Title
The physical and functional interactions between the tumor suppressor p53 protein and the human homolog of the RAD54 DNA repair protein

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The Physical and Functional Interactions Between the Tumor Suppressor p53 Protein and the Human Homolog of the RAD54 DNA Repair Protein

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The tumor suppressor gene p53 is mutated in nearly half of all human cancers. p53 is thought to maintain genomic integrity via its involvement in multiple cellular functions, including cell-cycle arrest, apoptosis and DNA repair. Cells lacking functional wild-type p53 show increased genomic instability and chromosomal aberrations. We propose that p53 prevents these genomic abnormalities by interacting with the homologous DNA recombinational repair machinery. p53 is known to interact with several DNA helicases as well as to the recombinational repair protein RAD51. Our study investigated an interaction between p53 and the human homolog of the yeast DNA recombinational repair gene RAD54 (hHR54). We used GST-tagged p53 and GST-tagged p53 deletion mutants to characterize the binding of p53 to *in vitro* produced hHR54. hHR54 binds to p53 in a time dependent manner that is not dependent upon the presence of a nucleic acid intermediate. This binding is best localized near the C-terminal portion of p53 between residues 293-393. Studies using cell lysates from normal lymphoblasts have shown that p53 and hHR54 will co-precipitate, indicating that this interaction occurs *in vivo* as well. Our results clearly show a protein-protein interaction between p53 and hHR54. This interaction may form the basis for the non-transcriptional regulation of homologous recombination by p53.

INTRODUCTION

Mutations in the p53 tumor suppressor gene are present in about half of all human cancers (Hollstein et al., 1994; Hollstein et al., 1991; Levine et al., 1991; Greenblatt et al., 1994). Although its precise role in human carcinogenesis has yet to be elucidated, a vast array of investigations into the functions of p53 have implicated its involvement in multiple cellular processes that may be important in its role as the "guardian of the genome" (Lane et al., 1992; Fukasawa et al., 1996). Among the proposed functions for p53 are involvement in transcription, cell-cycle growth arrest, programmed cell death, and DNA damage response and repair, including nucleotide excision repair and homologous recombinational repair. (reviewed in Ko and Prives, 1996; Harris, 1996)

p53 exerts these heterologous functional effects by both transcriptionally dependent and independent mechanisms. For example, p53 can cause cell-cycle arrest in a transcriptionally dependent manner by upregulating the transcription of p21 (Kemp et al., 1993; El-Deiry et al., 1994) and this arrest may also be affected by the intracellular environment (Knippschild et al., 1995). p53-dependent apoptosis has been shown to utilize both transcriptionally dependent (Sabbatini et al., 1995) and independent (Rowan et al., 1996; Caelles et al., 1994; Wang et al., 1996; Haupt et al., 1995) mechanisms. Transcriptionally independent mechanisms are mediated by the formation of complexes between p53 and existing

native or foreign cellular molecules. Wild-type, but not mutant, p53 can directly regulate DNA replication *in vitro* by binding to DNA (Miller et al., 1995; Cox et al., 1995;) and p53 can repress transcription from the SV40 promoter by disrupting DNA-protein complexes (Perrem et al., 1995). MDM2, RPA, XPB, XPD, CSB, WT1 and RAD51 (Oliner et al., 1993; Dutta et al., 1993; Wang et al., 1995; Maheswaran et al., 1993; Sturzbecher et al., 1996) are all examples of proteins shown to form direct complexes with p53, and presumably affect cell function. The hepatitis B viral X protein (HBX), a putative oncoprotein, has been shown to inhibit p53 DNA binding and transcriptional activity by binding directly to the p53 protein (Wang et al., 1994). By the direct interaction with these cellular proteins, p53 can exert a transcriptionally-independent regulatory effect on various cellular processes.

One of the processes which p53 may affect by transcriptionally independent mechanisms is DNA repair. p53 binds to XPB and XPD (Wang et al., 1995; Leveillard et al., 1996). These proteins form part of the TFIIH complex, which is involved in RNA polymerase II mediated transcriptional activation as well as in the nucleotide excisional pathway of DNA repair (Drapkin et al., 1994; Weeda et al., 1990). p53 has been shown to complex to these proteins, as well as to the p62 TFIIH subunit, with both its N- and C- termini (Leveillard et al., 1996) and specifically inhibits XPB and XPD helicase activities (Wang et al., 1995; Leveillard et al., 1996). CSB, the gene defective in Cockayne's syndrome which is involved in strand-specific DNA repair

(Troelstra et al., 1992), also binds to p53 (Wang et al., 1995). The CSB, XPB and XPD proteins are DNA helicases and share the seven motifs characteristic of the SNF2 family of helicases. Other evidence linking p53 to DNA repair includes the observation that keratinocytes from p53-mutant transgenic mice have a decreased efficiency of DNA repair following UV irradiation (Li et al., 1996). Also, p53 binds to single stranded DNA ends as well as to mismatched DNA (Bakalkin et al., 1994; Lee et al., 1995). These findings all strongly implicate the involvement of p53 DNA repair pathways.

The participation of normal p53 in DNA repair pathways conforms to its proposed role in the maintenance of genomic integrity. It is this loss of stability that may initiate the processes involved in molecular oncogenic transformation in some cells which are lacking normal p53 function. Further evidence has linked p53 to the homologous recombinational (HR) machinery, which is involved in both meiotic recombination, somatic cell recombination, as well as in DNA recombinational repair.

In rhesus monkey cells which express endogenous p53, SV40 T-antigen can bind to p53 and increase the rate of HR. This increased HR is abrogated by a T-antigen point mutation which eliminates its interaction with p53 (Wiesmuller et al., 1996). p53 deficient mouse embryo fibroblasts have increased genomic instability and chromatid aberrations following bleomycin treatment, which induces double-stranded breaks, when compared to cells with wild-type p53 (Donner

and Preston, 1996). Furthermore, p53 is expressed at high levels in the testis during meiosis in early pachytene spermatocytes, implicating a role for p53 in HR during meiosis (Sjoblom and Lahdetie, 1996). Ataxia-telengectasia (AT) is a clinical syndrome which includes increased cancer susceptibilities among its effects. AT cells demonstrate a deficiency in the induction of p53 in response to ionizing radiation (Khanna and Lavin, 1993)(Khanna et al., 1995), an increased apoptotic response to ionizing radiation (Meyn et al., 1994), and a high level of intrachromosomal recombination (Meyn, 1993). Meyn and his colleagues (Meyn et al., 1994) also demonstrated that normal cells which are transfected with a dominant negative form of p53 will also develop a high rate of spontaneous recombination. Finally, it has been shown that p53 interacts with hHRAD51, a human homolog of the yeast RAD51 gene family member which is involved in yeast homologous recombination (Sturzbecher et al., 1996; Buchop et al., in press). These data all suggest that p53 may play a role in preventing promiscuous DNA homologous recombination.

