

# Analysis of mutant p53 for MAR-DNA binding: determining the dominant-oncogenic function of mutant p53

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## Summary

At least some mutant p53 proteins not simply have lost the wild-type p53 specific tumor suppressor function, but exhibit oncogenic functions on their own. Recently we showed that binding of mutant p53 to MAR/SAR elements is an activity specific for mutant p53 and clearly distinguishable from the previously reported DNA-binding activities of p53. Since MAR/SAR elements are considered to be important regulatory elements for a variety of nuclear processes, the interaction of mutant p53 with MAR/SAR elements might form the molecular basis for oncogenic potential of mutant p53. By employing different binding assays (the target-bound DNA binding assay, the South-western blotting technique and an adapted liquid phase binding assay), we studied MAR/SAR binding of various p53 proteins to different MAR/SAR elements. Murine mutant p53 bound different MAR/SAR elements with an approximately 1,000-fold higher affinity than murine wild-type p53. Analysis of MAR/SAR binding of human wild-type and mutant p53 proteins revealed also high affinity MAR/SAR binding of several human p53 mutant proteins (175 Arg→His, 273 Arg→Pro), but not of human wild-type p53, confirming that MAR binding is a general property of mutant p53. By antibody interference analysis using a panel of different p53-specific monoclonal antibodies and by deletion mutant analysis the MAR/SAR binding domain on mutant p53 was mapped, revealing a bipartite domain consisting of the mutated core region and the C-terminal 60 amino acids.

## I. Introduction

Mutations in the p53 gene constitute the most frequent alteration in a single gene in human cancer (Soussi et al., 1994). Wild-type (wt) p53 is a tumor suppressor, whose main function is to preserve the integrity of the genome as a cell cycle checkpoint protein. Thereby p53 not only mediates DNA damage response, growth arrest or apoptosis by modulating cellular transcription, it also exhibits a variety of other biochemical activities, which are directly related to its function as major control element in preserving the integrity of the cells' genetic information. 90% of all mutations in the p53 gene are single missense point mutations. These mutations are localized mostly in the p53 core domain, which mediates most of the biochemical activities of wild-type p53. Consequently, these mutations serve to inactivate the

tumor suppressor functions of p53, and the expression of mutant p53 often is considered being equivalent to a p53 "null" situation.

However, considering the many different activities exerted by wild-type (wt) p53, it is quite astounding that a single point mutation in the p53 molecule should totally eliminate p53 function. Furthermore, point mutations are a rather unique way for inactivating a tumor suppressor. All of the other known tumor suppressors are inactivated mostly by loss of functional gene expression, resulting either from gene truncations or deletions, or promoter inactivation (Soussi et al., 1994). This, and the fact that there is a strong selection for the maintenance of mutant p53 expression, provoked the idea that mutant p53 not simply is an inactivated tumor suppressor, but exerts oncogenic functions on its own (Deppert et al., 1990, Dittmer et al., 1993, Michalowitz et al., 1991, Levine et

al., 1995). This view very well corresponds to the initial characterization of p53 as an oncogene, when all of the available p53 cDNAs directed the expression of mutant p53 proteins.

The notion that mutant p53 proteins can exhibit endogenous dominant- oncogenic functions of their own (Deppert et al., 1990, Dittmer et al., 1993, Michalowitz et al., 1991, Levine et al., 1995, Zambetti et al., 1993) is strongly supported by a variety of experimental observations, perhaps most remarkably by the findings that mutant p53 not only leads to full transformation of the weakly Abelson murine leukemia virus transformed L12 cells (Shaulsky et al., 1991), but also increases the metastatic capacity of cells of a p53-deficient murine bladder carcinoma cell line (Pohl et al., 1988). As another example, expression of mutant p53 in p53-negative human SAOS-2 or murine BALB/c (10/3) cells resulted in increased proliferation rates and higher tumorigenicity of these cells (Dittmer et al., 1993).

The molecular basis for this gain of function of mutant p53 is still elusive. Mutant p53 has retained some of the biochemical activities of the wt p53 protein, like non-sequence-specific RNA or DNA binding and specific binding to RNA with extensive secondary structures (Mosner et al., 1995; Steinmeyer et al., 1988). Furthermore, mutant p53 binds to various cellular proteins which may lead to deregulation of cellular functions. Alterations in gene expression by mutant p53, e.g. upregulation of the *mdr1* gene (Strauss et al., 1995), have been consistently reported; however, upregulation of a particular gene by a transfected mutant p53 was observed in one type of cell but was totally absent in another one (Deppert et al., 1996).

