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**OPTIMIZATION OF P53 MUTANT PROTEIN ISOLATION
AND ITS DNA BINDING PROPERTIES**

OPTIMALIZACE IZOLACE MUTANTNÍHO PROTEINU P53 A JEHO DNA VAZEBNÉ VLASTNOSTI

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ABSTRACT

P53 protein is one of the most important molecules in the human body. It is responsible for several cellular processes such as DNA repair, cell cycle or induction of apoptosis. P53 is also known as “guardian of the genome”. DNA binding ability of p53 is important for the normal cell development and growth. Different mutation of *TP53* gene, may cause lost of p53 binding ability and its tumor suppressor function that may lead to the cancer development.

The theoretical part of this diploma thesis, is focused on the characterization of main properties, function and mechanism of p53 protein activation and description of local secondary DNA structures. The main goal of the experimental part was production of four mutant forms of p53 protein and wild-type p53 protein and studying its binding properties with different local secondary DNA structures. Using Gateway cloning system, four expression vectors were prepared and used for protein production in the bacterial expression system. In total, four mutant and one wild-type p53 protein were prepared and their DNA binding properties were evaluated by Electrophoretic Mobility-Shift Assay. The results suggest different DNA-binding properties of wild-type p53 and mutant forms of this protein. All studied mutant proteins lost ability to bind DNA sequence specifically, whereas non-specific interaction with DNA were sustained for three out of four mutant forms. One of the studied mutant proteins showed preference for superhelical form of DNA.

ABSTRAKT

Protein p53 je jednou z nejdůležitějších molekul v lidském těle. P53 reguluje celou řadu procesů v buňce, jako je například oprava DNA, buněčný cyklus nebo indukce apoptózy. Protein p53 je známý i jako „strážce genomu“. DNA vazebné schopnosti proteinu p53 jsou důležité pro normální vývoj a růst buňky. Mutace genu pro p53 mohou vést ke ztrátě jeho DNA vazebných vlastností a funkce nádorového supresoru, což může způsobit rozvoj rakoviny.

Teoretická část této diplomové práce je zaměřena na popis vlastností, funkce a mechanismus aktivace proteinu p53 a popis lokálních sekundárních struktur DNA. Hlavním cílem experimentální části byla produkce čtyř mutantních forem proteinů p53 a wild-type p53 proteinu a studium jejich vazebných vlastností s různými lokálními sekundárními strukturami DNA. Pomocí Gateway klonovacího systému byly připraveny čtyři expresní vektory, které byly použity pro produkci proteinů v bakteriálním expresním systému. Celkem byly úspěšně připraveny čtyři mutantní formy a wild-type p53 protein. Jejich vazebné vlastnosti byly studovány gelovou retardační analýzou. Výsledky naznačují různé DNA-vazebné vlastnosti wild-type p53 a studovaných mutantních forem tohoto proteinu. Všechny mutantní proteiny ztratily schopnost sekvenčně specificky vázat DNA, zatímco nespecifická interakce s DNA byla pozorována u tří ze čtyř mutantních forem. Jeden ze studovaných mutantních proteinů se vázal jenom na superhelikální formu DNA.

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DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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student's signature

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ABBREVIATIONS

- A – adenine;
- AMP – ampicillin;
- APC – adenomatous polyposis coli protein;
- ARF – the ADP-ribosylation factor;
- ATM – a protein that is activated by DNA breaks, ATM serine/threonine kinase, “ataxia telangiectasia mutation”;
- ATR – a protein that detects DNA damage and is responsible for cell cycle arrest at a checkpoint (ataxia telangiectasia and Rad3-related protein)
- *att* – specific DNA sequence of components that recombine in the BP or LR reaction, respectively ("attachment sites");
- B-DNA – nucleic acid double helix, the most common double helical DNA structure;
- BRCA1 – Breast cancer type 1 susceptibility protein;
- C – cytosine;
- CAM – chloramphenicol;
- Chk1, Chk2 – checkpoint kinase (1 and 2);
- CTD – C-terminal domain of p53;
- DNA-PK – the DNA-dependent protein kinase;
- dsDNA – double stranded DNA;
- *E. coli* – *Escherichia coli*;
- E1B – adenovirus protein, responsible for blocking apoptosis in adenovirus-infected cells;
- E3 – pyruvate dehydrogenase protein X component;
- E6 – oncogenic human papillomavirus infection protein;
- EDTA – ethylenediaminetetraacetic acid;
- EMSA – electrophoretic mobility shift assay;
- G – guanine;
- GOF – gain of function protein;
- HAUSP – herpesvirus-associated ubiquitin-specific protease, antagonist of MDM2;
- HisTag – polyhistidine-tag;
- IgG – antibody type Immunoglobulin G
- IHF – Integration Host Factor;
- INKA4 – family of cyclin-dependent kinase inhibitors (hence their name **IN**hibitors of **CDK4**);
- Int – Integrase enzyme;
- L2, L3 – two flexible loops, which are proposed to be sites for DNA binding.
- LB – lysogeny broth;
- linDNA – linear DNA
- MDM2 – “Mouse double minute 2 homolog”, enzyme with ubiquitin ligase E3 activity, p53 protein level regulator;
- OD – tetramerization domain of p53;
- p21 – protein which is stimulated by the p53 protein and which ensures cell cycle regulation;

- PCR – polymerise chain reaction;
- PRR – proline-rich region of p53;
- RASSF1A – ras association domain-containing protein 1 is a protein that in humans is encoded by the *RASSF1* gene;
- RB – retinoblastoma protein;
- RE – responsive element, short consensus sequence of DNA, with gene promoter that is able to bind specific transcription factors and regulate transcription of genes;
- S40 – simian vacuolating virus 40;
- scDNA – superhelical DNA
- SDS-PAGE – sodium dodecyl sulfate–polyacrylamide gel electrophoresis;
- T – thymine;
- TAD – the transactivation domain of p53;
- TF – transcriptional factors;
- *TP53* – gene that encodes p53 protein;
- Xis – excisionase enzyme.

1 INTRODUCTION

Protein p53 is known as one of the most important molecules in the human body. The main role of the p53 protein is its tumor suppressor function, due to this fact it is also called “the guardian of the genome”. Together with the p63 and p73 proteins, they form family of p53 proteins, where all proteins play important regulatory roles [1].

The p53 protein is responsible for transmission of different stress-inducing signals. The p53 plays role in the DNA repair, cell cycle arrest or induction of apoptosis. Whereas in the normale cells, p53 is maintained in the low concentration, most tumor cells manifest accumulation of p53 protein [2].

The wild-type p53 protein is critically important for normal cells growth and development. Mutations of the p53 protein were found in more than 50% cases of cancer. P53 mutations were reported to occur in almost every type of cancer [3]. Majority of p53 mutations are missense mutation with a single nucleotide substitution. Mostly p53 mutations are located in the DNA-binding domain that can lead to the loss of p53 binding ability to interact with specific DNA sequence motifs and transcription of p53 regulated genes. As a result of different mutations, such as nonsense, frame shift or deletions, protein is inactivated or no protein is produced [4].

2 THEORETICAL PART

2.1 P53 Protein

The p53 protein is a tumor suppressor that controls cell cycle, genomic integrity and apoptosis. Steady-state level of the p53 protein is always maintained inside a cell under normal conditions. In the case of any stress, p53 activates and starts to accumulate in the nucleus. It starts to regulate transcription of target genes, by interaction with its specific DNA response elements. As a consequence of p53 activation cells can undergo cell cycle arrest, DNA repair, altered metabolism, senescence and apoptosis [1].

2.2 Structure of p53 Protein

P53 is a homo-tetramer, each subunit consisting of 393 amino acids. P53 contains transactivation domain, DNA-binding domain (DBD) and oligomerization domain that mediates tetramerization [5]. The transactivation domain, made out of two subdomains (TAD1 and TAD2), is located in the N-terminal region (residues 1-61) and is followed by proline-rich region (PRR), that is known for its apoptotic activity (Figure 1) [6]. Both TAD1 and TAD2 are responsible for transcription induction of target genes and for interaction with other proteins. N-terminal domain also contains high amount of aspartic and glutamic acids and frequently undergo post-translation modifications, such as phosphorylation. DNA-binding domain is located between amino acids residues 94 and 292 and is connected with tetramerization domains (OD) through a flexible linker region. DNA-binding core domain has in its structure central immunoglobulin-like β -sandwich scaffold and DNA-binding surface, made from loop-sheet-helix motif and two large loops (L2 and L3). DBD domain contains one zinc atom, which interacts with the DNA [7]. The C-terminal domain (CTD) (residues 356-393) is responsible for the down-regulation of the activity of central DNA-binding domain [6].

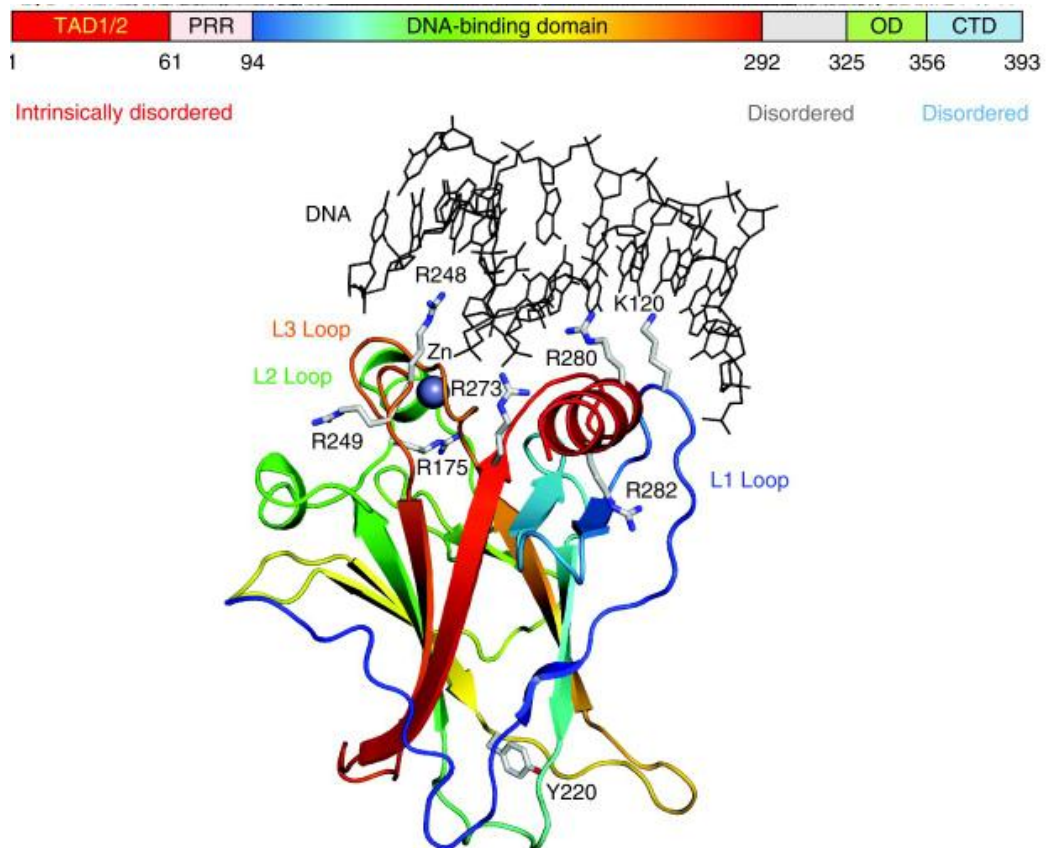


Figure 1 : Domain structure of p53. P53 has natively unfolded amino-terminal transactivation domain (TAD), consisting of two subdomains TAD1 and TAD2, followed by a proline-rich region (PRR). The DNA-binding domain and tetramerization domain (OD) are connected through linker region. The regulatory at carboxyl terminus is intrinsically disordered [7].

2.3 TP53 Gene

The *TP53* gene encodes a tumor suppressor protein p53. The *TP53* gene is located on the short arm of chromosome 17 at position 13.1 (17p13.1) (Figure 2). More precisely, the *TP53* gene is located from base pair 7,571,719 to base pair 7,590,867 on chromosome 17 [8]. The genome size of the *TP53* is about 20 kb. It spans 10 introns and 11 exons. Because the presence of two alternative promoters and due to alternative splicing, alternative initiation of translation, and alternative promoter usage the human p53 gene expresses not only 1 but 12 different p53 proteins (isoforms) with alternative C-terminal domain and N-terminal domains [2].