In order to further investigate the mechanisms by which p53 may influence the DNA recombinational system, we sought to characterize the interaction between p53 and hHR54, a newly cloned human homolog of the yeast DNA recombinational repair gene. The RAD family defines several groups of yeast genes and gene products whose mutation or inactivation causes increased radio sensitivity and radiation-induced cell death in yeast (Glassner and Mortimer, 1994). The RAD 51 and RAD54 genes are part of the RAD52 epistasis group which has been found to be involved in yeast homologous

recombinational (HR). This group has functions homologous to that of the RecA protein in bacteria (Ogawa et al., 1993).

As mentioned above, Stürzbecher, et al (1996) have demonstrated that p53 interacts with the human homolog of Rad51. They demonstrated both an *in vitro* and *in vivo* interaction. Furthermore, the *in vitro* binding has been localized to two specific sites on the p53 protein (Buchhop et al., in press). Also, p53 can inhibit RecA biochemical activities (Sturzbecher et al., 1996). Thiese data are the first evidence of p53 directly interacting with members of the RAD52 epistasis group and implies a direct means for p53 participation in the modulation of DNA recombinational activities.

RAD54 is another member of the RAD52 epistasis group and it shares a homologous damage-response sequence with RAD51 (Basile et al., 1992). Deletion of either of these genes yields a substantial reduction in DNA recombinational repair activity (Liefshitz et al., 1995). RAD54 is a member of the SNF2 helicase family (Eisen et al, 1995; Emery et al., 1991) and has been shown to allow access to DNA for repair following double-stranded breaks induced by irradiation or cross-linking agents (Glassner and Mortimer, 1994; Averbeck and Averbeck, 1994). Recently, the human homologue of RAD54 (hHR54) has been cloned and studied. This study demonstrated that hHR54 is a member of the SNF2/SWI2 family of DNA dependent ATPases, is localized to the nucleus, is expressed in organs of germ cell and lymphoid development, and is expressed at high levels during the early G1 phase of the cell cycle. Furthermore,

hHR54 can partially complement the functional deficit in *S. cerevisiae* Δrad54 cells, implying that hHR54 has functional homology to RAD54 as well (Kanaar et al., 1996).

We propose that p53 interacts with hHR54 and that this interaction is further evidence linking p53 to homologous recombinational activities in human cells. Using both *in vivo* and *in vitro* methods, we demonstrated a physical interaction between p53 and hHR54. Also, we were able to map the binding of hHR54 on the p53 protein. These findings demonstrate that p53 may have a transcriptionally independent effect on the recombinational repair machinery via a direct interaction with the hHR54 protein.

MATERIALS AND METHODS

Plasmid and Recombinant Protein Preparation

hHR54 was provided by HJ Hoijermakers (Rotterdam, Netherlands) as a 2.6 kB insert in a pSLM vector. The insert was isolated using a partial digestion method and ligated into a pcDNA3 vector (Invitrogen, San Diego, CA), which contains both T7 and CMV promoter regions. This hHR54 plasmid was used for both *in vitro* and *in vivo* expression of the hHR54 protein.

GST-p53 wild-type encodes GST fused to human wild-type p53. GST-p53 N5, NTD, 2C, 3C, 25 and 35, kindly provided by B. Stillman (Cold Spring Harbor Laboratory), encode GST fused to residues 2-293, 2-124, 94-393, 155-393, 94-293, and 155-293 respectively. Recombinant GST-fused proteins were produced in *E coli* and purified on glutathione-agarose beads according to the manufacturer (Sigma). The proteins attached to the beads were stored in PBS with 1% Triton X-100 at 4° for up to two months. Protein concentrations were determined by Coomassie blue staining of SDS-PAGE gels and comparison to molecular weight standards (Bio-Rad).

In Vitro Binding

HHR54 was produced *in vitro* using the TnT rabbit reticulolysate system (Promega), radiolabelled with ^{35}S cysteine per the manufacturers instructions. Immediately following protein production,

5 ul of the HHR54 lysate was incubated with GST-p53 (wild-type or deletion mutant) for the specified time period in IP buffer (120 mM NaCl, 50 mM Tris pH 8.0 and 0.5% NP-40) on a rocker at room temperature. Then the beads were washed five times with at least 25 volumes of IP buffer. The outcome was analyzed by denaturing the protein in 1x gel loading buffer and heating at 95° for ten minutes. Samples were run on a 10% SDS-PAGE gel. The gel was then fixed in 50% methanol/ 10% acetic acid, enhanced in 1.5M sodium salicylate, dried, and exposed overnight at -70°C to autoradiographic film. Binding was measured as the amount of radiolabelled hHR54 in the binding lanes as compared to a control direct translation product.

Transfection

Calu-6 cells were maintained at 37°C in a media consisting of RPMI 1640 (Gibco-BRL), 10%FBS, glutamine and penicillin/streptomycin. 1 or 3x 10^6 cells were seeded onto 100mm dishes and cells were transfected when 60 to 80% confluent. Transfection was done using the lipofectin system (Promega) with either hHR54, p53 or pCMV-NEO(vector control) DNA. Cells were maintained in lipofectin solution for 5 hours and then switched back to normal media. Cells were washed twice with PBS and lysed. For luciferase experiments, a p53-responsive luciferase gene was also transfected and the luciferase activity was measured on a luminometer twenty-four hours post transfection, immediately after cell lysis.

In vivo binding assay

Calu-6 cells were transfected with p53 and hHR54 expression vectors as above. Forty-eight hours post-transfection, these cells, normal lymphoblasts (NL) or NL exposed to 500 Rads ionizing radiation were put in serum-free methionine-free DMEM (Biolabs) supplemented with ^{35}S labeled methionine/cysteine mix (NEN Dupont) for two hours. Cells were then lysed in IP buffer containing protease inhibitors (1 mM PMSF, 10 ug/ml Leupeptin, 10 ug/ml Pepstatin A, and 10 ug/ml Aprotinin) and centrifuged at 4°C at 80000 rpm for 60 minutes. Anti-hHR54 antibody or normal rabbit serum was then incubated with the soluble lysate for 60 minutes at 4°C and precipitated using Protein A/G-linked agarose beads. Proteins were then released by denaturing with SDS-sample loading buffer and heated at 90°C for 10 minutes. Protein was renatured in twenty volumes of IP buffer for 60' at 4°C. then a second IP was performed using antibody DO-1 to precipitate p53. Samples were visualized on a 10% gel as above.

RESULTS

hHR54 binds specifically to p53

In order to determine if p53 does participate in homologous recombinational activities via an interaction with hHR54, we proposed that there is a demonstrable physical interaction between the two proteins. To test this hypothesis, radiolabelled *in vitro* translated hHR54 was incubated with *E. coli* produced, GST-tagged wild-type p53, which had been conjugated to GSH-linked agarose beads. Figure 1A demonstrates that hHR54 precipitated with the GST-p53, but not with the GST-linked or unconjugated beads. This result shows a clear specific interaction between GST-p53 and hHR54 in an *in vitro* system.