This clearly indicates that stimulation of gene expression by mutant p53 must be due to a different mechanism than the wt p53 specific transactivator function, especially since most mutant p53 proteins have lost this ability, due to loss of sequence-specific DNA binding (Deppert et al., 1994). Interestingly, however, additional mutations in the transactivator domain of mutant p53 also abolish the ability of mutant p53 to upregulate the expression of certain reporter genes (Li et al., 1995). The most likely interpretation of these results thus seemed that mutant p53 still is able to interact with the cellular transcription machinery, but interacts with DNA in a different way than wt p53. Nevertheless this interaction must be specific, as there obviously is only a limited number of genes which are regulated by mutant p53.

## II. DNA binding properties of murine wt and mutant p53.

To find a specific interaction of mutant p53 with DNA which differs from that of wt p53, our laboratory has analyzed in detail the DNA binding properties of murine wt and mutant p53. Using DNA as a model substrate for a DNA which, due to its length and complexity, contains abundant sequence elements for sequence specific interactions, as well as structural elements for more complex interactions of a protein with DNA, we were able to demonstrate that highly purified mutant p53 from MethA cells, a methylcholanthrene induced mouse tumor cell line (DeLeo et al., 1977), binds to the 1,215 bp *AluI* fragment of DNA using a target-bound DNA-binding assay (**Figure 1**).

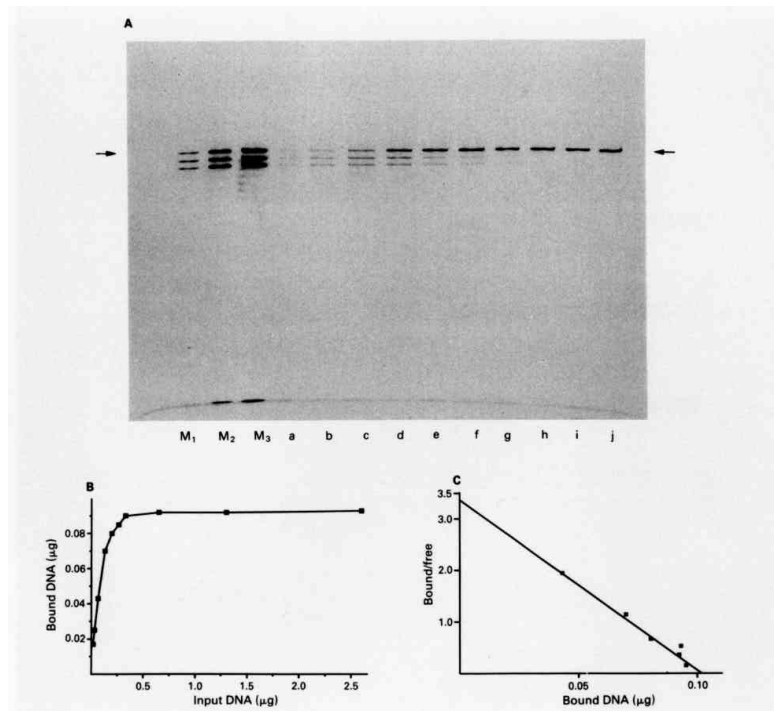
Computer analysis of this fragment then showed that it had both sequence and structure similarities with nuclear matrix attachment region/scaffold attachment regions (MAR/SAR elements). Both mutant and wild-type p53 previously were shown to interact with nuclear substructures within the nucleus, the chromatin and the nuclear matrix, with mutant p53 binding even more strongly to the nuclear matrix than wt p53. Therefore the binding of mutant p53 to a DNA fragment with homology to MAR/SAR elements raised the possibility that mutant p53 indeed might interact with such DNA, and prompted us to analyze this interactions in more detail using the target-bound DNA-binding assay specifically developed to detect this interaction.