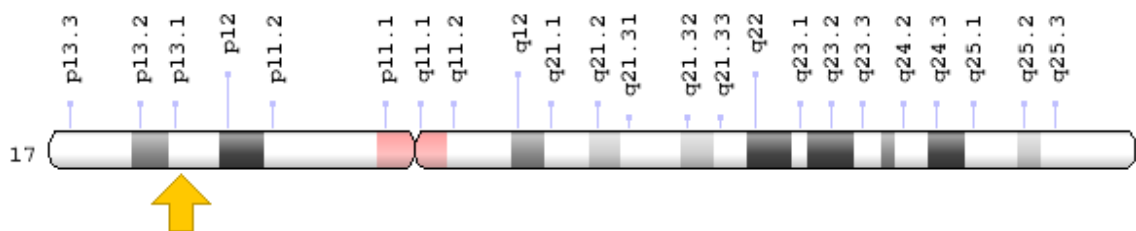


Figure 2 : Chromosomal location of the *TP53* gene on the short arm of chromosome 17 [8]. Orange arrow shows location of *TP53* gene on the chromosome.

2.4 Function of p53 Protein

Known as “guardian of the genome” the p53 protein is responsible for transmission of different stress-inducing signals to different antiproliferative cellular responses. Since DNA damage, oncogene activation or hypoxia is detected, p53 is activated which may result in cell-cycle arrest, cell senescence, DNA repair, apoptosis and metabolic adaptation [9]. Activation of the p53 could be described in three steps, stabilization of p53, sequence-specific DNA binding and transcriptional activation of target genes. Most of the time p53 is present in a low concentration, but as soon as any stress, that cause DNA damage, like UV light or ionizing radiation is detected, active p53 production starts [10]. In the cells exposed to potent stress signals, p53 induce senescence or apoptosis, whereas in the conditions of low-level stress p53 may institute cell-cycle arrest and activate DNA-repair mechanisms.

Steady level of p53 under normal conditions is primarily achieved through preventing its binding with MDM2, a negative regulator that causes degradation of p53. In response to stress, for example certain chemotherapeutic agents, p53 is posttranslational modified by phosphorylation of the amino terminus of p53 at specific amino acids, by various kinases, including ATM, ATR, DNA-PK, Chk1 and Chk2. Also, p53 could be stabilized by its binding with, p53-MDM2 antagonist, the tumor suppressor p14^{ARF} (p19^{ARF} in the mouse) [11]. P53 tetramers bind as dimers of dimers to sequence-specific p53 response elements, which are defined as two DNA half sites of RRRCWWGYYY (P-purine, W-adenine or thymine, Y-pyrimidine) with a spacer of 0-13 base pairs between half sites. Those sites are often localized in the promoter or first introns of p53 target genes. As soon as p53 binds to DNA, transcription of protein-coding genes starts [10].

2.5 Regulation of p53

Regulation of p53 plays critical role in the normal cell growth and development. Activation of p53 is regulated by different factors and mechanisms, like MDM2 interaction or posttranslational modifications of p53.

MDM2 in normal conditions is responsible for p53 degradation, through the ubiquitin-proteasome system. MDM2 makes a negative feedback loop with p53, because of its transcriptional induction by p53. Phosphorylation of N-terminus domain at Ser15 (mouse Ser18) and Ser20 (mouse Ser23) is responsible for stabilization of p53 by inhibiting the interaction between p53 and MDM2. In response to different stress or DNA damage Ser15 and Ser20 are phosphorylated by ATM, ATR, DNA-PK, Chk1 and Chk2 [12]. The tumor suppressor ARF (alternative reading frame protein expressed from INK4a locus) in the cases of oncogenic stimuli binds to MDM2 and inhibiting its E3 ligase activity, that leads to p53 accumulation, p53 dependent growth arrest or apoptosis [13]. In the cases of ribosomal stress, the ubiquitination of p53 by MDM2 is stopped by ribosomal proteins (L11, L5, L23, S14), and ubiquitination and degradation of MDMX are enhanced. In that case interaction between MDM2 and p53 is not blocked. In response to stress, that causes DNA damage, the MDM2-p53 interaction is inhibited by phosphorylation of Ser395 and Tyr394 on MDM2 by kinases ATM and c-Abl as schematically shown in Figure 3 [13], [14].

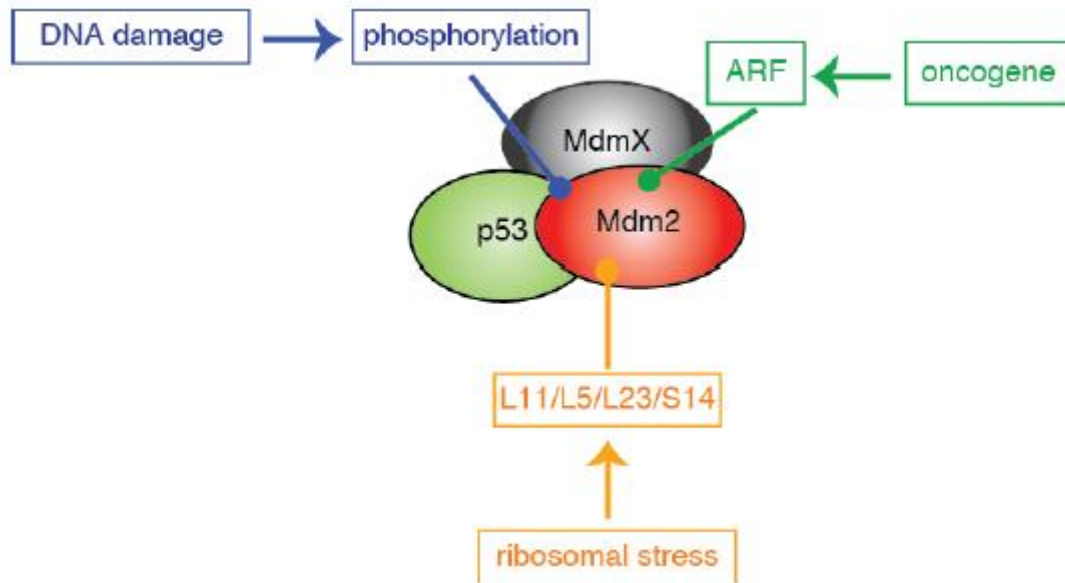


Figure 3 : P53 stabilization in response to different stress. In response to oncogenic stimulus ARF binds to MDM2 inhibiting its E3 activity and causing p53 accumulation. In response to ribosomal stress, ribosomal proteins (L11, L5, L23, S14) binds to MDM2 and block the ubiquitination of p53. In the case of DNA damage, ATM and ATR cause serine and threonine residues phosphorylation at the N-terminus and C-terminus domain of p53, and block its interaction with MDM2 [14].

Degradation of MDM2 is also an important mechanism for p53 stabilization. MDM2 has self-ubiquitination activity, as an E3 ligase. Deubiquitinase HAUSP enzyme reacts and deubiquitinates with MDM2 and p53, during a no-stress period. The stability of the MDM2-HAUSP complex is dependent on the proteins Daxx and RASSF1A. Dissociation of Daxx or RASSF1A, in response to stress, causes MDM2 auto-ubiquitination and degradation. Balance between these proteins is necessary; a low concentration of HAUSP prevents p53 deubiquitination, but in the case of HAUSP ablation, p53 is stabilized. These factors state the importance of MDM2 in the regulation of p53 function [13].

2.6 Mutation of p53 Protein

The wild-type p53 is a well-known tumor suppressor and is critically important for normal cell growth and development. Mutation of p53 was detected in many cases of cancer diseases. The majority of tumor suppressor genes, like *RB*, *APC* and *BRCA1* are inactivated by deletions or truncating mutations in tumors, but *TP53* is mostly inactivated by missense mutation with a single nucleotide substitution [3]. P53 mutations were reported to occur in almost every type of cancer at rates: esophageal – 45.5%, colorectal – 43.3%, lung – 37.2%, gastric – 32.4%, liver – 31.2%, breast – 22.8% and prostate cancer – 16.9% [15].

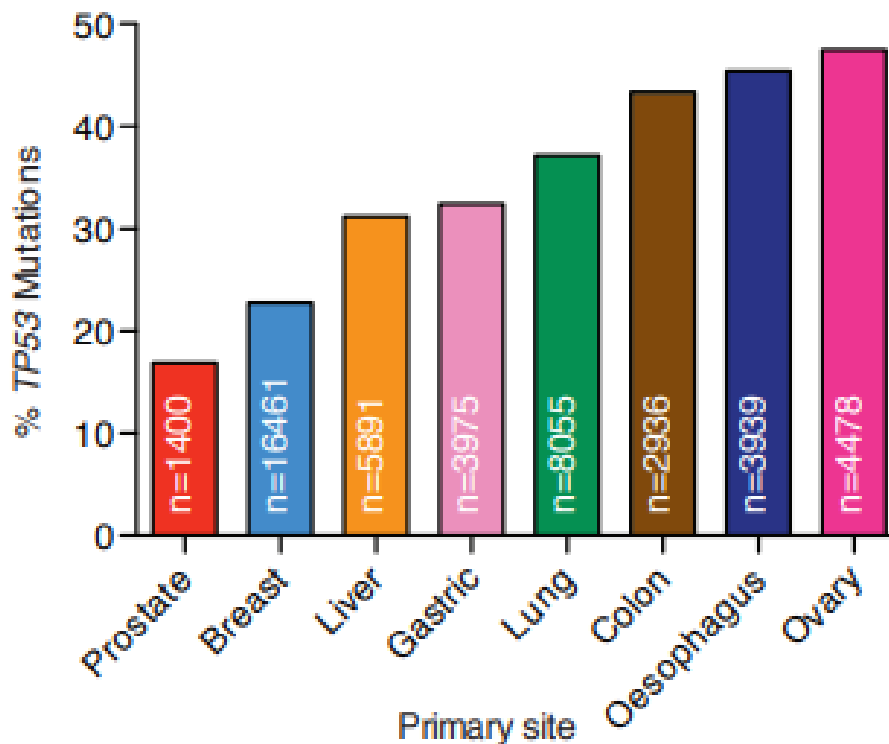


Figure 4 : TP53 mutation [14]. N=number of mutations. Generated from IARC database sequencing data. Version R20, July 2019.

The mutated p53 often lost its activity that leads to tumor growth. But there are also known cases where function of p53 is lost because of interaction with other protein or modulation of p53-related signaling pathways. Some proteins of DNA viruses can bind to p53 molecule and inactivate it, like E1B protein of adenovirus, large T antigen of SV40 virus or E6 protein of papillomavirus [16]. High level of MDM2 as a consequence of gene amplification, enhanced transcription or translation, was detected in the tumors with functional p53. With that high amount of MDM2, p53 is over-degraded [17]. In aggressive neuroblastoma, the overexpression of transcriptional factor H-Twist causes inhibition of the ARF-p53 signal pathway and therefore also inhibition in an apoptotic response to amplification of *N-Myc* oncogene [18].

P53 mutations are located most often at DNA-binding domain, between amino acids residues 102 – 292 leading to decrease of p53 binding affinity and as result also inhibits transcription of p53 regulated genes. Depending on the type of mutation, for example nonsense, frame shift mutation or deletions, inactivated protein is produced or no protein is produced. These types of mutations represent only 10% of total p53 mutations, the remaining mutations includes missense mutation producing and faulty protein [19].

Almost 80% of missense mutations are located at the DNA-binding domain. There are several “hot spot” residues identified in this domain, such as R175, G245, R249, R273 and R282. P53 mutations occurring in the DNA binding domain are classified into two groups: structural mutants or contact mutants. In DNA-contact mutants the amino acids that are critical for its binding properties are replaced. This group contains such mutation as Arg - 248 - Gln (R148Q), Arg -273- His (R273H) and Arg - 282 – Trp (R282W). These mutations

lead to p53 inactivation because high selectivity binding dismiss and residual affinity is too small for efficient transactivation. The missense substitution in structural mutants causes unfolded structure or altered conformation, such as Arg - 175 - His (R175H), Tyr - 220 - Cys (Y220C), Gly - 245 - Ser (G245S) and Arg - 249 - Ser (R249S). This group of mutants has replaced amino acid that is important for stability of protein structure [20]. This type of mutation can have different influence on protein activity. Some substitutions of amino acid that causes changes in local conformation might not affect its binding properties, but mutation of amino acid that interacts with zinc ion or forms β -sandwich, could cause destabilization of the entire central domain [21].

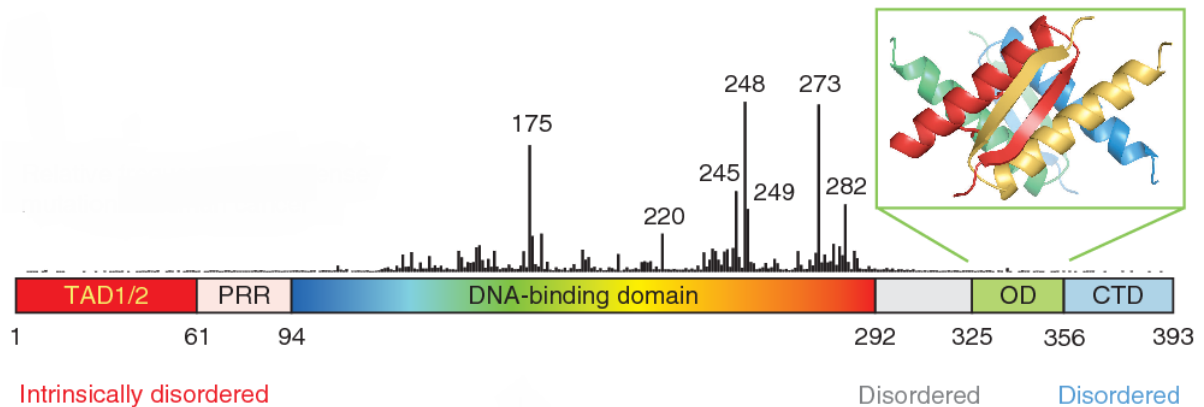


Figure 5 : Relative frequency of missense mutation in human cancer [7].