To investigate the ability of hHR54 and p53 to interact *in vivo*, we used radiolabelled normal lymphoblast cell lysates subjected to a double-immunoprecipitation. The first immunoprecipitation was with either rabbit anti-hHR54 antibody or with preimmune rabbit serum. The second immunoprecipitation was with DO-1 anti-p53 antibody, and the radiolabelled product was run on a standard SDS-PAGE gel. The two results were compared to look for the ability of hHR54 antibody to precipitate more p53 than background as an indication for *in vivo* p53-hHR54 complex formation. Figure 1B demonstrates the result of this experiment, showing that more p53 precipitated with the anti-hHR54 antibody than with the normal rabbit serum. Irradiating the cells did not affect the amount of complex formation, however both groups

received a significant amount of radiation from the radiolabelling. This result indicates that there is an *in vivo* interaction between hHR54 and p53 and that a complex of these proteins can be immunoprecipitated.

A second experiment was done to characterize the time course of the *in vitro* interaction. GST-p53 and *in vitro* translated, radiolabelled hHR54 were incubated at room temperature for time periods ranging from fifteen minutes to eight hours. Figure 2A demonstrates a time-dependent course of binding. The binding efficiency plateaus at one hour and again at three hours of incubation time.

As mentioned above, it is known that p53 can efficiently bind to DNA as one of its multiple interactions. hHR54 is a putative DNA helicase and its homolog in yeast is known to interact with DNA in allowing access to homologous sequences in the recombinational repair machinery. Therefore, it is important to determine that the interaction between p53 and hHR54 is not due to a nucleic acid intermediate. To test this possibility, the *in vitro* translated hHR54 lysate mixture was treated with DNAse, RNAse or both for thirty minutes following a normal protein translation. This lysate, as well as untreated lysate, was then incubated with the purified GST-p53. The results in figure 2B show no negative effect of DNAse or RNAse treatment on the hHR54-p53 interaction, implying that these proteins interact directly and not via a nucleic acid intermediate.

The C-terminal domain of p53 is necessary for the binding of hHR54.

p53 can be divided into three topographical domains based on function. The N-terminal domain is active in p53 transactivational activities. The multifunctional C-terminal domain possesses oligomerization, nuclear localization and possibly apoptosis-inducing activities (Ishioka et al., 1995; Jeffrey et al., 1995). The mid-portion of the protein is the DNA binding domain and is where most of the cancer-associated p53 point mutations are found (Cho et al., 1994). Localizing the site of the hHR54 interaction with p53 may help to indicate a functional correlate for their physical interaction. For these experiments, a panel of p53 deletion mutants was used. NC is the full length transcript, residues 1-393. N5 and NTD have C-terminal deletions and respectively include residues 1-203 and residues 1-124. 2C and 3C have N-terminal deletions, and they respectively include residues 94-393 and residues 155-393. The 25 and 35 mutants are residues 94-293 and 155-293, respectively. They are the result of combining the N5 deletion and the 2C or 3C deletion.

In these experiments, equal amounts of GST-tagged p53 or p53 deletion mutants were incubated for equal time periods with aliquots of radiolabelled hHR54. Figure 3 demonstrates that hHR54 binds preferably between residues 293 and 393 of p53, demonstrated by the shaded bar in the schematic drawing of p53 and the deletion mutants. All of the mutants with an intact C-terminus show strong binding, however the other mutants still show binding above background levels. This finding would indicate that hHR54 binds strongly near the multifunctional C-terminal region. The anti-p53

antibody 421 (Ab421), the binding of which has been shown to increase p53 transactivational activities (Hupp et al., 1992; Hupp et al., 1993), binds to p53 in this region, at residues 371-380.

A second set of experiments was done using saturating amounts of Ab421 and the same panel of deletion mutants to demonstrate blocking of binding by the antibody. In this experiment, the panel of p53 and mutants was again incubated with radiolabelled hHR54 in either the presence of absence of Ab421. Figure 4 shows that for p53NC, the antibody diminishes binding. For N5, there is little binding either with or without the antibody present. For the 2C and 3C mutants, strong binding without the antibody is greatly diminished with the antibody present, indicating that hHR54 does indeed bind to the p53 protein near the C-terminus.

To show that this is an effect of the antibody itself and not a non-specific effect, a panel of p53 antibodies was incubated with GST-tagged p53NC and radiolabelled hHR54. The antibodies (and epitopes) used included: Ab421 (residues 371-380), DO-1 (21-25), 1801 (46-55), 240 and 1620 (which both bind only conformational p53 at mid-protein tertiary structures), TrpE, a bacterial protein antibody, was used as a negative control for non-specific activity. Figure 5 clearly demonstrates that Ab421 causes the greatest interference with binding. Ab1801 also decreases binding slightly, and less so do antibodies DO-1, 1620 and 240. TrpE did not decrease binding. These results support the concept of a C-terminal binding site. The fact that the other p53 antibodies did decrease

binding slightly may be due to steric hindrance from a large antibody sitting on the protein or from a conformational change induced by these antibodies binding to p53.

Elimination of the extreme N-terminus of p53 may result in a second binding site for hHR54.

Although the evidence points towards a strong C-terminal binding site, there appears to be another site involved in binding of hHR54 to p53. The binding can not be totally eliminated by removing the C-terminal or even the entire protein from residue 125 to the C-terminal. Furthermore, the time-course experiment suggests that there might be two time-courses of binding, as there appeared to be two plateaus, which may indicate a second binding site with a different binding affinity. Therefore, an expanded set of deletion mutants was used to screen for a possible second binding site. Figure 6 demonstrates the results, in which the 25 mutant has a high level of binding affinity, whereas the 35 mutant does not. Note that the N5 mutant, which includes the same N-terminal deletion as the 25 and 35 proteins, does not have a high level of binding. These results suggest the existence of a putative second binding site which mainly resides between residues 94-155. However this binding site must normally be obscured by the conformation present when the N-terminal is intact, since the N5 mutant has low-level binding. This site might only be exposed to bind to hHR54 when a conformational change has taken place. These results may also explain why antibody 1801 had

the greatest low-level hindrance of binding in the antibody panel experiment, as it sits closest to the putative second binding site.

Transfection experiments demonstrate an equivocal effect of hHR54 on p53 transactivational activities.

In this set of experiments, we attempted to look at a functional consequence of the hHR54-p53 interaction. The strongest hHR54 binding to p53 is at a site which competes with Ab421, and it is known that Ab421 can increase p53 transactivational activity. Therefore we attempted to investigate the effect of hHR54 on p53 transactivational activity. In this experiment, p53-null Calu-6 cells were transfected with p53, hHR54, neither or both. Also transfected into all of the cells was DNA encoding the firefly luciferase gene, which carries a p53-responsive transcriptional promoter. The amount of luciferase produced can then be determined by adding a substrate and detecting the amount of light produced using a luminometer. Figure 7 demonstrates the results of several experiments. In three out of four experiments, hHR54 did increase p53 transactivational activity, however in the fourth and some subsequent experiments (data not shown) the result was not substantiated.