Its main features are that p53 is doubly immunopurified: p53 is immunoprecipitated with the p53-specific monoclonal antibody PAb122 and protein A-Sepharose (PAS), then eluted by PAb122 epitope-specific peptide (Steinmeyer et al., 1988) and reprecipitated with another p53-specific monoclonal antibody, recognizing an N-terminal epitope of p53 (PAb248) and PAS. This second immune complex then is incubated with DNA under saturating conditions for both specific and competitor DNA, followed by rigorous washing to remove non-bound or non-specifically bound DNA. Specifically bound DNA and p53 are eluted separately and analysed by SDS-PAGE (Weißker et al., 1992).

These analyses revealed that binding of mutant p53 to the 1,215 bp *AluI* DNA fragment is a complex process, involving recognition of both structural and sequence determinants, as the fragment could not be narrowed down to any small consensus oligonucleotide. Binding was of high affinity ( $K_D$   $10^{-10}$  M), as shown by Scatchard analysis (**Figure 1**, Weißker et al., 1992). Further studies provided evidence that this type of complex DNA binding indeed reflected the affinity of MethA mutant p53 to MAR-DNA elements, as it could be extended to several bona fide MAR-DNA elements.

Despite the usefulness of the target-bound DNA binding assay for quantitatively assessing MAR-DNA

**Figure 1** Scatchard analysis of the mutant p53 MAR-DNA binding using the target-bound DNA binding assay. (A) Equal amounts of the doubly immunopurified MethA p53 (1 $\mu$ g) bound to PAb248 were incubated in the target-bound DNA-binding assay with increasing amounts of pA1215 restricted with *HindIII*, *BglII* and *EcoRI*. Lane a, 0.05  $\mu$ g, lane b, 0.1  $\mu$ g, lane c, 0.2  $\mu$ g, lane d, 0.4  $\mu$ g, lane e, 0.6  $\mu$ g, lane f, 0.8  $\mu$ g, lane g, 1  $\mu$ g, lane h, 2  $\mu$ g, lane i, 4  $\mu$ g, lane j, 8  $\mu$ g. Lanes M, marker DNA (1215-bp fragment, 1369-bp *EcoRI-BglII* pUC18 fragment, 1118-bp *BglII-BglII* pUC18 fragment): M<sub>1</sub>, 10 ng, M<sub>2</sub>, 50 ng, M<sub>3</sub>, 100 ng. DNA is marked with an arrow. (B) Binding curve of the Scatchard analysis of A. (C) Linear Scatchard plot of B (from Weißker et al., 1992)



binding by mutant p53, further comparative analyses of wt and mutant p53 MAR-DNA binding required the development of another assay system, as the target-bound DNA-binding assay had intrinsic limitations. For instance, this assay did not allow appropriate competition experiments, as the amount of competitor DNA required would be out of any experimentally feasible range. Thus it was difficult to discriminate in MAR-DNA binding by wt and mutant p53 between binding activities reflecting non-specific DNA binding by these proteins, and their specific MAR-DNA binding properties. Furthermore, the target-bound DNA binding assay also did not allow a direct comparative analysis of the MAR-DNA binding activity of deletion fragments of mutant p53 due to the necessity of binding the p53 proteins to a monoclonal antibody, and, last not least, steric interference of the affinity column material during the binding reaction could not be excluded.