2.7 Binding of p53 Protein to DNA

The p53 protein binds sequence-specifically to a DNA consensus sequence (consisting of two consecutive half-sites, with each half-site being formed by two head-to-head quarter-sites) is a tetramer. One p53 tetramer can binds to four DNA quarter-sites through each p53 subunit. The p53 ability of sequence-specifically binding to DNA is essential for its tumor suppressive activity [22].

2.7.1 P53 Protein Binding to DNA

Central (DBD) is responsible for specific binding of p53 to DNA. DBD recognizes consensus sequence. This small region of DNA is also called response elements (RE). Consensus sequence has two repeating ten bases long motifs 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0 – 13 bp [23]. In the real conditions p53 could also binds to sequences with one and more mutations of consensus sequence. P53 is capable to bind to sites in the genome consisting of one and a half decameric sequential motif or even in sites consisting of only one decameric sequential motif [23].

C-terminal domain is known for its non-specific binding to G-quadruplexes, triplexes or single chain breaks [25]. In the cases of specific sequence binding C-terminal domain plays role as a negative regulator by blocking DNA-binding domain. For example, in the *p21* regulation, C-terminal domain acts as an inhibitor. This effect was also studied during protein binding to the 66 bp linear DNA consisting consensus sequence, but in the case of circular DNA, protein activity increased due to C-terminal domain presence [26]. P53 preferentially binds to superhelical DNA than linear DNA. Comparing affinity of the p53 to the different

sequences derived from binding sites of gene promoters, p53 has higher affinity for sequences that encodes the cell cycle arrest, while affinity for sequences from genes associated with apoptosis is lower [27]. Non-specific interaction was also detected between p53 and G-quadruplex that consists out of tandem repeats in the telomeric DNA regions. Experiments using oligonucleotides showed that binding affinity between p53 protein and telomeric G-quadruplex increases with growing length of oligonucleotides [28].

2.7.2 Binding of Mutant p53 Protein

P53 mutants often lost their sequence specific binding properties and therefore also ability to target genes typical for wild-type protein. Nevertheless, mutated p53 is sometimes able to regulate different set of genes that are could be responsible for oncogenesis in cells. However, the specific consensus sequence for p53 mutant proteins were not detected [29].

So called “dominant negative effect” was observed in the activity of some mutant p53 proteins. It has been shown that mutated p53 suppress wild-type expression and function. Mutant p53 can form complex with wild-type protein, through heterodimerization, which may inhibit function of wild-type p53 through conformational changes or inhibiting the DNA-binding activity of wild-type p53 to target genes. Tumor suppressor mutations are recessive, therefore mutation of both alleles of tumor suppressor is usually required for carcinogenesis, while dominant negative effect is caused by mutants with only one mutated allele. This fact increases significance of dominant negative effect of mutated p53 [30]. Several experiments *in vivo* proved that carcinogenesis is induced even in the presence of wild-type p53 protein [31].

Mutant forms of p53 may adopt so called “gain-of-function” (GOF) activity and thus influence different signaling pathways. GOF activity of mutant p53 is dependent on the protein-protein interactions with transcription factors (TFs) and chromatin complexes and thus affecting target gene expression. Several studies have found out ability of TF or proteins to bind multiple mutant p53 missense proteins that leads to suggestion, that mutant p53 may influence converge on partnering proteins. Despite the site of p53 missense mutation, core set of effector genes, regulated by commonly bound TFs, may affect mutant p53 GOV activity [32].

It has been also shown that p53 protein with mutation on residue G245S and R273H specifically binds to sequences on the genomic DNA containing non-coding repetitions [33], [34]. In the case of protein, containing mutation at the 115 residues (H115N mutation), increasing specific and non-specific binding activity to dsDNA was detected, comparing to wild-type p53 [35].

2.8 Local Secondary DNA Structures

DNA structure was discovered by Watson J. and Crick F. in the 1953. DNA is a molecule made up out of two polynucleotide chains, coiled around each other and formed in a double helix (B-DNA). Genetic information that carried in the DNA is coded by four bases: cytosine (C), adenine (A), thymine (T) and guanine (G). Adenine pairs with thymine by two hydrogen bonds and guanine pairs with cytosine by three hydrogen bonds (Figure 6) [36].

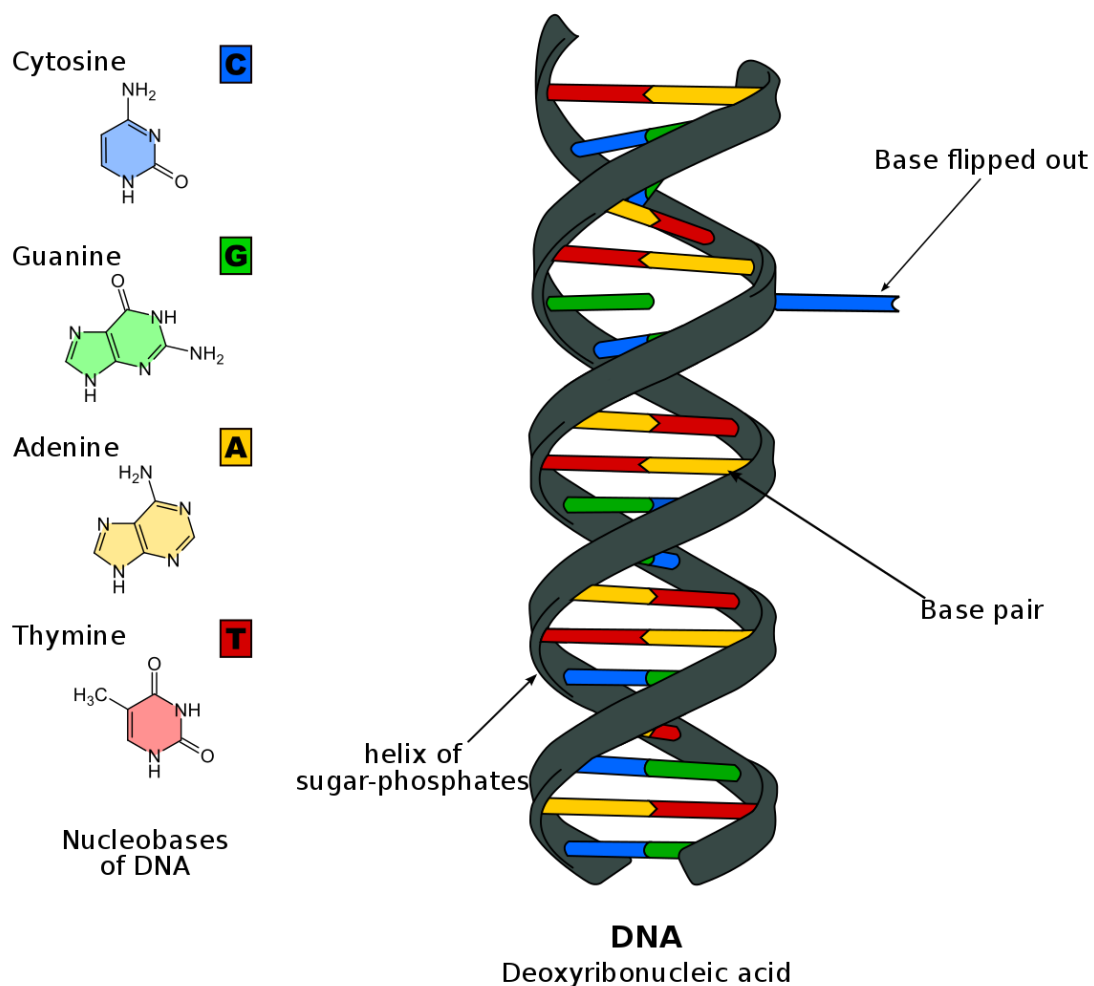


Figure 6 : Double-stranded DNA described by Watson J. and Crick F [36].

The main function of p53 protein is mediated by binding to DNA and recognizing its target sites. Structure of DNA plays important role in interactions with not only standard p53 protein, but also with mutated forms of p53 protein. Due to different mutation in DBD, mutant p53 lost its ability of sequence specific binding, suggesting that, mutant p53 need to interact with DNA mainly depending on structural recognition conformation of nucleic acids [37]. According to that, occurrence of alternative structures in the DNA increases ability of mutant p53 to specific binding [38].

Watson and Crick model of B-DNA is not the only one possible DNA secondary structure. Many other structures, sometimes called “non-canonical structures”, such as a triplex, quadruplex, i-motif, hairpin or cruciform can be formed and presented in genomes [39]. It was discovered that combination of local DNA structures is more energetically favorable than separated nitrogenous bases [40]. Local DNA structures are formed by rearrangement of the double stranded polynucleotide chains with specific base sequences as a result of secondary structures [41]. Local structures may impact some biological processes, such as its ability to regulate gene expression or cause instability in gene expression [42].

2.8.1 G-quadruplex

DNA G-quadruplex is non-canonical secondary structure formed in sequence containing consecutive runs of guanine. G-quartet is four-stranded structure, where stabilization is

provided by stacked guanine tetrads which are cyclically bonded to each other via eight hydrogen bonds according to Hoogsteen base pairing. The tetrads increase their stability of negative charged channel via univalent cation, usually using K^+ or Na^+ cation (Figure 7) [43].

Due to G-quadruplex high stability, structural versatility and functional diversity, it was used as building blocks and functional elements in synthetic biology. G-quadruplexes are responsible for different biological roles such as regulation of transcription, translation, DNA replication and RNA localization [44].

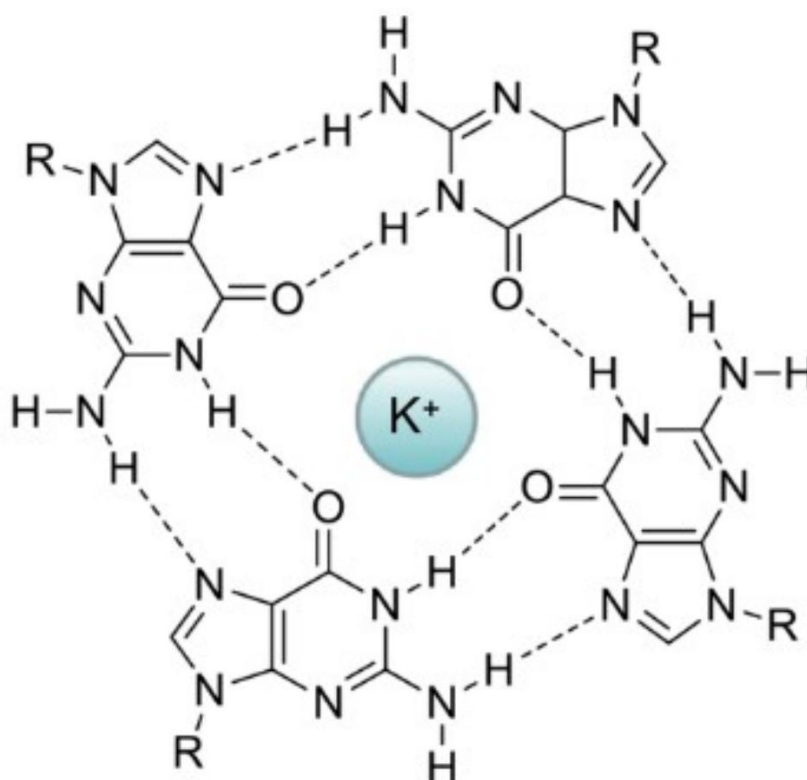


Figure 7 : G-quartet. Four guanines construct a G-quartet via Hoogsteen hydrogen bonds. Univalent metal cations (K^+ or Na^+) located in the central channel of the G-quart to stabilize the structure [45].

G-tetrads stacking up on top of one another are giving a rise to a G-quadruplex. Due to number of DNA molecules G-quadruplexes are divided into two groups, intramolecular and intermolecular. G-quadruplex formed from only one strand is called intramolecular, and G-quadruplex that is formed out of more than one strand is termed intermolecular. Intermolecular G-quadruplexes can be divided according to the number of strands into bimolecular (dimeric), trimolecular (trimeric) or tetramolecular (tetrameric). Depending on the strand, G-quadruplexes could be homomultimeric, with identical strands, or heteromultimeric, with nonidentical strands [44].

