-1: A paradigm for distinct apoptotic pathways. Oncogene **9**: 1791-1798.

Seto E., Usheva A., Zambetti GP., Momand J., Horikoshin N., Weinmann R., Levine AJ., Shenk T. 1992. Wild-type p53 binds to the TATA-binding protein and represses transcription. Proc Natl Acad Sci. USA. **89(245):** 12028-32.

Sengupta S., Vonesch J.L., Waltzinger C., Zheng H., Wasyluck B. 2000. Negative crosstalk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells. EMBO J. **19**: 6051-6064.

Shaulsky G., Ben-Ze'ev A., Rotter V. 1990. Subcellular distribution of the p53 protein during the cell cycle of Balb/c 3T3 cells. Oncogene, **5**: 1707-1711.

Shaulsky G., Goldfinger N., Rotter V. 1991. Alterations in tumor development in vivo mediated by expression of wild-type of mutant p53 proteins. Cancer Res., **51**: 5232-5237.

Shaulsky G., Goldfinger N., Tosky M.S., Levine A., Rotter V. 1991. Nuclear localization is essential for the activity of p53 protein. Oncogene, **6**: 2055-2065.

Shaw P., Freeman J., Bovey R., Iggo R. 1996. Regulation of specific DNA binding by p53: evidence for a role of O-glycosylation and charged residues at the carboxy-terminus. Oncogene, **12**: 921-930.

Sheikh M.S., Burns T.F., Huang Y., Wu G.S., Amudson S., Brooks K.S., Fornace A.J., El-Deiry W.S. 1998. P53-dependent and independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. Cancer Res. **58**: 1593-1598.

Sherr C.J. 1996. Cancer Cell Cycles. Science **274**:1672-1677.

Sherr C.J.,1998. Tumor surveillance via the ARF-p53 pathway. Genes Dev. **12**: 2984-2991.

Sherr C.J., Weber J.D. 2000. The ARF/p53 pathway. Curr Opin Genet Dev **10**: 94-99.

Sheikh MS., Antinore MJ., Huan Y., Fornace AJ. Jr. 1998. Ultraviolet-irradiation-induced apoptosis is mediated via ligand independent activation of tumor necrosis factor receptor 1. Oncogene. **17(20):** 2555-63.

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

Shieh S.Y., Ahn J., Tamai K., Taya Y., Prives C. 2000. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylates p53 at multiple DNA damage-inducible sites. Genes Dev., **14**: 289-300.

Shikama N., Lee C.W., France S. Delavaine L., Lyon J., Krstic-Demonacos M., La Thangue N.B. 1999. A novel cofactor for p300 that regulates the p53 response. Mol Cell **4**: 365-376.

Sidransky D., Mikkelsen T., Schwechheimer K., Rosenblum M.L., Cavanee W., Vogelstein B. 1992. Clonal expansion of p53 mutant cells associated with brain tumor progression. Nature (London) **355**: 846-847.

Sigal A., Rotter V. 2000. Oncogenic mutations of the p53 tumor suppressor: the Demons of the guardian of the genome. Cancer Res. **60(24):** 6788-6802.

Siliciano J.D., Canman C.E., Taya Y., Sakaguchi K., Apella E., Kastan M.B. 1997. DNA damage induces phosphorylation of the amino terminus of p53. Gens Dev. **11**:3471-3481.

Sionov RV., Moalleem E., Berger M., Kazaz A., Gerlitz O., Ben- Meriah Y., Oren M., Haupt Y.. 1999. C-Abl neutralizes the inhibitory effect of MDM2 on p5333. J. Biol. Chem. **274**:8371-8374.

Sionov R.V., Haupt Y. 1999. The cellular response to p53: the decision between life and death. Oncogene **18**: 6145-6157.

Soussit T. Legros Y., Lubin R., Ory K., Schlichtholz B. 1994. Multifactorial analysis of p53 alterations in human cancer: a review. Int. J. Cancer, **57**:1-9.

Spillare E.A., Robles A.I., Wang X.W., Shen J.C. Yu C.E., Schellenberg G.D., Harris C.C. 1999. p53- mediated apoptosis is attenuated in Werner syndrome cells. Genes Dev. **13:** 1355-1360.

Srinivasula S.M., Ahmad M., Fernandes-Alnemri T., Alnemri E.S. 1998. Autoactivation of procaspase-9 Apaf-1 mediated oligomerization. Mol. Cell, **1**: 949-957.

Steeg H. 2001. The role of nucleotide excision repair and loss of p53 in mutagenesis and carcinogenesis. Toxicol Lett. **120(1-3):** 209-19.

Stenger J.E., Mayr G.A., Mann K., Tetmeyer P. 1992. Formation of stable p53 homotetramers and multiples of tetramers. Mol. Carcinog. **5 (2)**: 102-106.