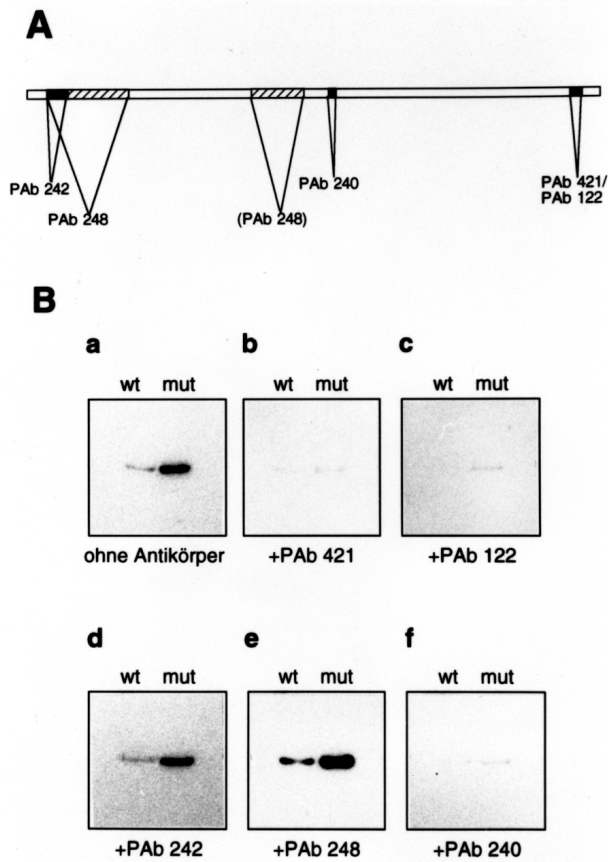
Therefore, we adapted an alternative binding assay, the South-western blotting technique, for the analysis of MAR-DNA binding by wt and mutant p53. After separation of the p53 proteins by SDS polyacrylamide gel electrophoresis, the proteins were transferred onto a nitrocellulose membrane, and renatured on this membrane. The membrane then was incubated with excess radioactively labeled MAR-DNA in the presence of unlabeled non-specific competitor DNA, washed

extensively, and the DNA bound by p53 was visualized by autoradiography.

These analyses, using the *XbaI*/MAR/SAR fragment of the murine immunoglobulin heavy chain gene enhance locus (Cockerill et al., 1987), revealed that this binding was specific for mutant p53. The affinity of MethA p53 to MAR-DNA was approximately 1,000-fold higher than that of wt p53 (Müller et al., 1996). By antibody interference analysis using a panel of different p53-specific monoclonal antibodies (Figure 2) and deletion mutant binding studies (Figure 3), we mapped the MAR/SAR binding region on mutant p53 to a bipartite domain consisting of the mutated core region and the C-terminal 60 amino acids (Müller et al., 1996).

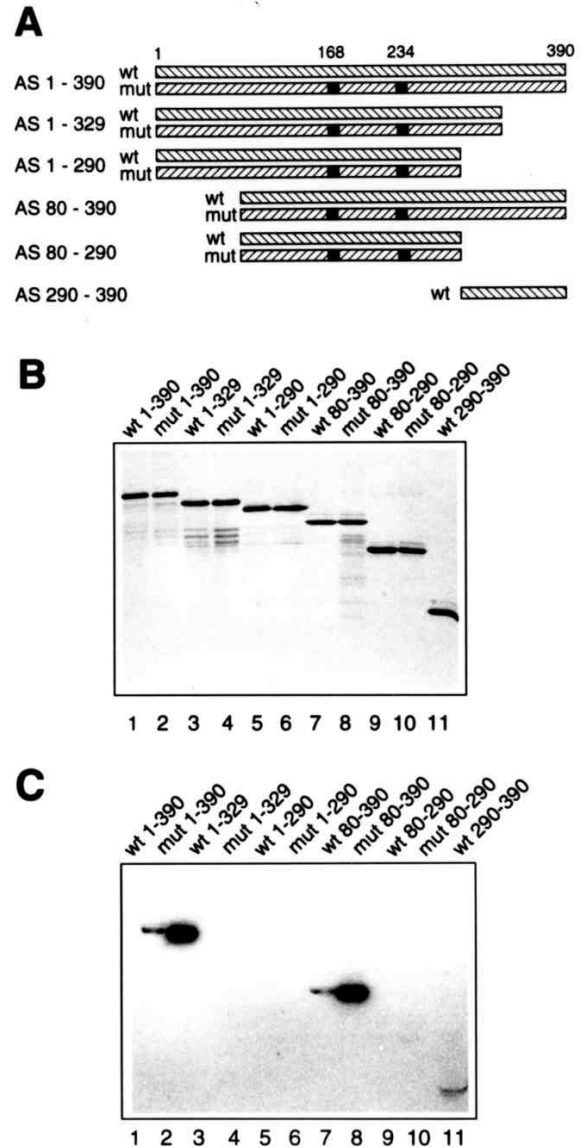
Thus both the non-sequence specific DNA binding domain localized on the C-terminus of p53, as well as the core domain of p53 mediate MAR/SAR binding synergistically (Müller et al., 1996), thereby clearly discriminating this activity from sequence-specific DNA-binding by wt p53 (mediated by the core domain), and from non-sequence specific DNA binding of both wt and mutant p53 (mediated by the C-terminus).