Intermolecular G-quadruplexes are under highest research interest, due to its potential to form single-stranded telomeres and oncogenic promoters' regions. Intramolecular G-

quadruplexes have better ability of forming in the physiological conditions than intermolecular. They are also found to be DNA sequence specific, exhibiting great conformation and capping structures [46].

G-quadruplexes are also divided into different isoforms whether the strands are oriented parallel to each other, anti-parallel or have hybrid form (Figure 8). Factors, that could affect G-quadruplex conformation, include number of bonded guanines, the nucleotide sequence between tracks of Gs, type of incorporated cation or ion concentration [47]. For intramolecular G-quadruplexes with anti-parallel orientation is typical non-parallel orientation of guanine sections to each other, on the other hand, for parallel orientation of G-quadruplexes all of guanine sites are parallel [48].

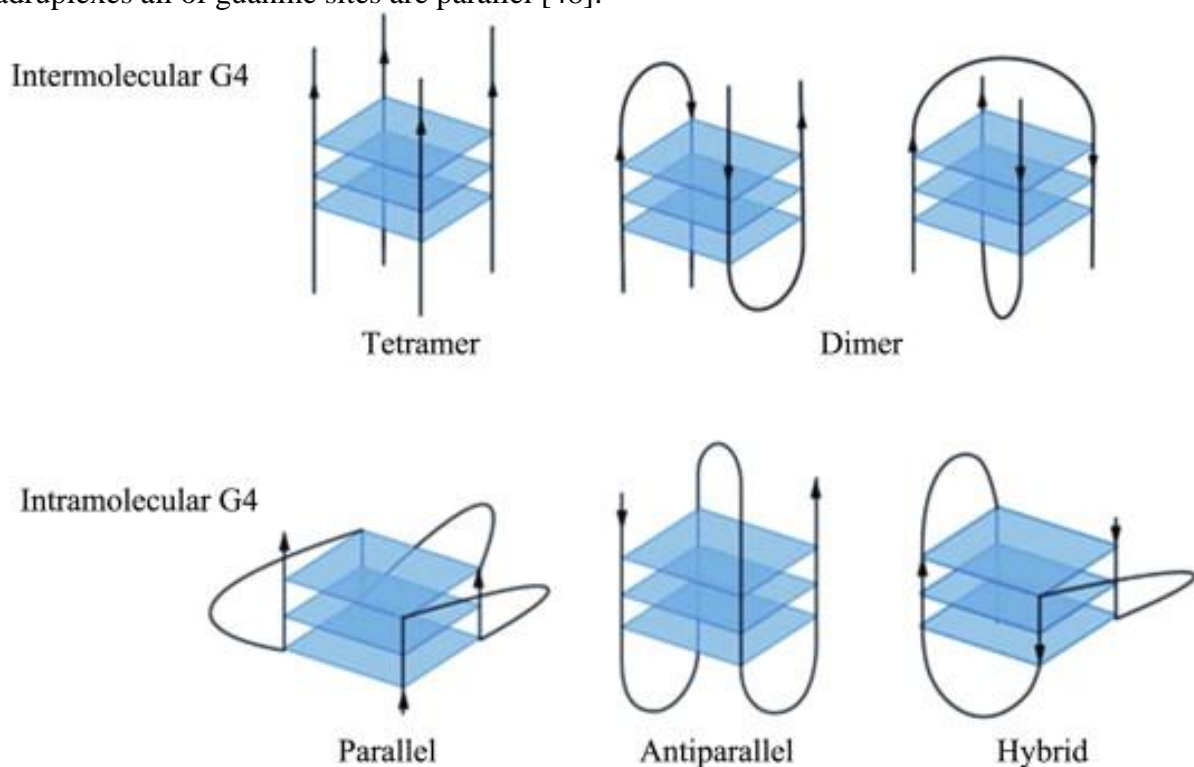


Figure 8 : Various types of intramolecular and intermolecular G-quadruplexes and its orientation [49].

G-quadruplexes were detected *in vivo* at human chromosomal ends by G-quadruplex-specific antibodies [50].

Because of ability of p53 to bind to single stranded DNA, p53 protein has high affinity to interact with single stranded overhangs of telomeric DNA and is probably responsible for stability of chromosomal ends [51]. Almost 40% of genes have G-quadruplexes in their promoter sequences that indicate that G-quadruplexes may regulate gene expression at the transcriptional level [52].

2.8.2 Cruciform

Cruciform structures could arise from sequences with inverted repeats of six or more nucleotides. The composition of the repeats is important for example due to the higher stability of the cytosine-guanine pair. Due to the low stability of A-T pairs, sequences like ATA and TAT are mostly located in the central parts of the sequences, where pair splits and

form “central bubble” of future cruciform [53]. Then free bases start to pair between the fibers, closing of “bubble” leads to formation of the cruciform structure (Figure 9) [54].

The cruciform could be divided into two classes. First class of cruciform, unfolded, has square conformation with a 4-fold symmetry, where adjacent arms are nearly perpendicular to each other. The second class, folded conformation, has disposal of adjacent arms from an acute angle with the main DNA strands [55].

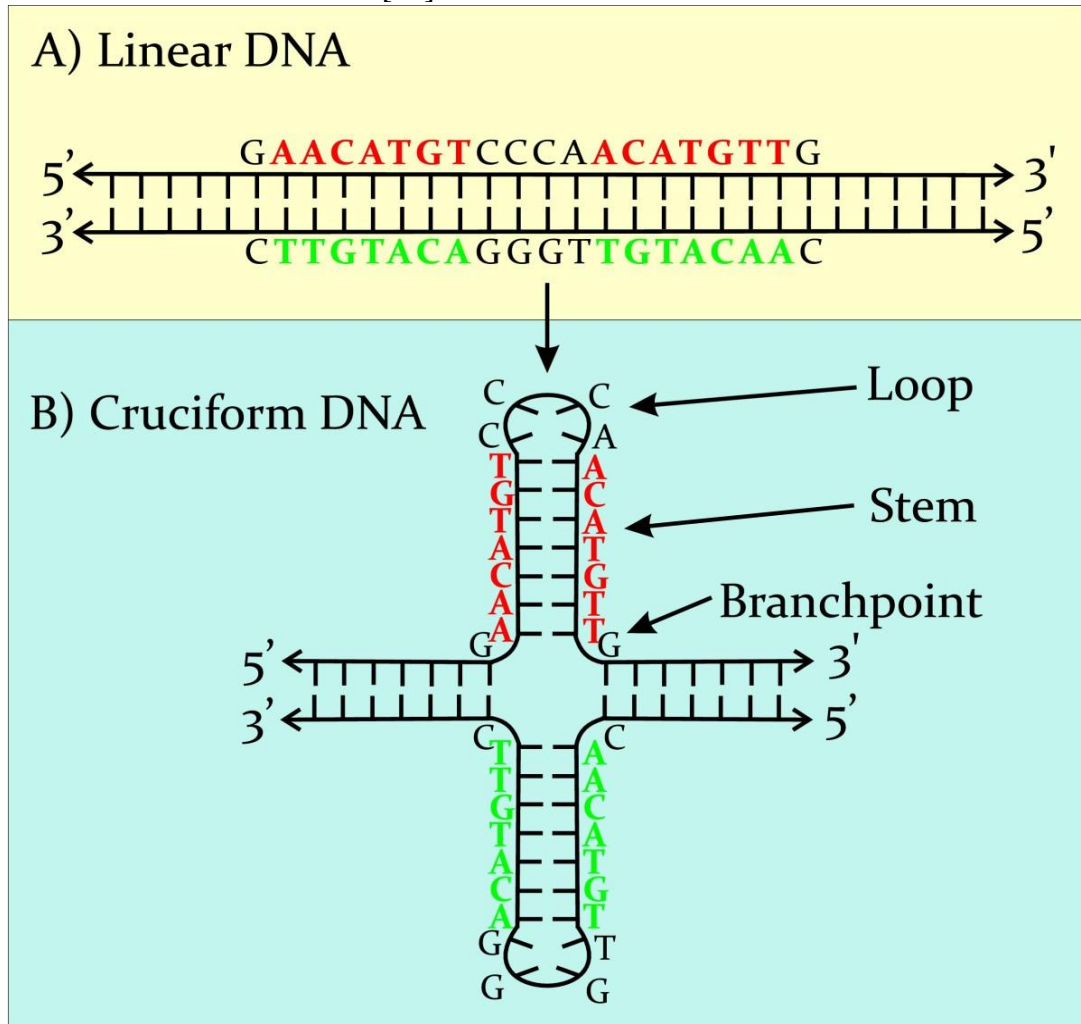


Figure 9 : Changes associated with transition from the linear to cruciform state in the p53 target sequence from the p21 promoter. The promoter sequence contains a 20 bp p53 target sequence with 7 bp long inverted repeat (red), (A) as linear DNA and (B) as an inverted repeat as a cruciform structure. In the cruciform structure, the p53 target sequence is presented as stems and loops [53].

Cruciform DNA was found in both prokaryotes and eukaryotes and has a role in the transcription of DNA, double strand repair, DNA translocation and recombination. Those structures are likely to be associated with the development of some diseases, including cancer, as they may interact with regulatory proteins like p53, PERP-1 or BRCA proteins [53].

2.9 Gateway Cloning System

Gateway is commercial cloning system based on recombination between two homologous sequences at specific attachment sites (*att*) using enzyme from bacteriophage λ . The phage λ encoded protein Int (Integrase) that allows phage incorporation into the *E. coli* chromosome

and switching between the lytic and lysogenic cycles. The main principle of this method is a reversible recombinant reaction, therefore some steps of traditional cloning, such as usage of restriction enzyme and PCR purifying after ligation, are no longer required. The advantage of this method is that once the desired gene has been inserted into the Gateway vector, it can be transferred to many other expression vectors, which increases the flexibility of the method. Moreover, this method is simple, fast, carried out at room temperature and has high efficiency (approx. 99%).

The Gateway cloning technology is based on two reactions: BP reaction and LR reaction [56].

2.9.1 BR Reaction

BP reaction is the first step of the Gateway cloning technique. During BP reaction PCR product that is bounded by two *attB1* and *attB2* sites is transferred into donor vector with *attP1* and *attP2* sites (Figure 10). Advantage of the BP reaction is that *attB1* only reacts with *attP1* resulting *attL1* and *attR1* sites, the same reaction is between *attB2* and *attP2* resulting *attL2* and *attR2*. As a result, entry clone with wanted DNA sequences and *attL* sites is formed. Donor vector that is used in this technique carries the *ccdB* gene that is responsible for preventing the survival of the cells carrying the empty vector. Product of the *ccdB* gene is toxic for *E. coli*, it interacts with the bacterial enzyme gyrase that is responsible for proper DNA winding during replication. Due to DNA breaks replication becomes impossible. BP reaction is driven by enzymatic mix BP Clonase, containing *Int* and IHF (*E. coli* protein Integration Host Factor). The donor vector itself contains kanamycin resistance gene. Between *att* sites not only *ccdB* toxin sequence is located, but also the chloramphenicol resistance gene, which resulting entry clone lacks. Resistance only to kanamycin is used for more precise clone selection [56], [57].

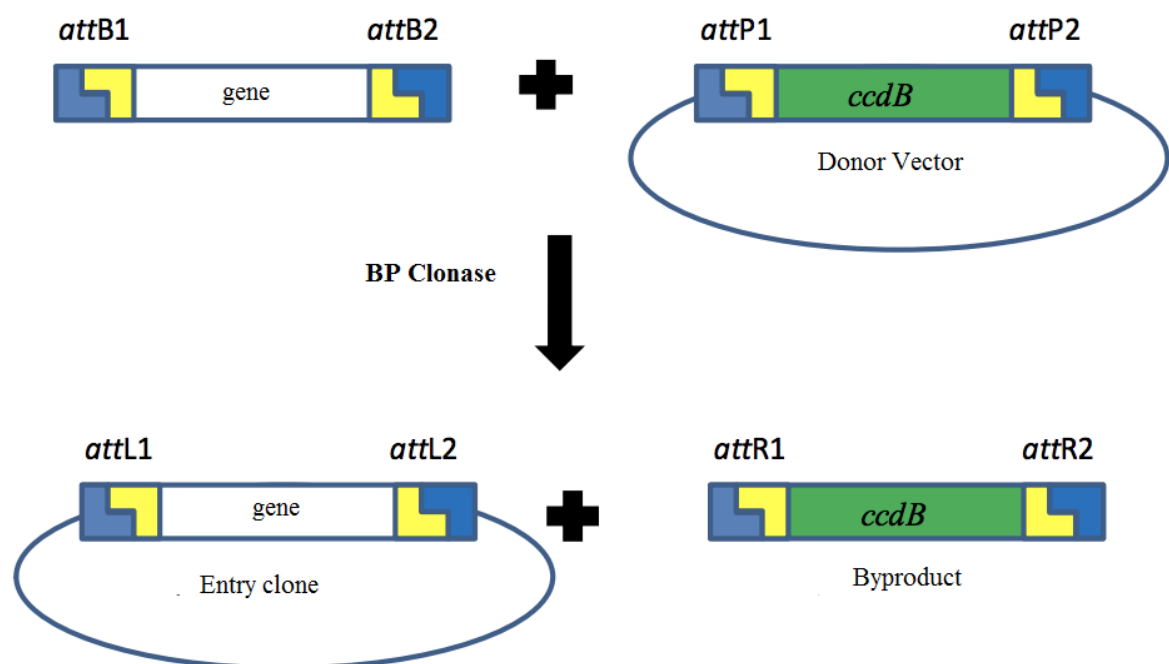


Figure 10 : Cloning the gene of interest into an entry vector using the BP reaction [58].