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

Stommel J.M., Marchenko N.D., Jimenez G.S., Moll UM., Hope T.J., Walhl G.M. 1999. A leucine-rich nuclear export signal in the p53 tetramerization domain: Regulation of subcellular localization and p53 activity by NES masking. EMBO J., **18**: 1660-1672.

Stuart ET., Haffner R., Oren M., Gruss P. 1995. Loss of p53 function through Pax-madiated transcriptional repression. EMBO J **22**:5638-5645.

Subler M., Martin D., Deb S., 1992. Inhibition of viral and cellular promoters by human wild-type p53. J. Virol **66**:4757-4762.

Sun X., Shimizu H., Yamamoto K. 1995 Identification of a novel p53 promoter element involved in genotoxic stress-inducible p53 gene expression. Mol. Cell. Biol. **15**: 4489-4496.

Sun Y., Oberley LW. 1996. Redox regulation of transcriptional activators. Free Radiac Biol Med. **21(3):** 335-48.

Takenaka I., Morin F., Seizinger B.R., Klleey N. 1995. Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases. J. Biol. Chem., **270:** 5405-5411.

Tan TH., Wallis J., Levine A.J. 1986. Identification of the p53 protein domain involved in formation of the simian virus 40 large T-antigen-p53 protein complex. J. virol., **59**: 574-583.

Tang H.Y., Zhao K., Pizzolato J.F., Fonarev M., Langer J.C., Manfredi J.J. 1998. Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. J. Biol. Chem., **273**: 29156-29163.

Tang W., Willers H., Powell S.N. 1999. p53 directly enhances rejoining of DNA double-strands breaks with cohesive ends in gamma-irradiated mouse fibroblasts. Cancer Res. **59**: 2562-2565.

Teysier F., Bay J.O., Dionet C., Verrelle P. 1999. Cell cycle regulation after exposure to ionizing radiation. Bull Cancer. April **86(4):** 345-57.

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

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

Truant R., Antunovic J., Greenblat J., Prives C., Cromlish JA. 1995. Direct interaction of the hepatitis B virus HBX protein with p53 leads to inhibition by HBX of p53 response element-directed transcription. J. Virol. **69(3):** 1851-9.

Ueba T., Nosaka T., Takahashii JA., Shibata F., Florkiewickz RZ., Vogelstein B., Oda Y., Kikuchi H., Hatanaka M. 1994. Transcriptional regulation of basic fibroblast growth factor gene by p53 in human glioblastoma and hepatocellular carcinoma cells. Proc. Natl. Acad. Sci. USA. **91**: 9009-9013.

Ueda H., Ullrich SJ., Gangemii JD., Kappel CA., Ngo L., Feitelson MA., Jay G. 1995. Functional inactivation but not structural mutation of p53 causes liver cancer. Nature Genet., **90**: 41-47.

Ueno M., Masutani H., Arai R.J., Yamauchi A., Hirota K., Sakai T., Inamoto T., Yamaoka Y., Yodoi J., Nikaiddoi T. 1999. Thioredoxin-dependent redox regulation of p53-mediated p21 activation. J. Biol. Chem., **274**:35809-35815.

Unger T., Nau MM., Segal S., Minna JD. 1992. p53 a transdominal regulator of transcription whose function is ablated by mutations occurring in human cancers. EMBO J. **11**: 1383-1390.

Unger T., Juven-Gershon T., Moallem E., Berger M., Vogttsionov R.., Lozano G., Oren M. Haupt J.. 1999. Critical role for Ser 20 of human p53 in the negative regulation of p53 by MDM2. EMBO J. **18**: 1805-1814.

Unger T., Sionov R.V., Moallem E., Yee C.L., Howley P.M., Haupt Y. 1999. Mutations in serines 15 and 20 of human p53 impair its apoptotic activity. Oncogene, **18**: 3205-3212.

Utrera R., Collavin L., Lazarevic D., Delia D., Schneider C. 1998. A novel p53-inducible gene coding for a microtubule –localized protein with G2-phase-specific expression. EMBO J., **17**: 5015-5025.

Valenzuela MT., Nunez MI., Villalobos H., Siles E., McMillan TJ., Pedraza V., Ruiz de Almodovar JM. 1997. A comparison of p533 and p16 expression in human tumor cells treated with hyperthermia or ionizing radiation.. Int J Cancer. **72(2):** 307- 12.

Vaziri H., West M.D., Allsopp R.C., Davison T.S., Wu Y.S., Arrowsmith C.H., Poirier G.G., Benchimol S. 1997. ATM-dependent telomerase loss in aging human diploid fibroblasts and DNA damage leads to the post-translational activation of p53 protein involving poly (ADP-ribose) polymerase. EMBO J. **16**: 6018-6033.