DISCUSSION

This study demonstrates that p53 has a direct physical interaction with hHR54. This interaction is specific and is demonstrated both *in vitro* as well as *in vivo*. The binding takes place at two putative binding sites on the p53 protein, and can be interfered with specifically by antibodies which bind to those sites on the p53 protein. Thus we have shown a possible physical link between p53 and the control of homologous DNA recombinational repair processes.

The tumor suppression functions of p53 have been extensively studied, showing the involvement of p53 in several aspects of the life cycle of a cell. It is known that DNA damage activates p53 which in turn can cause apoptosis or can transactivate p21 leading to G1 arrest (reviewed in Bates and Vousden, 1996). Also, high levels of p53 inhibit the PCNA promoter, decreasing cell proliferation rates(Shivakumar et al., 1995). This slowing of the cell cycle is thought necessary in allowing time for DNA repair to occur and to assure that progression through the cell cycle is held in check until an intact genome is present. p53 can also cause a G2/M phase arrest (Agarwal et al., 1995) and cells lacking p53 which undergo mitosis will have dysfunctional chromosomal segregation resulting in polyploid cells (Cross et al., 1995), indicating again that p53 functions to maintain genetic stability, perhaps by causing a time delay via cell cycle arrest.

Aside from allowing time for DNA repair to occur, recent evidence suggests that p53 can more directly affect DNA repair. As noted

above, evidence links p53 directly with TFIIH and its subunits. Functional evidence shows that p53 inhibits the helicase functions of XPD and XPB (Wang et al., 1995; Leveillard et al., 1996). Thus a direct role for p53 in nucleotide excision repair is likely.

This study shows that p53 interacts with the human homolog of RAD54 (hHR54). In yeast, the RAD52 epistasis group has been shown to be necessary for maintaining DNA recombinational repair (Aguilera, 1995; Hays et al., 1995; Game, 1993). DNA homologous recombination is necessary to repair damage in which both strands of the DNA are damaged, because the normal template used for repair is also damaged (Shinohara and Ogawa, 1995). Homologous recombination is necessary in meiosis and some somatic cells to allow for genetic variability (Bezzubova and Buerstedde, 1994). The notion of p53 regulating recombination is a logical conclusion from evidence that cells which lack normal p53 tend to have increased recombinational activity. It also helps explain why p53 mutations can lead to oncogenic transformation due to increased genomic instability. From Sturzbecher's experiments (1996), it appears that p53 acts to inhibit DNA recombination. This inhibitory activity would be valuable in preventing promiscuous recombination which may result in oncogenic DNA transformations.

In yeast, RAD54 specifically aids in the recombination of, and is preferentially induced by agents which result in, double stranded DNA breaks (Moore et al., 1993; Averbeck and Averbeck, 1994). The recently cloned hHR54 has been shown to restore recombinational

function in yeast cells lacking RAD54. Thus there is an implication that hHR54 may also be involved in recombinational activities. It has already been shown (Sturzbecher et al., 1996) that p53 interacts with RAD51 and its human homolog and that p53 can inhibit RecA recombinational activity. Our study strengthens the evidence that p53 is directly involved in modulating homologous recombinational DNA repair in a transcriptionally independent fashion via its interaction with the hHR51 and hHR54 proteins.

hHR54 is a member of the SNF2 helicase family, sharing homology in all seven of the helicase domains. Several of the other DNA repair proteins to which p53 binds also are members of the helicase family, including XPB, XPD, CSB and the Werner's and Bloom's syndrome proteins. Furthermore, p53 has been shown to bind both single-stranded and double-stranded DNA as well as to catalyze the renaturation of complementary single-stranded DNA fragments (Bakalkin et al., 1994). Thus it appears that one way in which p53 may decrease recombination is by inhibiting access to and unwinding of homologous areas of DNA. Specifically, this inhibition is due to a direct physical interaction with DNA helicases.

Our finding of two binding sites on the p53 protein for hHR54 is similar to the mapping studies done with RAD51 binding to p53 (Buchhop et al., submitted). The curious demonstration of a masked binding site revealed only by removal of the N-terminus indicates that a conformational change is necessary to reveal the second binding site. The crystal structures of the core DNA-binding domain (Cho et al.,

1995) and the tetramerization domain (Jeffrey et al., 1995) have both been published, the structure of the N-terminal aspect of the p53 protein has not yet been determined. It is possible that the N-terminal may have several conformations, which would be consistent with our finding that removal of the N-terminal reveals a normally obscured binding site. This binding site may also be revealed by a purely conformational change at the N-terminal.

The relative importance and the time course of binding to each site *in vivo* may be important in determining the nature of the interaction between hHR54 and p53. The first binding site may be active in early stages following DNA damage in which both p53 and hHR54 are upregulated. This binding site is near the C-terminus of p53, and antibody binding to this site has been shown to increase p53 DNA binding and transactivational activities (Hupp et al., 1995; Hupp et al., 1992). Perhaps the interaction at this site leads to further p53 activation and cell cycle arrest, allowing hHR54 to begin recombinational repair. The second binding site may act in the opposite way, later on in the process, prohibiting the overzealous recombination, which may result in significant genetic aberrations, by sequestering excess hHR54. Studies which investigate the specific time course of expression of hHR54 versus p53 after DNA damage as well as the physical interactions at these times may shed light on this hypothesis. Also, assaying the effect of selected p53 point mutations on hHR54 recombinational activities could indicate the specific nature of the interaction.

In our *in vivo* studies, we demonstrated hHR54-p53 complex co-immunoprecipitation using a very sensitive assay. However, as figure 1B clearly demonstrates, only a very small fraction of total cellular p53 was precipitated with the hHR54. The low yield is likely secondary to the fact that the hHR54 antibody has a relatively weak affinity for the hHR54 protein as well as the fact that there are low levels of hHR54 protein in the cells. We were not able to demonstrate the reverse experiment, in which we could identify hHR54 after a p53 immunoprecipitation, again because of the weakness of our antibody. The use of a tagged hHR54 protein would be helpful in improving the validity of these results. Furthermore, the hHR54 antibody we had was not able to identify hHR54 protein *in situ* when using immunofluorescence. That experiment was attempted in an effort to demonstrate co-localization of the p53 and hHR54 proteins *in situ*. These experiments could be conducted with greater success using a tagged hHR54 construct, which would allow better identification and precipitation of the hHR54 protein.

In the transfection experiments, we were not able to conclusively demonstrate an effect of hHR54 on p53 transactivational activity, nor a lack thereof. When the cell lysates were examined for protein levels (data not shown) it was found that the levels of detectable hHR54 were not very high and there was marked variability from study to study. Furthermore, we could not find a correlation between the detectable levels and the effects on p53 transactivation. These technical difficulties made it impossible to conclude a functional consequence of the p53-hHR54 interaction. Further studies could

investigate the effect of the interaction on other p53 activities, such as cell-cycle arrest or apoptosis. Also, the development of an hHR54-dependent recombinational assay may allow for the investigation of the effects of the interaction on hHR54 activity.