2.9.2 LR Reaction

LR reaction is second step in the Gateway cloning system. Selection of destination vector depends on the type of expression system or the type of follow-up procedure. During LR reaction the gene of interest that is captured in the entry clone is subcloned into destination vector with the help of LR clonase mix, containing *Int*, IHF and *Xis* (Excisionase). Donor vector, which contains toxic gene *ccdB*, gene of resistance to chloramphenicol and ampicillin, interacts with its *attL1* and *attL2* sites with *attR1* and *attR2* sites of the entry clone and forms expression vector (Figure 11). *CcdB* gene is excised from destination vector and provides selection of empty clones, as a toxic byproduct, similar like it was during BP reaction [56].

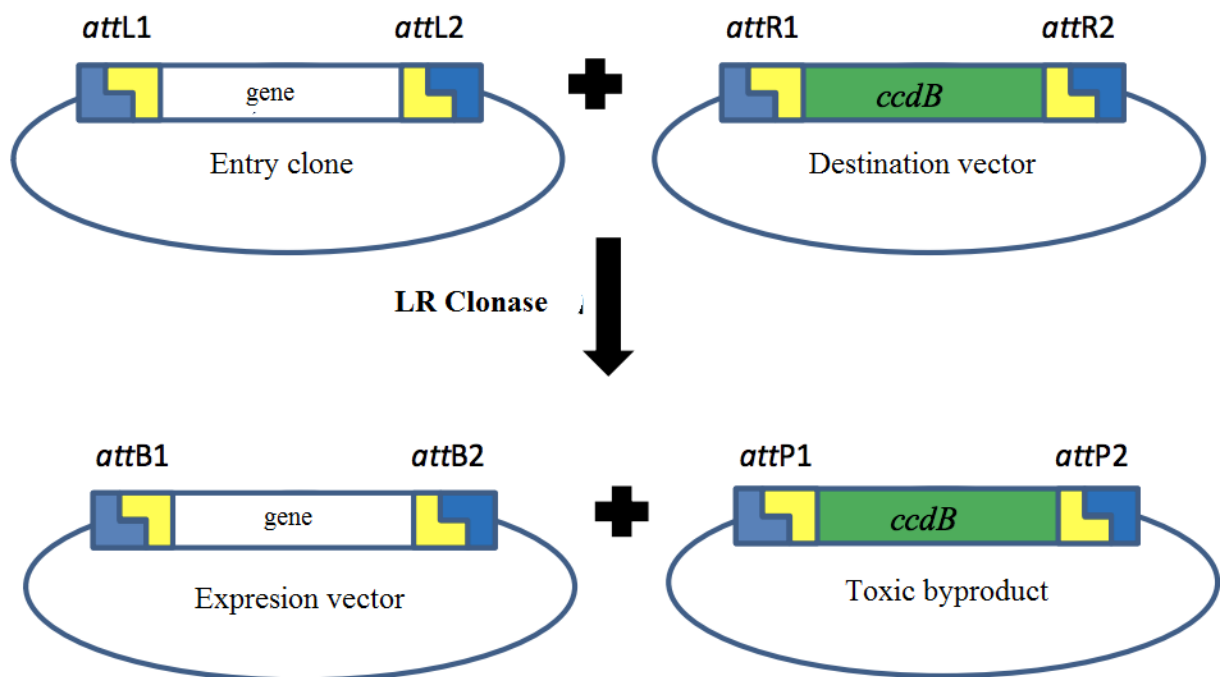


Figure 11 : Subcloning the gene of interest from the entry clone into a destination vector using the LR Reaction producing the expression clone [58].

3 AIMS OF THE THESIS

- Literary research on the topic
- Isolation of plasmids
- Preparation of competent cells
- Transformation
- Cultivation of bacterial cultures
- Isolation and purification of proteins
- Study of protein–DNA interaction

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Enzymes

Gateway LR Clonase II Enzyme Mix	Thermo Fischer Scientific
Proteinase K (2 µg/µl)	Thermo Fischer Scientific
<i>PvuII</i> -HF™	New England BioLabs

4.1.2 Primers

T7 forward primer (100µM)
T7 reverse primer (100µM)

4.1.3 Competent cells

BL21 CodonPlus Competent cells, <i>E. coli</i>	Agilent Technologies
One Shot STBL3 Chemically Competent <i>E. coli</i>	Thermo Fischer Scientific

4.1.4 Plasmids

Gateway pDONR221 Vector	Thermo Fischer Scientific
Gateway pDEST17 Vector	Thermo Fischer Scientific
pENTR-gus positive control	Thermo Fischer Scientific

4.1.5 Other materials

DNA Ladder 1 kb	New England Biolabs
DNA Ladder 100 bp	New England Biolabs
TALON Metal Affinity Resins	Clontech Laboratories
ECL Western Blot detection kit	GE Healthcare
Nucleic Acid Gel Stain GelRed, 10,000x	Biotium
Spin Plasmid mini kit NoLid® Nucleo	Macherey-Nagel
Isolate II Plasmid Midi Kit	Bioline
Column Econo-Pac Chromatography Columns 10 ml	Bio-Rad
Precision Plus Protein Standards Dual Color 10–250 kDa	Bio-Rad
Antibody Anti-polyHistidine (mouse), at a ration 1:1 000	Sigma-Aldrich
Antibody Anti-mouse IgG peroxidase, at a ration 1:10 000	Sigma-Aldrich
QIAquick® Nucleotide Removal Kit	Quiagen
Protease Inhibitor Cocktail cOmplete™	Sigma-Aldrich

4.1.6 Chemicals

Agar	Oxoid
Agarose	Serva
Acrylamide	AppliChem
Ampicilin (100 mg/ml)	BB Pharm
APS	Sigma-Aldrich

Bradford reagent	Serva
Butanol	Penta
CaCl ₂	Sigma-Aldrich
DTT	Duchefa Biochemie
EDTA	Lachema
Ethanol	Penta
Glucose	Sigma-Aldrich
Glycerol	Serva
Glycine	Serva
Chloramphenicol (34 mg/ml)	Duchefa Biochemie
Imidazole	Sigma-Aldrich
IPTG	Sigma-Aldrich
KCl	Sigma-Aldrich
KH ₂ PO ₄	Duchefa Biochemie
Yeast Extract	Duchefa Biochemie
Boric Acid	Penta
Acetic Acid	Penta
Methanol	Penta
MgCl ₂	Sigma-Aldrich
MgSO ₄ .7H ₂ O	Lach-Ner
NaCl	Penta
PONCEU	Sigma-Aldrich
Sucrose	Serva
SDS	Serva
Skimmed milk powder	Laktino
TEMED	Sigma-Aldrich
Tris	Duchefa Biochemie
Trypton	Duchefa Biochemie
ZnSO ₄	Sigma-Aldrich
B-mercaptoethanol	Merck

4.1.7 List of used instruments, apparatus and aids

Amersham 680	GE Healthcare
Microjet Personal microwave autoclave	Enbio
Autoclave	Nuve
Biological thermostat BT-12M	Lab. instruments Prague
Centrifuge 5804R	Eppendorf
Centrifuge Avanti J-30	Beckman
Centrifuge Mini Spin plus	Eppendorf
Detection system ILU-1	Herolab
Detection system LAS 3000	FUJIFILM
Electrophoresis equipment Mini PROTEAN Tetra Cell system	Bio-Rad

Electrophoresis equipment Mini-Sub Cell GT	Bio-Rad
ELISA reader Synergy H1 Hybrid Multi-mode reader	BioTek
Incubator Innova 44	New Brunswick Scientific
Pellet mixer	VWR
Microwave oven SMW3717	Sencor
Programmable Rotator Multi RS-60	Biosan
Sonicator	Dynatech
Spectrophotometer NanoDrop ND-1000	Termo Scientific
Spectrophotometer Specord 200 Plus	Analyst Jena
Thermoblock QBT2	Grant Instruments
Thermoblock Thermomixer comfort 1.5 ml	Eppendorf
Transilluminator UVT-28MP	Herolab
Orbital shaker incubator ES-20	Biosan shaker
Scales TE 412	Sartorius
UV Water Purification System	Synergy
Vortex Genie-2	Scientific Industries
Vortex IKA 4 digital	IKA
Power supply Pac 300	Bio-Rad Power
Power supply Pac 1000	Bio-Rad Power
Power supply Pac Basic	Bio-Rad Power

4.1.8 Preparation of solutions, buffers, media

Acrylamide for SDS-PAGE, 5%, 100 ml	12,5 ml 40% acrylamide (acrylamide: bisacrylamide 19:1); 12,5 ml 1M Tris (pH 6,8); 0,5 ml 20% SDS; filled up with Mili-Q H ₂ O to 100 ml
Acrylamide for SDS-PAGE, 10%, 300 ml	75 ml 40% acrylamide (acrylamide: bisacrylamide 19:1); 112 ml 1M Tris (pH 8,8); 1,5 ml 20% SDS; filled up with Mili-Q H ₂ O to 300 ml
Blotting buffer, 10x 1 l	30,3 g Tris; 145 g glycine; supplemented with Mili-Q H ₂ O up to 1l Working solution = 1 part buffer; 1 part methanol; 8 parts of Mili-Q H ₂ O
Elution buffer	500mM Imidazole; 20 mM Tris (pH 7,6); 500mM NaCl; 1 mM β-mercaptoethanol; 5% glycerol; filled up with Mili-Q H ₂ O
LB medium	1% (w/v) tryptone; 1% (w/v) NaCl; 0,5% (w/v) yeast extract; filled up with Mili-Q H ₂ O Solid LB = LB medium + 1,4% agar;
Lysis buffer	5mM Imidazole; 20 mM Tris (pH 7,6); 500mM NaCl; 0,1% Triton X-100; 1mM β-mercaptoethanol; filled up with Mili-Q H ₂ O; pH 8

Milk, 5%, 1 l	5 g skimmed milk; 100 ml 10x PBS; filled up with Mili-Q H ₂ O to 1 l
CSB application buffer, 5x	0,25M Tris; 8% SDS; 40% glycerol; 8% β-mercaptoethanol; bromophenol blue; filled up with Mili-Q H ₂ O
LB loading buffer, 6x	40% sucrose; 0,2% bromophenol blue; 0,2% xylencyan violet; filled up with Mili-Q H ₂ O
Native acrylamide 16%, 40 ml	16 ml 40% acrylamide (acrylamide: bisacrylamide 19:1); 2 ml 10x TBE; filled up with Mili-Q H ₂ O to 40 ml
Native acrylamide 4%, 100 ml	10 ml 40% acrylamide (acrylamide: bisacrylamide 19:1); 5 ml 10x TBE; filled up with Mili-Q H ₂ O to 100 ml
PBS, 10x 1 l	80 g NaCl; 2 g KCl; 14.4 g Na ₂ HPO ₄ ; 2,4 g KH ₂ PO ₄ ; filled up with Mili-Q H ₂ O to 1 l
Wash buffer	10mM Imidazole; 20 mM Tris (pH 7,6); 500mM NaCl; 1 mM β-mercaptoethanol; 5% glycerol; filled up with Mili-Q H ₂ O
SDS-PAGE buffer 10x	30,3 g Tris; 144.2 g glycine; 10 g SDS; filled up with Mili-Q H ₂ O
SOC media	2% tryptone; 0,5% yeast extract; 1 ml 1M NaCl; 2,5 ml 1M KCl; 1 ml 1mM MgSO ₄ ; 2 ml 1M glucose; 1 ml 1M MgCl ₂ ; filled up with Mili-Q H ₂ O
TAE buffer, 50x, 1 l	242 g Tris; 18,6 g EDTA; 57,1 ml acetic acid; filled up with Mili-Q H ₂ O to 1 l; filtered through a 0,22 μm filter
TBE buffer, 10x, 1 l	108 g Tris; 40 ml 0.5M EDTA (pH 8,0); 55 g boric acid; filled up with Mili-Q H ₂ O to 1 l; filtered through a 0,22 μm filter
TE buffer, 1x	10 mM Tris-HCl; 1 mM EDTA (pH 8,0); filled up with Mili-Q H ₂ O
Binding buffer (used for protein isolation)	5mM Imidazole; 20 mM Tris (pH 7,6); 500mM NaCl; 1 mM β-mercaptoethanol; 5% glycerol; filled up with Mili-Q H ₂ O
Binding buffer, 20x	50mM KCl; 5 mM Tris (pH 7,6); 0.5mM EDTA; 0,01% Triton; filled up with Mili-Q H ₂ O

4.2 Methods

4.2.1 Gateway Cloning

BP Reaction

In the BP reaction interested gene is amplified with help of an *attB* tagged primer pair. PCR product, including *attB* sites, combines with donor vector, including *attP* sites, forming an entry clone with *attL* sites. For BP reaction as a donor vector was used pDONR221 (Figure 12). Interested PCR fragment inserted into donor place and formed entry clone of interest. During BR reaction, from *attP* sites of the donor vector was cleaved sequence that encodes a toxic *ccdB* product. In the Gateway cloning system, this toxic product provides negative selection – cells that contain successfully recombined gene are going to survive unlike cells without recombination. This property works with antibiotic resistance as negative selection [59].