Venot C., Maratrat M., Dureuil Conseiller E., Bracco L., Debussche L. 1998. The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. EMBO J., **17**: 4668-4679.

Vogelstein, B. and Kinzler,K.W. 1992. p53 function and dysfunction. Cell **70**:523-526.

Vogelstein B., Lane D., Levine AJ. 2000. Surfing the p53 network. Nature, **408**: 307-310.

Waga S., Hsannon G.J., Bech D., Stillmman B. 1994. The p21 inhibitor of cyclin-dependent kinase3s controls DNA replication by interaction with PCNA. Nature, **369**: 574-578.

Wagner A.J., Kokontis J.M.,Hay N. 1994. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21wafl/cipl. Genes Dev. **8**:2817-2830.

Waldman T., Kinzler K.W., Vogelstein B. 1995. p21 is necessary for the p53-mediated G1 arrest in human cancers. Cancer Res. **55**: 5187-5190.

Walker D.R., Bond J.P., Tarone R.E., Harris C.C., Makalowski W., Boguski M.S., Greenblatt M.S. 1999. Evolutionary conservation and somatic mutation hotspot maps of p53: correlation with p53 protein structural and functional features. Oncogene. **19**: 211-218.

Walker K.K., Levine A.J. 1996. Identification of a novel p53 functional domain that is necessary for efficient growth suppression. Proc. Ntl. Acad. Sci. USA, **93**: 15335-15340.

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

Wang X-J., Greenhalgh D.A., Jiang A., He D., Zhong L., Brinkley B.R., Roop D.R. 1998. Analysis of centrosome abnormalities and angiogenesis in epidermal-targeted p53173H mutant and p53 knockout mice after chemical carcinogenesis: evidence for a gain of function. Mol. Carcinog., **23**: 185-192.

Wang Y., Reed M., Wang P., Stenger J.E., Myar G., Anderson M.E., Schwedes J.F., Tegtmeyer P. 1993. P53 domains: Identification and characterization of two autonomous DNA-binding regions. Genes Develop., **7**: 2575-2586.

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

Waterman J.L., Shenk J.L., Halazonetis T.D. 1995. The dihedral symmetry of the p53 tetramerization domain mandates a conformational switch upon DNA binding. EMBO J., 14: 512-519.

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

Weber J.D., Taylor L.J., Roussel M.F., Sherr C.J., Bar-Sagl D. 1999. Nucleolar Art sequesters Mdm2 ND activates p53. Natl. Cell biol., **1**: 20-26.

Webley K., Bond J.A.,Jones C.J., Craig A., Hupp T., Wynford-Thomas D. 2000. Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage. Mol. Cell. Biol. **20**: 2803-2808.

Webster N.J.G., Resnik J,L., Reichart D,B., Strauss B., Haas M., Seely B,L 1996. Repression of the insulin receptor promoter by the tumor suppressor gene product p53: a possible mechanism for receptor overexpression in breast cancer. Cancer Res. **56**: 2781-2788.

Webster G.A., Perkins N.D. 1999. Transcriptional cross talk between NH-kappaB and p53. Mol. Cell. Biol. **19**:3485-3495.

Werner H., Karnieli E., Rauscher F.J., LeRoith D. 1996. Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene. Proc. Natl Aced. Sci. USA **93**: 8318-8323.

Wiesmuller L., Cammenga J., Deppert W.W. 1996. In vivo assay of p53 function in homologous recombination between simian virus 40 chromosomes. J. Virol. **70**: 737-744.

Wolf D., Harris N., Rotter V., 1984. Reconstitution of p53 expression in a non-producer Ab-MuLV-transformed cell line by transfection of functional p53 gene. Cell, **38**: 119-126.

Wolkowicz R., Peled A., Elkind N.B., Rotter V. 1995. Augmented DNA-binding activity of p53 protein encoded by a carboxyl-terminal alternatively spliced mRNA is blocked by p53 protein encoded by the regularly spliced form. Proc. Natl. Acad. Sci. USA, **92**: 6842-6846.

Woods DB., Vousden KH. 2001. Regulation of p53 function. Experimental Cell Research, **264**: 56-66.

Woo RA., McLure KG., Lees-Miller SP., Rancourt DE., Lee PW. 1998. DNA-dependent protein kinase acts upstream of p53 in response to DNA damage. Nature. **394(6694):** 700-4.

Wu G.S., 1998. Induction of the TRAIL receptor KILLER/DR5 in p53-dependent apoptosis but not growth arrest. Oncogene **18**: 6411-6418.

Wu GS., Burns TFC., Zhan Y., Alnemrii ES., El-Deiry WS. 1999. Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. Cancer Res. **59(12):** 2770-5.

Wu X., Bayle J.H., Olson D., Levine A.J. 1993. The p53-mdm2 autoregulatory feedback loop. Genes Dev. **7**:1126-1132.