CONCLUSION

Our findings demonstrate that p53 binds to hHR54 both *in vivo* and *in vitro*. Although we could not demonstrate a functional correlate of the interaction, this interaction is significant physical evidence that p53 may be involved in the homologous DNA recombinational repair machinery.

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Figure Legends

Figure 1. hHR54 binds specifically to p53.

A. Radiolabeled, *in vitro* produced hHR54 was incubated for 30 minutes with either GST-linked p53 attached to agarose beads (lane 2), GST linked to the beads (lane 3) or plain beads (lane 4). The beads and associated proteins were precipitated by centrifugation. The proteins were washed with PBS and then released by denaturing with SDS-Page sample loading buffer, and run on a 10% SDS-Page gel visualized by autoradiography. Lane 1 molecular ladder, Lane 5 20% input.

B. Normal human lymphoblasts (NL) or lymphoblasts irradiated with 500 Rads (IRR NL) were incubated in serum-free, methionine-free DMEM supplemented with 150 uCi/ml of ^{35}S labeled methionine for two hours. The cells were then lysed and precleared with protein A/G linked agarose beads. Then 10 ul of either anti-p53 antibody DO-1 (Lanes 1,4), normal rabbit serum (Lanes 2,5) or anti-hHR54 antibody (Lanes 3,6) were added to the lysates and then precipitated on protein A/G linked agarose beads. The precipitated was washed and then denatured and the beads were cleared away. The remaining protein was renatured in 25 volumes of IP buffer. Then a second immunoprecipitation was done in all tubes using 20ul of DO-1 anti-p53 antibody. The proteins were collected on protein A/G agarose beads, and run on an SDS-Page gel, and visualized by autoradiography.

Figure 2. hHR54 binds p53 in a time-dependent manner.

A. Radiolabeled, in vitro produced hHR54 was incubated with GST-linked p53 for the indicated time periods. The complexes were precipitated onto glutathione -linked beads via centrifugation. The proteins were denatured, run on an SDS-PAGE gel and visualized by autoradiography. 20% input demonstrates 20% of total hHR54 per tube directly loaded onto the gel.

B. A graph showing the percent of hHR54 bound, as compared to 100% of input, versus the amount of time incubated with GST-p53. The percent bound was figured as a ratio of amount of protein visualized per time period divided by five time the amount in the 20% input lane. Amounts were quantified using a densitometer.

C. Radiolabeled, in vitro produced hHR54 was treated for thirty minutes with buffer (lanes 1-2), DNAse (lanes 3-4), RNAse (lanes 5-6),or both (lanes 7-8). The hHR54 was then incubated with GST-p53 for two hours (lanes 2,4,6,8), then the protein complexes were precipitated, denatured and run on an SDS-Page gel. Lanes 1,3,5,7 are 20% input of hHR54 from each respective treatment condition.

Figure 3. The C-terminal region is necessary for the hHR54-p53 interaction.

Radiolabeled, *in vitro* produced hHR54 was incubated with equal amounts of GST-p53NC, GST-p53N5, GST-p532C, GST-p533C, GSTp53NTD or GST-linked beads for two hours. To the right are schematic drawings of each p53 protein demonstrating which portions of the protein are present in each mutant. Numbers indicate amino acid residues. Black boxes indicate conserved regions. Gray boxes represent proposed binding sites. Input: direct loading of 20% of the radiolabeled hHR54 used for each experiment.

Figure 4. Antibody 421 inhibits binding in the deletion mutants.

Radiolabeled, *in vitro* produced hHR54 was incubated with GST-p53 NC (lanes 1-2), GST-p53 N5 (lanes 3-4), GST-p53 2C, (lanes 5-6), GST-p53 3C (lanes 7-8) or GST-linked agarose beads (lane 9), and either no antibody (lanes 1,3,5,7,9) or saturating amounts of antibody 421 (lanes 2,4,6,8) for two hours. The proteins complexing with p53 were collected by centrifugation and visualized by autoradiography of an SDS-Page gel. Input, 20% of the hHR54 used per experiment was directly loaded onto the gel.

Figure 5. p53 antibodies selectively inhibit binding.

A. Radiolabeled, *in vitro* produced hHR54 was incubated for two hours with GST-p53NC and either no antibody (lane 1), or saturating levels of the indicated anti-p53 antibody (lanes 2-6) or anti-bacterial TrpE antibody (lane 7). Lane 8, hHR54 was incubated with GST-linked agarose beads. Input, 20% of the hHR54 used per experiment was directly loaded onto the gel.

B. Schematic drawing indicating the epitopes for each anti-p53 antibody. Antibodies 240 and 1620 are conformational only, and their epitopes depend upon an intact tertiary structure of the p53 protein.

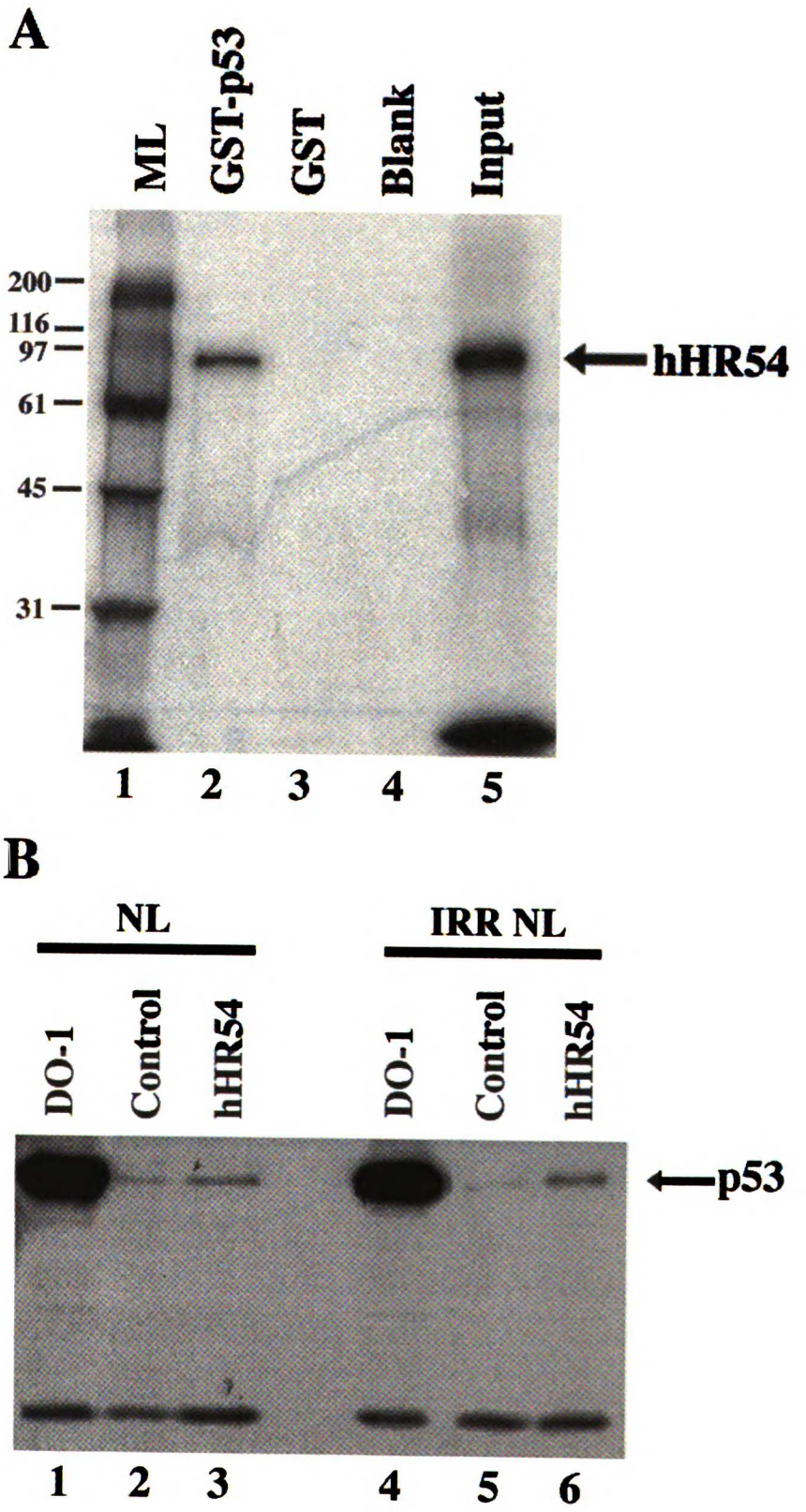
Figure 6. Further deletion mutant mapping reveals a possible second binding site.