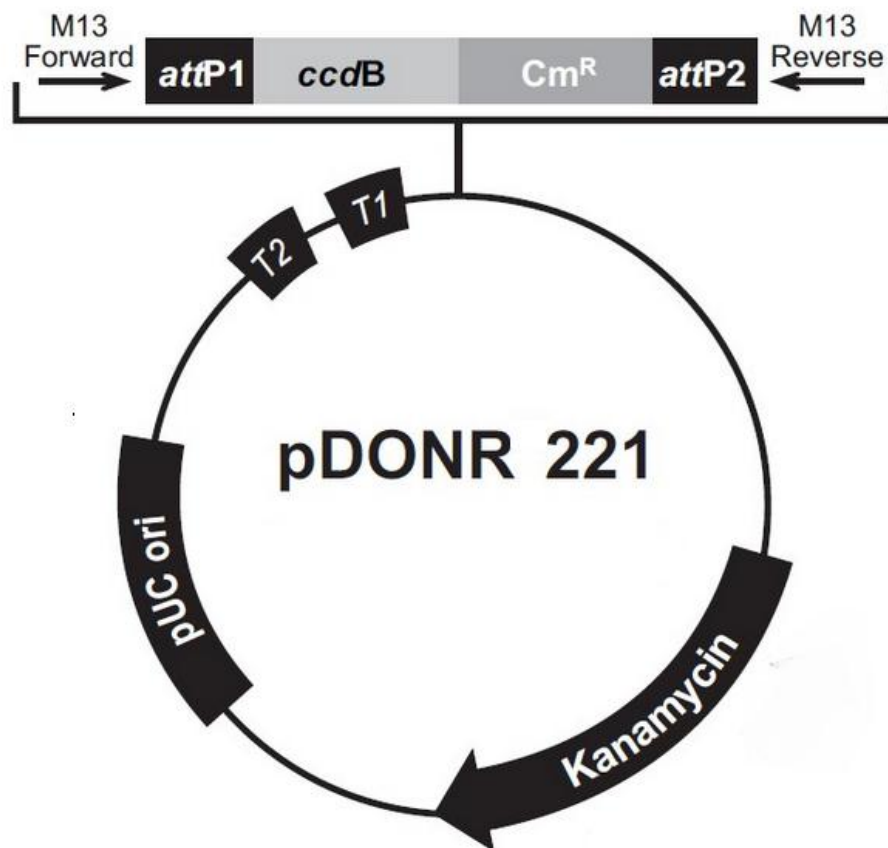


Figure 12 : Map of used donor vector pDONR221, with showed *attP* sites.

Entry clones after BP reactions were donated by Thorstena Stiewa from Marburg, Germany for our experiments (Table 1:) [60].

Table 1: Used entry clones

Full name of entry clone	Short name of entry clone	Concentration [ng/μl]
pENTRD-p53-S183AS185A	A	225,25
pENTR-p53RR-A344A	B	272,04
pENTR-p53EE-L344A	C	249,44
pENTRD-p53-S183DS185D	D	305,16

4.2.1.1 LR Reaction

LR reaction is the second step of Gateway cloning system. Prepared in the BP reaction entry clone interacts with destination vector. Interested gene is transferred from entry clone and inserted to destination vector by LR clonase, reaction is going between *attL* sites of entry clone and *attR* sites in destination vector. After this step two molecules are prepared, expression clone, containing the DNA segment of interest and the other molecule is by-product. Prepared expression clone has wanted DNA segment inserted instead of *ccdB* gen, so the cells are going to survive. For this step as a destination vector was used pDEST17 (Figure 13) due to presence of 6xHisTag sequence in it, that is important during affinity protein isolation and their western transfer.

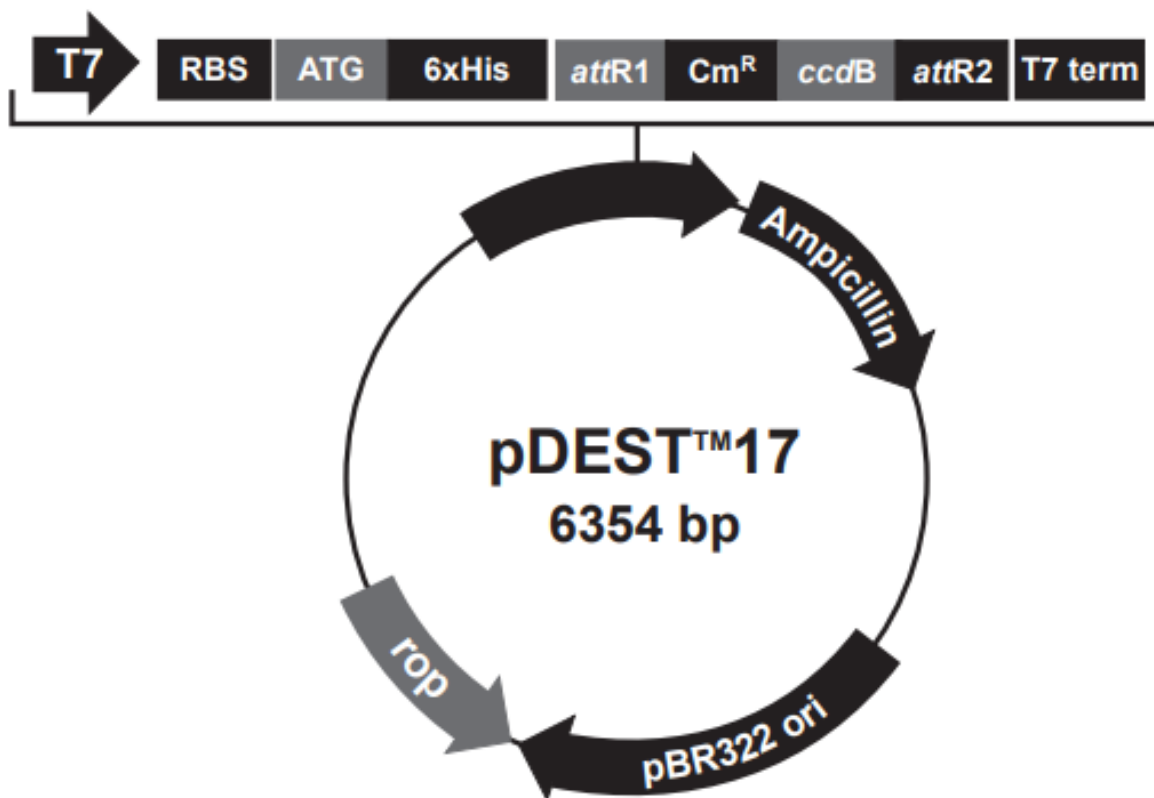


Figure 13 : Map of used destination vector pDEST171, with showed attR sites.

For LR reaction 150 ng of entry reacted with 300 ng of destination vector. Entry clone, destination vector, TE buffer were mixed to the total volume of 8 μ l. Last 2 μ l of LR clonase were added to the samples. Samples were left overnight at 25°C. For positive control 100 ng of commercial plasmid pENTR-gus were added instead of entry clone.

Next day 1 μ l of proteinase K were added to all samples and samples were incubated for 10 minutes at 37°C.

4.2.2 Transformation

4.2.2.1 Preparation of Competent Cells

First, 10 ml of bacterial culture were cultivated in liquid LB media overnight at 37°C. Next, 1 ml of culture was transferred to 50 ml of LB media. Culture was then incubated at 37°C at 120 rpm till the absorbance at 595 nm reached 0,5-0,7. After incubation culture was transfer to 50 ml pre-cooled tubes and left on ice for 10 minutes. Then tubes were centrifuge 6000x g for 10 minutes at 4°C. Supernatant was removed and discarded and the cells pellets were resuspended in 10 ml of pre-cooled 0,1M CaCl₂. Solution was incubated on ice for 25 minutes. After incubation tubes were centrifuge at 6000x g for 10 minutes at 4°C. Supernatant was removed and discard. The cells pellet was resuspended in 1 ml of 0,1M CaCl₂. To the cells were added 0,2 of 70% sterile glycerol. After gentle mixing, solution was pipetted into 1,5ml tubes and frozen with dry ice. The cells were stored at -80°C.

4.2.2.2 Transformation

After LB reaction plasmids were transformed to *STBL3* competent cells. Cell culture was thawed on ice, 50 µl of competent cells were mixed with 8 µl of each plasmid after LR reaction. Culture was incubated on ice for 30 minutes. After incubation, samples were transferred to the thermostat for 40 seconds at 42°C. Then samples were transferred on ice and incubated for 2 minutes. Next 1 ml of SOC media pre-heated to 37°C were added to all samples. Cell culture was incubated for 1 hour at 37°C at 250 rpm. After incubation tubes were centrifuge at 1000x g for 2 minutes and part of supernatant was discarded and removed. Pellet was resuspended in the small amount of SOC media and spreaded onto the petri dish with LB agar with AMP (100 µg/ml). The petri dishes were incubated overnight at 37°C.

The same steps were applied for transformation for protein production. For protein production used BL21 CodonPlus competent cells and 10-100 ng of plasmid DNA were. LB agar contained ampicilin (AMP) (100 µg/ml) and chloramphenicol (CAM) (34 µg/ml).

Next day colonies were grown on new petri dish because of appropriate antibiotic (AMP for *STBL3* and AMP and CAM for *BL21*) selection. Using the streak-plate technique and incubated for 18 hours at 37°C.

4.2.3 DNA Plasmid Isolation

Always one pure colony from streak-plate was inoculated to 5 ml of LB media with AMP (100 ng/ml) and incubated for 12 hours at 37°C at 120 rpm. The next day 500 µl of grown culture were collected into 1,5 ml tube, mixed with 713 µl of 70% glycerol, freezeed with dry ice and stored at -80°C.

The rest of the grown culture was centrifuge at 11 000x g for 60 seconds. Plasmid DNA was isolated with NoLid® Nucleo Spin Plasmid kit.

Protocol:

- 5 ml of the grown culture were centrifuge at 11 000x g for 30 seconds. Supernatant was discarded and removed.
- Pellet were resuspended in 250 µl of Buffer A1.
- To the solution were added 250 µl of Buffer A2. Solution was gently mixed by inverting the tube 6-8 times. Solution was incubated for 5 minutes at room temperature.
- Then to solution were added 300 µl of Buffer A3 and solution was gently mixed by inverting the tube 6-8 times.
- Solution was centrifuged at 11 000 x g for 5 minutes at room temperature.
- NucleoSpin® Plasmid Column was placed in a 2 ml collection tube. 750 µl of supernatant were pipetted onto the column and tube was centrifuge at 11 000 x g for 1 minute. Supernatant was discarded and removed.
- 600 µl of Buffer A4 were pipetted onto the column, tube was centrifuge at 11 000 x g for 1 minute. Supernatant was discarded and removed.
- To dry silica membrane tube was centrifuged at 11 000 x g for 2 minutes.
- After centrifugation column was transferred to 1,5 ml tube and 50 µl of Buffer AE were pipetted onto the column. The column was incubated for 1 minute at room temperature. After incubation tube was centrifuged at 11 000 x g for 1 minute.
- Concentration and purity of isolated DNA was measured at NanoDrop 2000. DNA was also controlled on horizontal electrophoresis on 1% agar gel.

4.2.4 Sequencing

For sequencing 5 µl 80-100 ng/µl DNA were mixed with 5 µl 5µM primer. For expression clones T7 forward and T7 reverse primers were used. Samples were sanded to GATC Biotech for sequencing. Sanger sequencing was applied.

4.2.5 DNA Restriction Digestion

DNA for EMSA was digested with PVUII-HF™ restriction enzyme. Amount of PVUII-HF™ was calculated due to concentration of DNA that going to be digested (for 1 µg of DNA were used 2 U of enzyme). Solution of enzyme and DNA was mixed and incubated for 20 minutes at 37°C.

After digestion DNA was purified with QIAquick® Nucleotide Removal Kit.