An important question regarding the relevance of the MAR-DNA binding observed with murine MethA mutant p53 was, whether this interaction would be limited to this special mutant p53 or exhibited also by other mutant p53



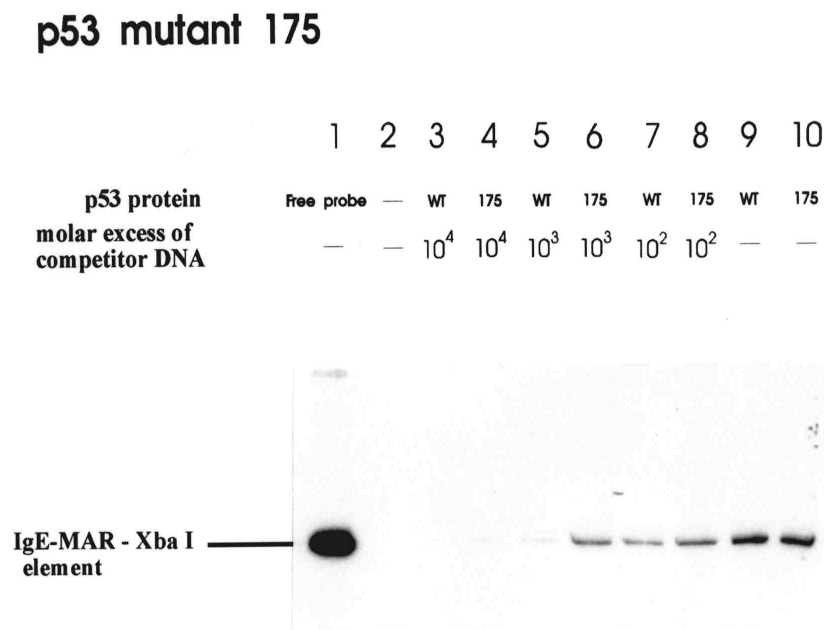
**Figure 2.** Mapping of the MAR/SAR binding domain of mutant p53 by antibody-interference using South-western blotting. (A) Schematic representation of the epitopes of various anti-p53 monoclonal antibodies on the p53 molecule. (B) The influence of the anti-p53 monoclonal antibodies indicated below each panel upon binding of the IgE-MAR element by wild-type and mutant p53 was monitored in South-western analyses. 2µg of purified wild-type and MethA mutant p53, respectively, were analysed in the presence of a  $3 \times 10^4$  fold excess of calf thymus genomic DNA. Antibody-incubation was performed prior to DNA-binding (From Muller et al, 1996).

proteins. Various murine mutant p53 proteins were selected, isolated from different cellular and recombinant sources and subsequently subjected to South-western binding analysis using the IgE-MAR element. In accordance with our earlier findings, all murine mutant p53 proteins in repeated experiments clearly showed high affinity binding to the IgE-MAR also in the presence of a



**Figure 3.** Mapping of the MAR/SAR binding domain of mutant p53 by deletion analysis using South-western blotting. (A) Schematic representation of the wild-type and MethA mutant p53 deletion-molecules, constructed according to the tripartite structure of the p53 molecule. (B) 1µg of the purified wild-type and MethA mutant p53 deletion fragments were subjected to SDS-PAGE and stained with Coomassie blue. (C) South-western analysis of the binding of 2 µg of each wild-type and MethA mutant p53 deletion fragment to the IgE MAR element in the presence of a  $3 \times 10^4$  fold molar excess of non-labeled calf thymus genomic DNA. (from Müller et al., 1996).

**Figure 4.** Analysis of MAR-DNA binding using the liquid phase binding assay. Mutant p53 protein 175 (aa175 Arg His) binds to the IgE-MAR element with higher affinity than wild-type p53. Equal amounts of wild-type p53 and mutant 175 p53 were added to binding buffer (SWB-buffer) including the radioactively labeled IgE-MAR-DNA fragments and increasing amounts of unlabeled competitor DNA and incubated for 30 min at room temperature. Subsequently, after incubation of the mixture with antibody PAb1018 and protein A-Sepharose (PAS) the protein-DNA complexes were washed and the bound IgE-MAR-DNA eluted. The eluates were lyophilized, dissolved and subjected to DNA-SDS-PAGE and visualised by autoradiography (manuscript in preparation).



high excess of non-specific competitor DNA, whereas murine wt p53 failed to bind to this MAR element under such conditions.