Radiolabeled, *in vitro* produced hHR54 was incubated with equal amounts of GST-p53 NC, GST-p53 N5, GSTp53 NTD, GST-p53 2C, GST-p53 3C, GST-p53 25, GST-p53 35 or GST-linked beads for two hours. To the right are schematic drawings of each p53 protein demonstrating which portions of the protein are present in each mutant. Numbers indicate amino acid residues. Black boxes indicate conserved regions. Light gray boxes represent the C-terminal proposed binding sites. Dark gray box represents the proposed second binding site.

Figure 7. hHR54 may increase p53 transactivational activities.

Calu-6 cells were co-transfected with a p53 responsive/p21 promoter-driven luciferase gene and CMV-driven plasmids containing the cDNA of hHR54 and p53 (RAD+p53+luc), p53 alone (p53+luc), hHR54 alone (Rad+luc), or vector alone (Luc only) using the Lipofectin system. 48 hours post-transfection, cells were lysed and examined for luciferase activity using a luminometer. Results are shown as four separate experiments (exp 1-4) and a mean + Standard Error bars (average).

Figure 1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GST-p53
GST
Blank
Input
200
116
97
61
45
31
hHR54
1
2
3
4
5
B
NL
IRR NL
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hHR54
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p53
1
2
3
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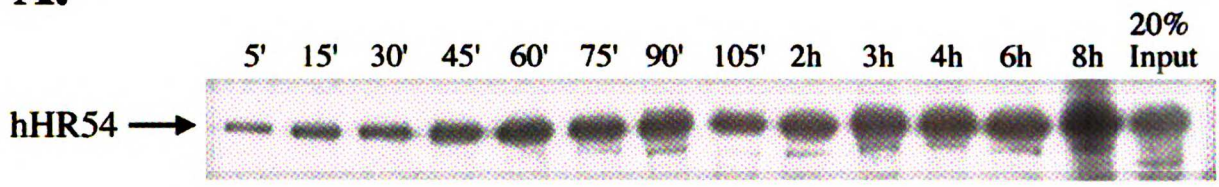
5
6

Figure 2

A.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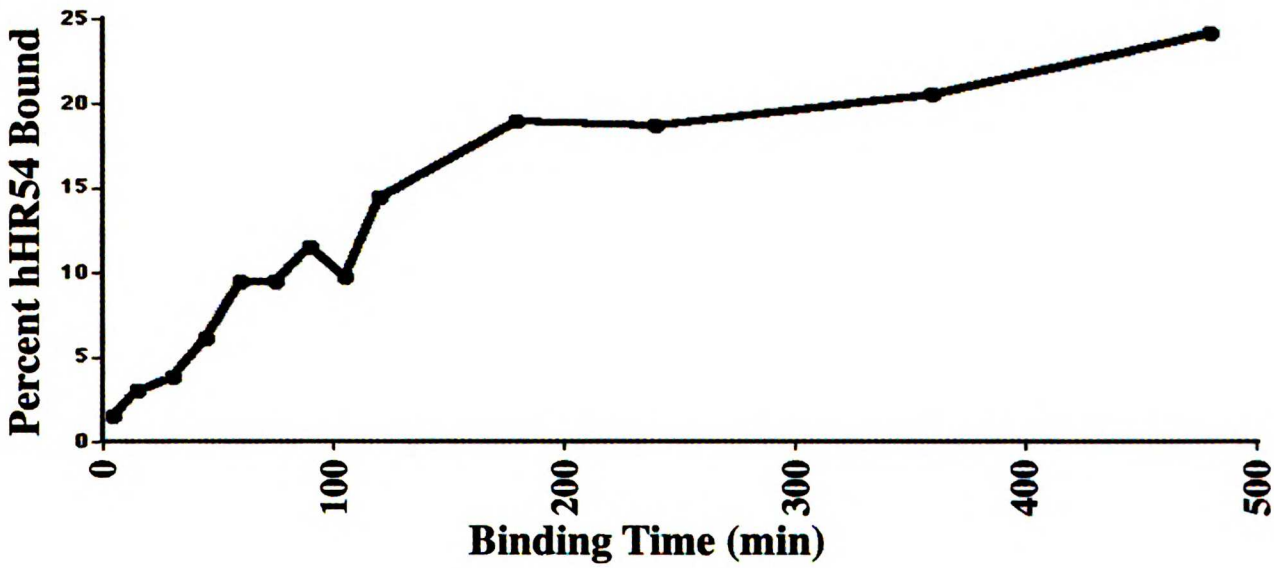
hHR54

B.