Wynford-Thomas D. 1999. Cellular Senescence and Cancer. J. Pathol. **187**:100-111.

Xiangwei W., Bayle JH., Olson D., Levine AJ. 1993. The p53-mdm2 autoregulatory feedback loop. Genes Dev. 7:1126-1132.

Xiao H., Pearson A., Coulombe B., Truant R., Zhang S., Regier J.L., Triezenberg S.J., Reinberg D., Flores O., Ingles C.J., et al. 1994. Binding of basal transcription factor TGIIH to the acidic activation domain of VP16 and p53. Mole. Cell. Biol., **14**:7013-7024.

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

Xiong J., Hannon GJ., Zhang H., Casso D., Kobayashi R., Beach D. 1993. P21 is a universal inhibitor of cyclin kinases. Nature, **366**: 701-704.

Yoonish-Rouach E., Resnitzky D., Lotem J., Sachs L., Kimchi A., Oren M. 1991. Wild-type p53 induces apoptosis of myeloid leukemia cells that is inhibited by interleukin-6. Nature. **352**: 345-347.

Yu C.L., Driggers P., Barrera-Hernandez G. Nunez S.B., Segars J.H., Cheng S. 1997. The tumor suppressor p53 is a negative regulator of estrogen receptor signaling pathways. Biochem. Biophys. Res. Commun., **239**: 617-620.

Yuan Z.M., Huang Y., Ishiko T. Nakada S., Utsugisawa T., Shioya H., Utsugisawa Y., Yokoyama K., Wichselbaum T., Shi Y., Kufe D. 1999. Role for p300 in stabilization of p53 in the response to DNA damage. J. Biol. Chem. **274**: 1883-1886.

Yuangang L., Kulesz-martin M. 2001. p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. Carcinogenesis, **22**:851-860.

Zaika A., Marchenko N., Moll U. M. 1999. Citoplasmically "sequestered" wild-type p53 protein is resistant to Mdm2-meidated degradation. J. Biol. Chem. **274**: 27474-27480.

Zambetti, GP., Levine. AJ. 1993. A comparison of the biological activities of wild-type and mutant p53. FASEB J., **7**, 855-65.

Zauberman A., Barak Y., Ragimov N., Levy N., Oren M. 1993. Sequence-specific DNA binding by p53- identification of target sites and lack of binding to p53-MDM2 complexes. EMBO J. **12**:2799-2808.

Zauberman A., Flushberg D., Haupt Y., Barak Y., Oren M. 1995. A functional p53-responsive intronic promoter is contained within the human mdm2 gene. Nucleic Acids Research, **23 (14):** 2584-2592.

Zhan J., Xiong Y., Yarbrough WG.1998. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppressor pathways. Cell **92**:725-734.

Zang Q., Chen IT., Antinore MJ., Fornace AJ. Jr. 1998. Tumor suppressor p53 can participate in transcriptional induction of the GADD45 promoter in the absence of direct DNA binding. Mol Cell Biol. **18(5):** 2768-78.

Zhan Q., Antinore M.J., Wang X.W., Carrier F., Smith M.L., Harris C.C., Fornace A.J. Jr. 1999. Association with Cdc2 and inhibition of Cdc2/ cyclin B1 kinase activity by the p53-regulated protein Gadd 45. Oncogene. **18(18):** 2892-900.

Zhang L., Zhan Q., Zhan S., Kkashannchi F., Fornace A.J., Jr. Seth P., Helman L.J. 1998. P53 regulates human insulin-like growth factor II gene expression through active P4 promoter in rhabdomyosarcoma cells. DNA Cell Biol, **17**: 125-131.

Zhang W., Guo W.Y.D., Deisseroth A.B. 1994. The requirement of the carboxyl terminus of p53 for DNA binding and transcriptional activation depends on the specific p53 binding DNA element. Oncogene, **9**: 2513-2521.

Zhang W., Lu Q., Xie Z.J. Mellgren R.L. 1997. Inhibition of the growth of WI-38 fibroblasts by benzyloxycarbonyl-Leu-Leu-Try diazomethyl ketone: Evidence that

cleavage of p53 by a calpain-like protease is necessary for G1 to S-phase transition. Oncogenne, **14**: 255-263.

Zhao K., Cahi X., Joohnston K., Clements A., Marmorstein R. 2001. Crystal structure of the mouse p53 core DNA-binding domain at 2.7 A resolution. J. Biol. Chem., **276(15):** 12120-7.

Zhao R., Gish K. Murphy M., Yin Y., Notterman D., Hoffman W., Tom E., Mack D., Levine A. 2000. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. Genes Dev. **14**: 981-993.

Zhou J., Ahn J., Wilson SH., Prives C. 2001. A role for p53 in base excision repair. EMBO J. **20(4):** 914-23.