### III. Studies with human p53 proteins

A disturbing result was obtained when we subjected different human p53 proteins to MAR-DNA binding analysis in South-western experiments. In contrast to murine mutant p53, which reproducibly bound to MAR-DNA in repeated experiments, we obtained quite varying results when human mutant p53 proteins were used, ranging from weak to no binding at all. Rather than assuming that MAR-DNA binding is a property specific for murine mutant p53, we considered the possibility that the apparent lack of a reproducible MAR-DNA binding by human mutant p53 reflected technical problems related to structural differences between human and murine p53.

Although human and murine p53 share extensive homologies, there are sequence and conformational differences between these proteins, already reflected by the fact that there are species-specific monoclonal antibodies for human and murine p53. The most important step in the South-western binding assay is a renaturation step, which is very critical for reconstructing the capability for DNA binding. Therefore, we suspected that problems in

refolding the human p53 proteins accounted for our difficulties to unequivocally demonstrate MAR-DNA binding for human mutant p53 proteins.

This forced us to develop an assay which did not require renaturation procedures. The liquid-phase binding assay fulfilled this criterion. In this assay, the desired MAR-DNA fragments were isolated and end-labeled using T4 polynucleotide kinase and (<sup>32</sup>-P) ATP by standard procedures and subjected to the binding assays including mutant p53 and unlabeled competitor DNAs. To avoid interference of the column material, p53 and the DNA were first incubated alone, and an N-terminal p53 specific monoclonal antibody (PAb248 for murine p53 and PAb1801 for human p53) and PAS were added later. Finally, the DNA-Protein-PAS complexes were washed and the bound DNA quantitatively eluted. The eluates were lyophilized, resuspended in sample buffer and separated by gel electrophoresis.

Application of this assay first for murine mutant p53 proteins in accordance with our earlier findings in repeated experiments clearly showed high affinity binding to the IgE-MAR also when high excess of non-specific DNA was added. Murine wt p53 again failed to bind to this MAR element under these conditions. When this assay then was applied to human wt and mutant p53, we in repeated experiments observed high affinity MAR-DNA binding

also of human mutant p53 proteins (**Figure 4**, 175 Arg His, not shown 273 Arg Pro), but not of human wt p53, thereby confirming the assumption that MAR-DNA binding is a general property of mutant p53.

#### **IV. Conclusions**

Many questions remain to be resolved before MAR-DNA binding of mutant p53 can be related to its oncogenic activities, and before the molecular consequences of such interactions are understood within tumor cells. Most importantly, we must identify the structural features within MAR-DNA which mediate the specific interaction of mutant p53 with these DNA elements. Although our understanding of the oncogenic effects of mutant p53 is still at the beginning, the exciting possibility emerges that by interfering with MAR-DNA binding of mutant p53 it might be possible to abrogate its oncogenic functions in the tumor cell. Considering the strong selection for the maintenance of mutant p53 expression in tumor cells, one can hope that elimination of mutant p53 function in tumor cells is detrimental to tumor cell growth and will lead to its destruction.

#### **V. Protocols**

##### **A. Target-bound p53 binding to MAR/SAR elements**

1. Immunoprecipitation of p53 (approximately 1 µg total) from extracts of MethA cells, or from High five insect cells infected with recombinant baculoviruses expressing the respective mutant p53 protein using PAb122.

2. Elution of p53 from the immune complex with a 100-fold molar excess of a PAb122 epitope-specific peptide, followed by reprecipitation of p53 with an antibody recognizing a different epitope on p53.

3. Target-bound DNA binding assay of the doubly immunopurified p53: The immune complexes were washed with binding buffer (10 mM MOPS, pH 7, 150 mM NaCl, 1 mM DTT, 0.5 mM MgCl<sub>2</sub>) and incubated with 8 µg of the respective DNA (restricted plasmid DNA containing the respective DNA fragment) in a total volume of 200 µl of binding buffer for 1 hr at 4°C. Immune complexes were washed three times with high-salt buffer (10 mM Tris-HCl, pH 7.8, 10 mM NaCl) to separate bound and free DNA.