Percent hHR54 Bound
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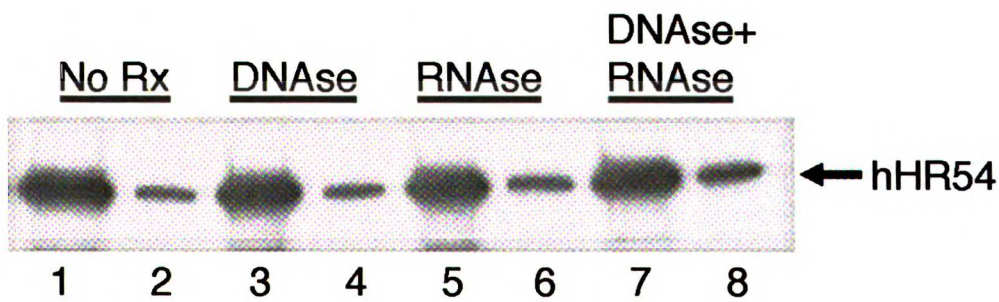
5
10
15
20
25
0
100
200
300
400
500
Binding Time (min)

C.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DNAse
RNAse
DNAse+ RNAse
hHR54
1 2 3 4 5 6 7 8

Figure 3

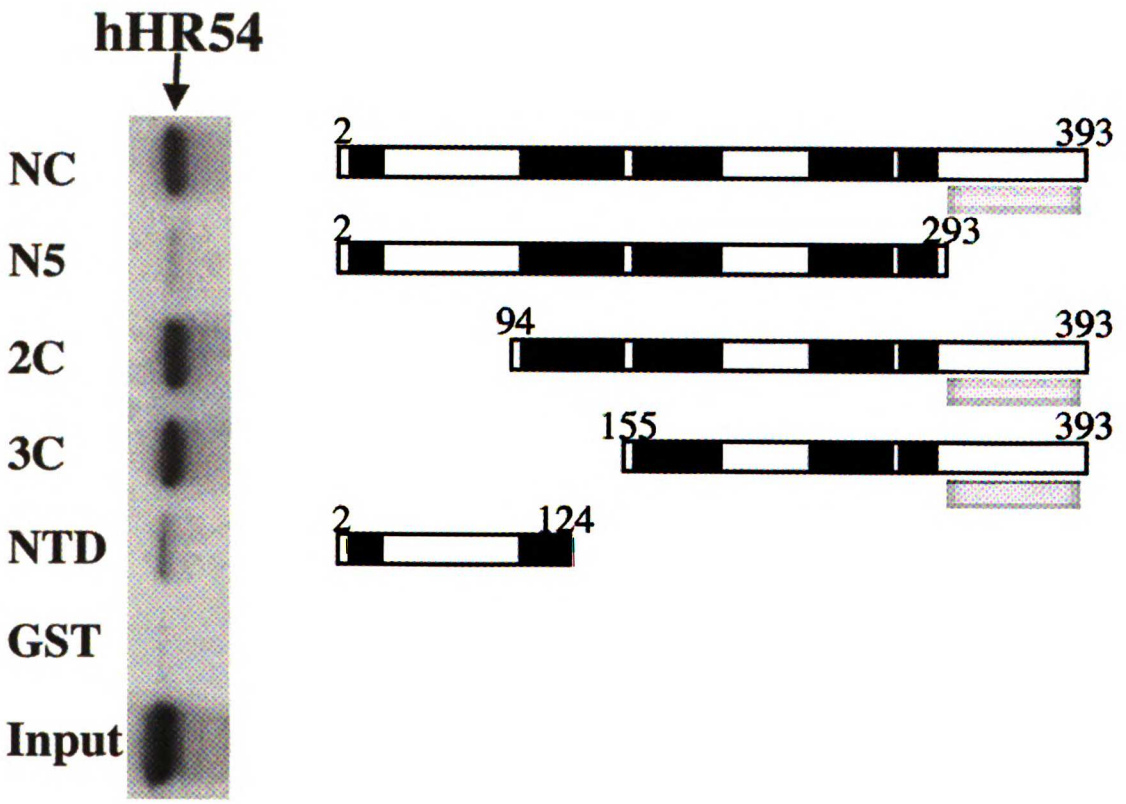


Figure 4

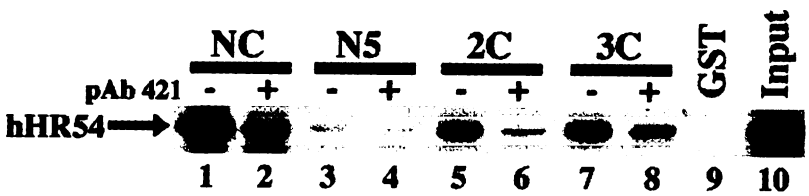
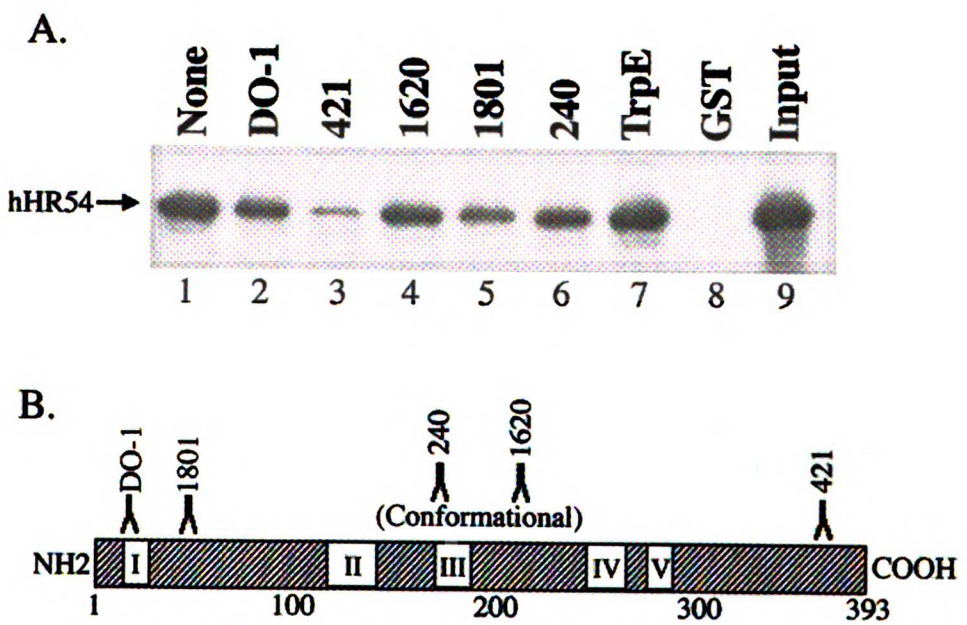