Protocol:

- Samples were mixed with 5 volumes of Buffer PNI to 1 volume of DNA.
- Samples were applied onto QIAquick spin column and centrifuged at 6000 x g for 1 minute. The flow-through was discarded and removed.
- 750 µl of Buffer PE were applied on to the column and centrifuge at 3800 x g for 1 minute. The flow-through was discarded and removed.
- Tube was centrifuge at 13 900 x g for 1 minute to dry the column. The flow-through was discarded and removed.
- QIAquick spin column was transferred into the new 1,5 ml tube. 100 – 200 µl of Buffer EB were applied onto the column and tube was centrifuge at 13 900 x g for 1 minute.
- DNA was analyzed on a 1% agar gel. DNA concentration was measured at NanoDrop 2000.

4.2.6 Protein Isolation and Purification

4.2.6.1 Transformation to BL21 and Cell Cultivation

Expression plasmids of four mutant forms of p53 protein were isolated and transformed to BL21 competent cells for protein production.

Small amount of culture was inoculated to 25 ml LB liquid media containing AMP and CAM (100 mg/ml). Culture was incubated at 37° at 120 rpm overnight. The next day culture was transferred to 1 l of liquid LB media with AMP and CAM (100 µg/ml) and left at 37° at 120 rpm until $OD_{600} = 0,7-1$ was detected. After the desired optical density was detected, the solution of ZnSO₄ was added to the culture to the 50µM concentration. 1 ml of culture was taken as a control sample for immunoblot analysis. To the culture 1 ml of 1M IPTG was added to induct protein production in the cells. The culture was incubated at 16°C at 120 rpm overnight. The next day 1 ml of culture was taken as a second control sample. After that culture was centrifuged on Beckman Avanti J-30 at 6500x g for 20 minutes at 4°C, culture was collected at the bottom of the tube and placed on ice.

4.2.6.2 Cell Lysis

The pellet was resuspended in the 40 ml of lysis buffer containing protease inhibitor and left on ice for 20 minutes. After incubation solution was sonicated ten times for 30 seconds with 30 seconds pauses at 4°C. Solution was centrifuged at 14 000x g for 30 minutes at 4°C.

Isolated protein was part of the supernatant from which 200 µl was taken for immunoblot analysis.

4.2.6.3 Protein Isolation

The protein isolation of p53 was carried out using affinity chromatography method. 40 ml of binding buffer were added to the 4 ml of TALON Metal Affinity Resin. Solution was mixed and centrifuged at 2000x g at 4°C for 5 minutes. Supernatant was removed and discarded. Culture lysate was added to the resin suspension and agitated on a platform shaker for 1 hour at 4°C. After incubation suspension was centrifuged at 2000x g for 5 minutes at 4°C. Supernatant was removed and discarded and 200 µl of supernatant was taken for immunoblot analysis. 40 ml of binding buffer were added to the pellet and solution was centrifuged at 2000x g for 5 minutes at 4°C. After centrifugation 200 µl of supernatant were taken for immunoblot analysis. Supernatant was removed and discarded. 40 ml of washing buffer were added to the pellet, gently mixed and centrifuged at 2000x g for 5 minutes at 4°C. After centrifugation 200 µl of supernatant were taken for immunoblot analysis. The solution was transferred to Econo-Pac column. After all washing buffer drained through column and ring settled down, elution started. 1 ml of elution buffer was added to the column, fraction was collected into the tube and frozen with dry ice, these steps were repeated 5 times, therefore 5 fractions were prepared. All fractions were stored at -80°C.

4.2.7 Measuring of Protein Concentration

Bradford protein assay was used to determine protein concentration of fractions. Samples for Bradford protein assay were prepared by mixing 100 µl of Bradford reagent, 396 µl of water and 4 µl of protein fraction. A series of protein standards were prepared from BSA source solution in the range 0,1 mg/ml to 2 mg/ml. As a blank elution buffer was used.

200 µl of each standard and unknown sample solution was pipetted into micropallet wells in two repeats. The absorbance values of all the samples and controls were measured after five minutes incubation at room temperature at 595 nm by ELISA reader. Using program Gen5 all protein concentrations were calculated and exported to the Microsoft Excel.

4.2.8 Concentration of protein

Concentration of all mutant proteins was increased after isolation using Amicon Ultra 0.5 mL Centrifugal Filters.

Protocol:

- 500 µl of sample were pipetted into the filter and filter was transferred to 1,5 ml tube.
- Rotation time of centrifugation was chosen from the table, provided by manufacturer company based on the required protein concentration. Samples were centrifuged from 15 to 30 minutes at 14 000x g at 4°C.
- After centrifugation, filter was transferred to the new 1,5 ml collecting tube and centrifuged at 2000x g at 4°C for 3 minutes.
- Concentrated protein was stored at -80°C.

4.3 SDS-PAGE

A method for the analysis of proteins by an electrophoresis is the polyacrylamide gel-based separation method. On each gel control samples taken during protein isolation for immunoblot analysis, all fractions of protein, molecular weight marker and positive control of wild-type p53 were loaded.

Glass plates were cleaned with ethanol and assembled casting stand. To the 10 ml of 12% solution for running gel were added 100 μ l of 10% APS and 30 μ l of TEMED. Running gel was poured into plates leaving about 1 cm at the top. Gel was covered with 80% butanol to prevent dehydration and allow polymerizing. After approximately 10 minutes butanol was removed with filter paper. 3 ml of 5% solution for stacking gel were mixed with 30 μ l of 10% APS and 9 μ l of TEMED and poured over running gel. Running unit was assembled and covered with 1x RB buffer.

Protein fractions, positive control and control samples were prepared by mixing 3 μ l of protein with 0,75 μ l 5x CSB. Samples, taken before and after induction, were centrifuge at 6000x g for 2 minutes. Supernatant was separated and mixed with 20 μ l 5x CSB and 80 μ l Mili-Q water. All samples were left for 5 minutes at 95°C, vortexed and shortly centrifuged.

All solutions and 3 μ l of weight marker were applied to each well. Gel ran at three phases: 15 minutes at 50V, 15 minutes at 100V and 60 minutes at 150V.

After electrophoreses ended, gel was transferred into glass container and dyed with ponceau staining. To get rid of the dye before Western blot, gel was covered with 1x PBS buffer for 5 minutes.

4.4 Western Blot

Western blot is method for protein analysis and detection. This method uses electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to sheet of nitrocellulose membrane, followed by immunodetection of proteins using antibodies with fluorescent or chemiluminescence detection.

Cassette holder was placed in the glass container and covered with 1x BB buffer. Foam pad was placed on one side of the cassette. Two sheets of filter paper were placed on the top of the pad. Gel after SDS-PAGE without glass was placed over the filter paper, and covered with nitrocellulose membrane. Two sheets of filter paper and foam pad were placed on the top of membrane. Cassette was closed and placed in the transfer tank with ice cassette to cool the gel. 1x Blotting buffer was added to the tank. Gel ran 90 minutes at 150 mA.

4.4.1 Immunodetection of Isolated Protein

After Western blot nitrocellulose membrane was transferred to the glass container, covered with 5% milk and left for 20 minutes at room temperature. During this incubation casein from 5% solution of milk binds to the membrane everywhere except bands of, so antibody is going to bind only to protein but not to the membrane.

After binding, milk was discarded. Bottom of clean glass container was covered with parafilm. On the parafilm 1 ml of primary antibody diluted in 5% milk at a 1:1000 ratio was added. Membrane was placed in the glass container the way so side with bonded protein was

in the contact with primary antibody. Glass container was placed in the fridge overnight at 4°C.

Next day primary antibody was collected and membrane was washed 5 times with 1x PBS buffer for 5 minutes. After wash membrane was incubated in the secondary antibody Anti-mouse IgG with peroxidase diluted 1:10 000 in 5% milk for 1 hour at 10 rpm.

After incubation secondary antibody was collected and membrane was washed 5 times with 1x PBS buffer for 5 minutes. Then membrane was covered with solution prepared by mixing 200 µl of solution A with 200 µl solution B from ECL Western Blot kit. Membrane was placed on a plastic wrap. All bubbles were gently smoothed. Chemiluminescence was detected on LAS-3000.

4.4.2 Electrophoretic Mobility Shift Assay (EMSA)

The gel electrophoresis mobility shift assay is used to detect protein complex with nucleic acids.

Samples for EMSA were prepared by mixing 300 ng of DNA, 1x Binding buffer with DTT and Mili-Q H₂O to total volume 15 µl. At the end protein was added to the sample at increasing molar ratios (1:0, 1:1, 1:2, 1:4, 1:8, 1:16). Samples were left on ice for 20 minutes. To all samples 3 µl of 3x LB were added.

For EMSA 1% agarose gel with 0,5x TBE buffer was prepared. Gel was transferred to the electrophoresis tank and filled with 0,5x TBE buffer.

All samples were applied to each well. Gel with superhelix DNA ran for 180 minutes at 90 V at 4 °C, gel with digested DNA ran for 90 minutes at 90 V at room temperature. After electrophoresis, gel was dyed for 25 minutes in the solution prepared by mixing 30 µl GelRed and 150 ml water. Signal was detected on transilluminator.

5 RESULTS

5.1 Gateway Cloning

The prepared construct using PCR method was used in the first step of the Gateway cloning reaction, the BP reaction. Entry clone, prepared during BP reaction, can be cloned into different types of expression systems (bacterial, yeast, mammalian, etc.) depending on the destination vector that is going to be used. In this work, the pDEST17 vector was used in the LR reaction. The pDEST17 vector added polyhistidine sequence (HisTag) to the N-terminal domain of protein, that allows affinity protein isolation from *E. coli*.

5.1.1 LR Reaction

The entry clones were donated by Thorsten Stiewe from Marburg, Germany for our experiments were used for LR reaction (4.2.1.1). After LR reaction samples were transformed into *STBL3* competent cells and select on medium with ampicillin for verification of success. After successful transformation and selection, cells were inoculated into liquid LB media for plasmid isolation. As soon as isolation was finished, plasmid DNA was prepared for sequencing to verify successful cloning. For sequencing T7 reverse and T7 forward primers were used. During LR reaction 4 expression clones were successfully prepared (Table 2:). The names of expression clones are derived from the pDEST17 destination vector and the names of used entry clones.

Table 2: Prepared expression clones

Construct	Sequencing
pDEST17/A	OK
pDEST17/B	OK
pDEST17/C	OK
pDEST17/D	OK

For illustration of individual steps during Gateway cloning agarose electrophoresis was made in 1x TAE buffer. Figure 14 shows a 1% agarose gel, result of BP reaction in the lane number 2 – entry clone (pENTR221/D), at the lane number 3 to 5 results of LR reaction expression vectors (pDEST17/D1, pDEST17/D2, pDEST17/D3), and at the lane 6 is destination vector pDEST17. DNA of pDEST17/D was isolated several times, due to previous unsuccessful LR reactions.

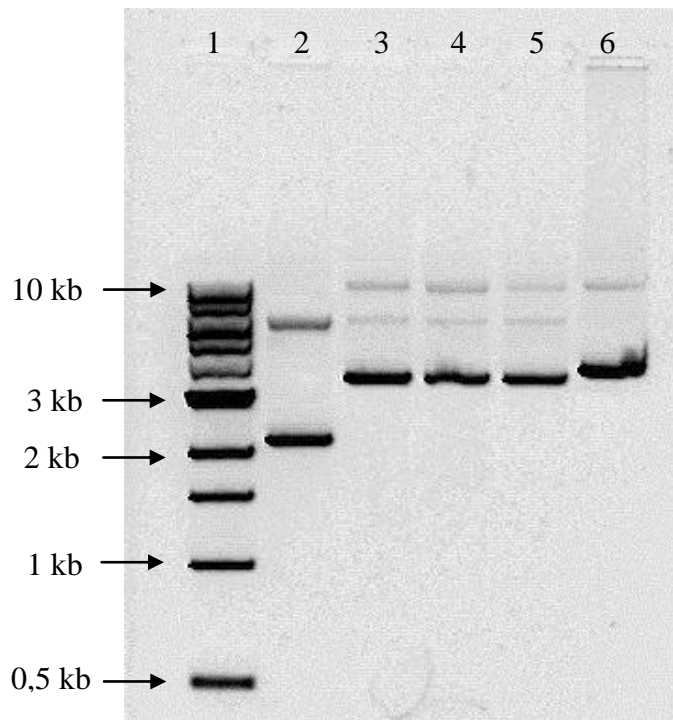


Figure 14 : Result of DNA plasmid isolation at 1% agarose gel. Samples contained 100 ng of DNA. Lane 1: 1 kb DNA ladder: lane 2: entry clone pENTR221/D: lane 3 to 5: expression vectors pDEST17/D1, pDEST17/D2, pDEST17/D3; lane 6: destination vector pDEST17.