4. Two-step elution of bound DNA and p53 and SDS-PAGE: DNA fragments bound to p53 immune complexes were quantitatively eluted with 500 µl of 100 mM ammonium hydrogen carbonate, pH 9.5 for 45 min at 35°C. Proteins were eluted with 50 µl SDS sample buffer and subjected to SDS-PAGE.

5. The DNA eluates were lyophilized and dissolved in 50 µl of gel loading buffer (water, 10% glycerol, bromophenol blue). Samples of 5 µl were subjected to SDS-PAGE and visualised by silver staining. Marker proteins of known concentration, electrophoresed on the same gel, served as standards.

##### **B. South-western DNA binding assay**

1. Purified protein was subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane soaked in transfer buffer (20mM Tris-acetate pH 8.3, 0.1% SDS, 20% 2-propanol) at 60V for 2 h.

2. Proteins were fixed on the filters with 50% 2-propanol.

3. Filters were washed with demineralized water and incubated 2 times for 30 min in renaturation buffer I (50mM NaCl, 10mM Tris HCl pH 7, 2mM EDTA, 0.1mM DTT, 4M urea, 1% TritonX100) and renaturation buffer II (as renaturation buffer I, without TritonX 100), respectively.

4. A 30 min incubation at 30°C in renaturation buffer III (50mM NaCl, 10mM Tris HCl pH 7.0, 1mM EDTA, 6mM MgCl<sub>2</sub>, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 1 µg/ml DnaK, 0.5mM DTT, 1mM ATP) strongly enhanced renaturation, but was optional for MAR/SAR binding.

5. After renaturation the membranes were equilibrated and saturated in DNA binding buffer with genomic calf thymus DNA and bovine serum albumin (SWB: 50mM NaCl, 10mM Tris-HCl pH 7.0, 1mM EDTA, 6mM MgCl<sub>2</sub>, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 100 µg/ml, calf thymus DNA with an average fragment length of 10<sup>3</sup> to 10<sup>4</sup> bp (Sigma)). Filters were incubated in a total volume of 5 ml binding buffer with 5x 10<sup>6</sup> cpm of the specific DNA probe (MAR/SAR DNA elements), which was radioactively labeled by primer extension.

6. After 4 h the membrane was washed 3 times with DNA binding buffer and subjected to autoradiography.

##### **C. Liquid phase binding assay**

1. p53 was isolated from extracts of MethA cells, from High five insect cells infected with recombinant baculoviruses or from bacteria expressing the respective wild-type or mutant p53 protein using antibody PAb248 columns. PAS-antibody-p53-complexes were washed with buffer A (30 mM KPi, pH 8.0, 50 mM KCl, 1 mM EDTA, 2 mM DTT) and eluted with buffer A including 1 M KCl. and subsequently with buffer B (100 mM KPi, pH 12, 1M KCl, 1 mM EDTA, 2 mM DTT), followed by immediate neutralisation with KH<sub>2</sub>PO<sub>4</sub>. Aliquots of the eluates were subjected to SDS-PAGE and protein concentrations were determined after Coomassie blue staining.

2. The desired MAR-DNA fragments were isolated by restriction digest and gel electrophoretic separation, purified from the gel and end-labeled using T4 polynucleotide kinase and (<sup>32</sup>-P) ATP by standard procedures.

3. Afterwards equal amounts of each p53 preparation were added to the binding buffer (SWB-buffer, see Southwestern DNA binding assay) including the desired radioactively labeled MAR-DNA fragments and unlabeled competitor DNA and incubated for 30 min at room temperature.

4. Subsequently, antibodies PAb248 or PAb1810 and PAS were added and shaken for 30 min at room temperature.

5. The DNA-protein-antibody complexes were washed three times with SWB-buffer.

6. DNA fragments bound to p53 immune-complexes were quantitatively eluted with 500 µl of 100 mM ammonium hydrogen carbonate, pH 9.5 for 45 min at 35°C.

7. The eluates were lyophilized and dissolved in 20 µl of gel loading buffer (water, 10% glycerol, bromophenol blue). Samples are subjected to DNA-SDS-PAGE and visualized by autoradiography.

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