Figure 5


A.
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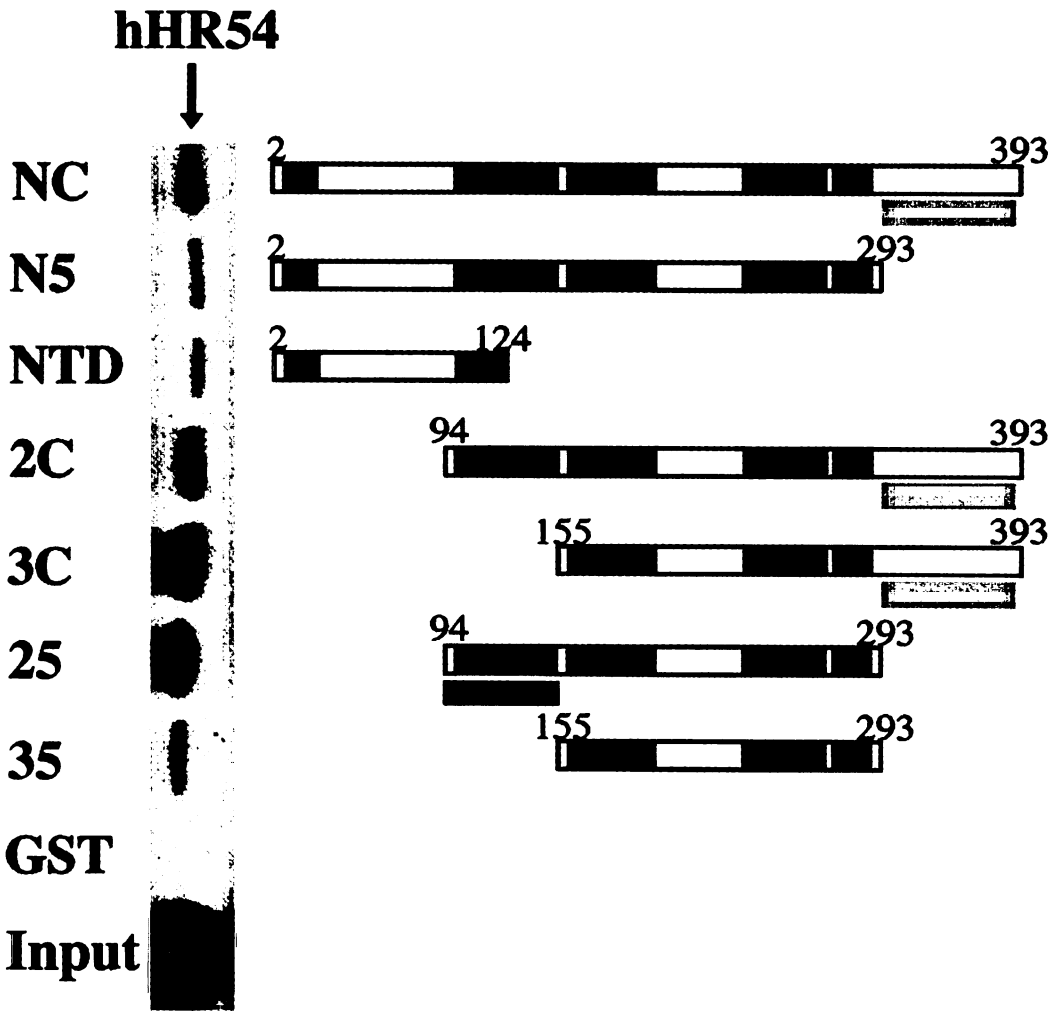
1801
240
TrpE
GST
Input
hHR54
1 2 3 4 5 6 7 8 9
B.
DO-1
1801
240
1620
(Conformational)
421
NH2
I
II
III
IV
V
COOH
1
100
200
300
393

Figure 6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NC
N5
NTD
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3C
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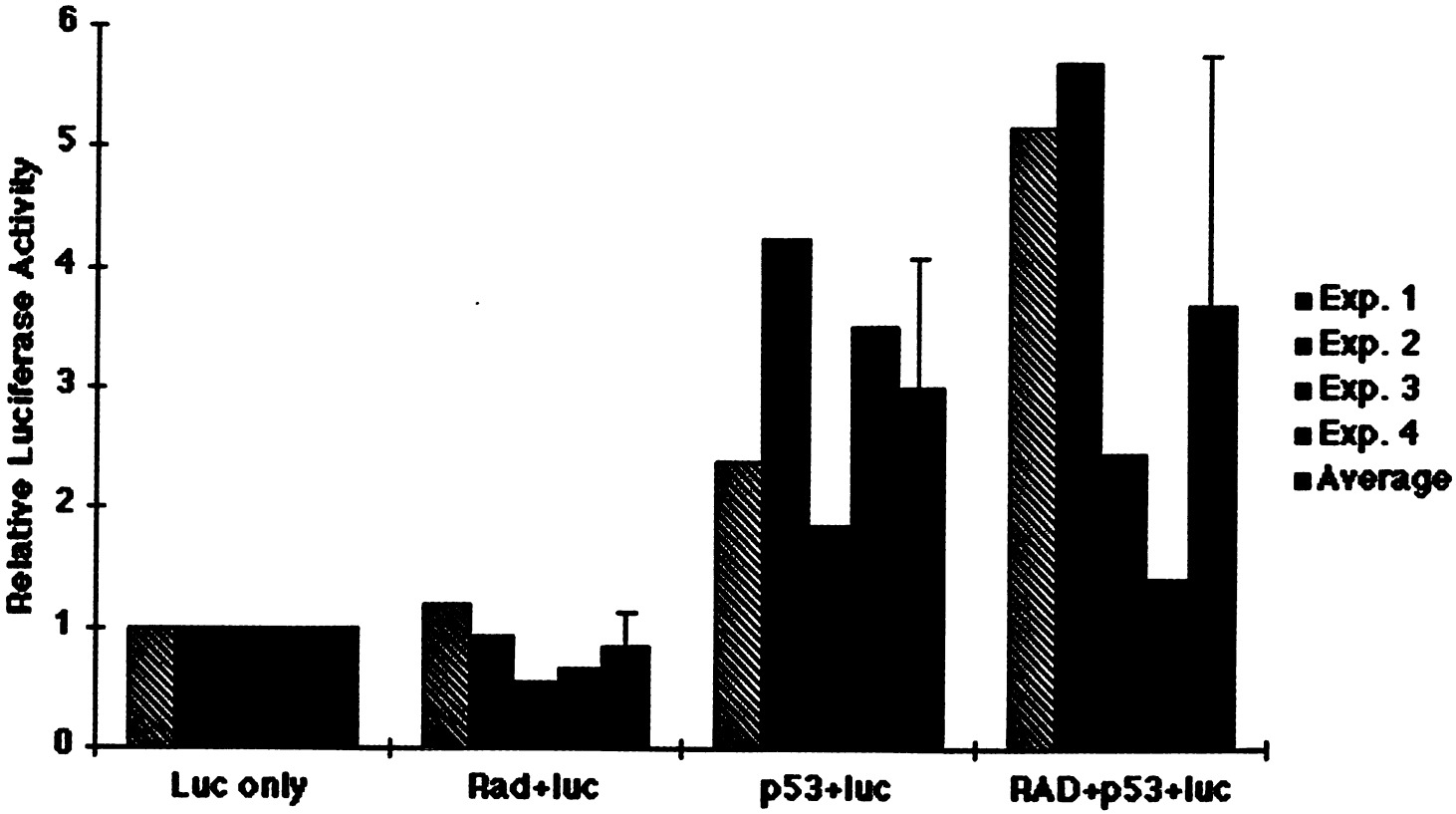
293
155
293

Figure 7


Relative Luciferase Activity
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5
4
3
2
1
0
Luc only
Rad+luc
p53+luc
RAD+p53+luc
Exp. 1
Exp. 2
Exp. 3
Exp. 4
Average

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