The difference in sizes of plasmids can be clearly seen on the gel. The sizes of plasmids during Gateway procedure has changed. This phenomenon can be explained by the fact that each vector has different number of base pairs and different *ccdB* gene size.

5.2 DNA Isolation

Isolated DNA was verified by horizontal agarose gel electrophoresis in 1x TAE buffer. Gel contained a fluorescent dye GelRed that bound to DNA during electrophoresis. Signal was detected with transilluminator.

5.3 DNA Restriction Digestion

DNA fragments were restriction digested with *PvuII*-HF™ restriction enzyme (4.2.5). In order to digest cloned fragment with different local non-B DNA secondary structures. With this method it is possible to distinguish between nonspecific interactions with linear long fragment and specific recognition of responsive element of non-B DNA secondary structures by p53 protein. After digestion DNA was purified with QIAquick® Nucleotide Removal Kit. Restriction digestion of DNA was verified by horizontal agarose gel electrophoresis in 1x TAE buffer. Gel contained a fluorescent dye GelRed that bound to DNA during electrophoresis. Signal was detected with transilluminator. Figure 15 shows a 1% agarose gel, at the lane 1 and 10 are DNA leaders 1 kb and 100 bp. Plasmids in superhelical (scDNA) form followed by their cleaved form are visualized in Figure 15.

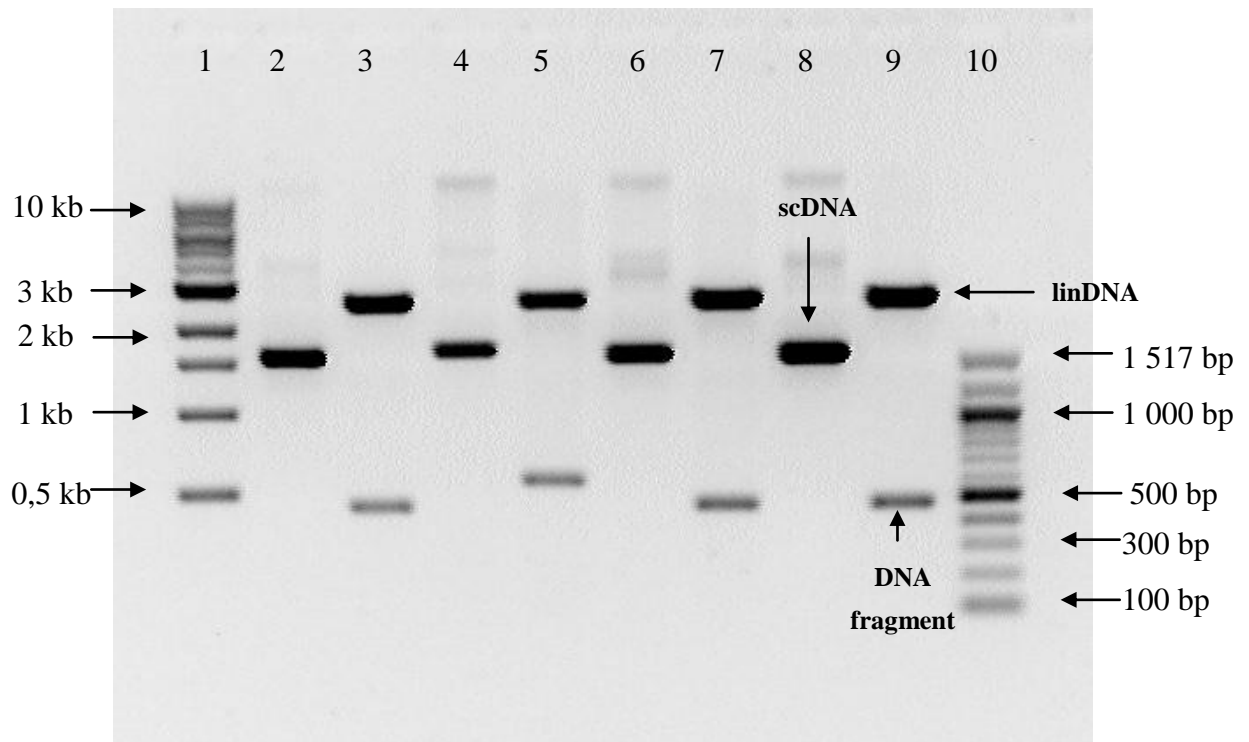


Figure 15 : Result of DNA plasmid isolation and restriction digestion at 1% agarose gel. Samples contained 100 ng of DNA. Lane 1: 1 kb DNA ladder; lane 2: pBleskript; lane 3: pBlueskript/PvuII-HF; lane 4: Mycpu21 3x; lane 5: Mycpu21 3x/PvuII-HF; lane 6: pPGM2; lane 7: pPGM2/PvuII-HF; lane 8: pCFNO; lane 9: pCFNO/PvuII-HF; lane 10: 100 bp DNA ladder. ScDNA – superhelical DNA, linDNA – linear DNA.

A change in the mobility of the sc form of DNA and the digested form is well observed on the displayed gel forms of individual plasmids. All digested plasmids are in good quality for subsequent binding experiments with isoforms of p53.

Table 3: Sequences cloned into non-B DNA plasmids structures

Plasmid	Cloned sequence	Type of non-B DNA structure	Presence of p53 RE
Mycpu21 3x	AGCTTGAGGGTGGGGAGGGTGGGGAAA	G-quadruplex	No
pPGM2	AGACATGCCT AGGCATGTCT	Cruciform	Yes
pCFNO	CATGATGTGA TCACATCATG	Cruciform	No

5.4 Measuring of Protein Concentration

After successful sequencing of four expression clones, plasmid DNA were transformed into BL21 competent cells and protein production started (4.2.6). As a positive control p53 wild-type protein was also produced. Five elution fractions were prepared from each protein. Each fraction was spectrophotometric measured using Bradford protein assay at 595 nm. Protein concentrations are shown in Table 4:

Table 4: Protein concentration measured using bradford protein assay

Elution	Concentration [mg/ml]				Wild-type p53
	A	B	C	D	
1	<0,02	<0,02	<0,02	<0,02	0,99
2	0,11	0,34	0,08	0,06	2,88
3	0,22	0,14	0,06	0,08	2,18
4	0,13	0,11	<0,02	<0,02	0,74
5	0,09	<0,02	<0,02	<0,02	<0,06

5.5 SDS-PAGE and Western Blot

After protein isolation and measuring of protein concentration of each fraction, all fractions were used for detection at polyacrylamide gel with sodium dodecyl sulphate (4.3). All controls samples, which were taken during protein induction and isolation were also used for SDS-PAGE and immunodetection. Control samples before induction, after induction, and during the washing steps were taken to monitor proper recovery and purification – to detect residual protein in the wash supernatant or residual protein in the supernatant after binding the protein to the affinity separation particles.

One polyacrylamide gel was prepared, this gel was after electrophoretic separation used for western transfer (4.4). During western blot proteins were detected with specific DO-I antibody, so as result only p53 proteins were detected. As a positive control wild-type p53 protein was used.

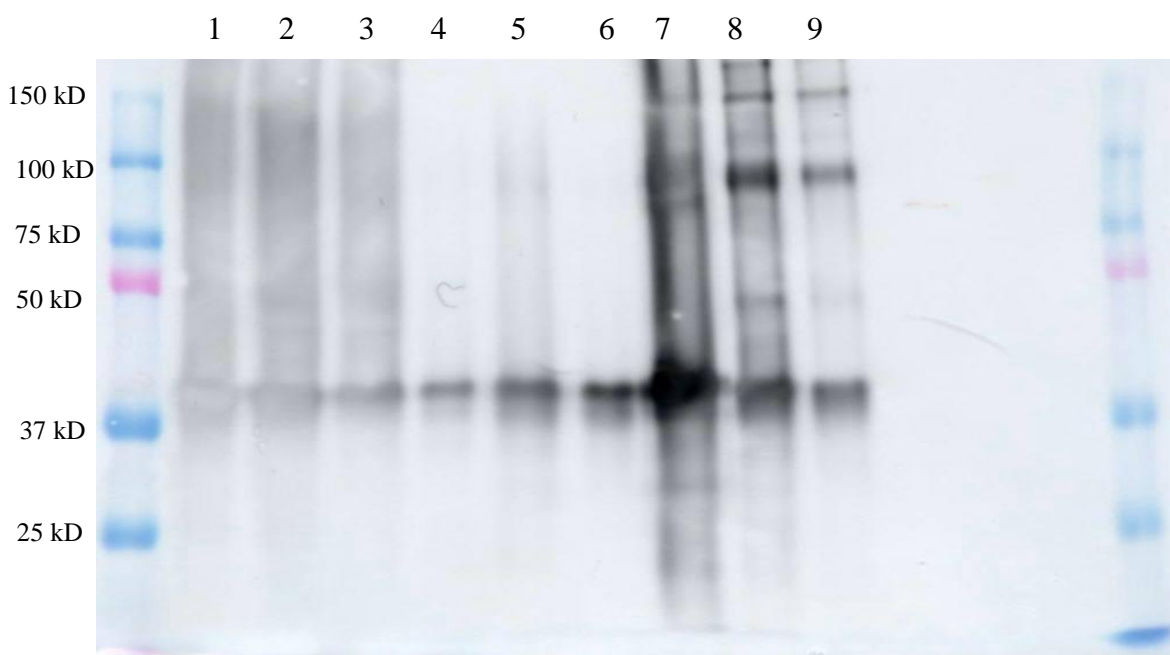


Figure 16 : Western blot analysis of wild-type p53. Lane 1: control sample before induction; lane 2: control sample after induction; lanes 3 to 5: control samples taken during cells lysis and protein isolation; lanes 6 to 9: fractions of isolated protein.

Western blot showed a presence of wild-type p53 protein by chemiluminescence signal on the membrane. For western blot detection DO-I antibody (primary, 1:1000 dilution) and Anti-mouse IgG peroxidase conjugated antibody (secondary, goat, 1:10 000 dilution) were used (Figure 16). In lanes 6 to 9 was wild-type p53 protein fraction of appropriate size visibly detected, 43 kDa.

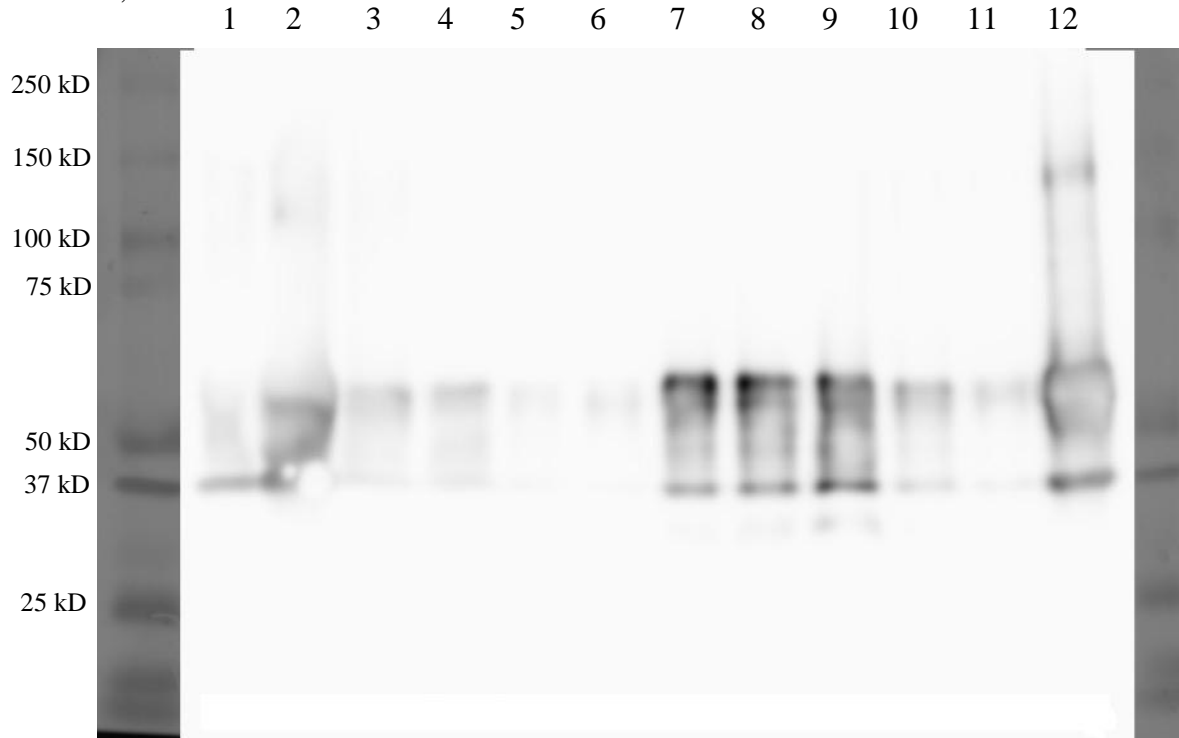


Figure 17 : Western blot analysis of mutant p53/A. Lane 1: control sample before induction; lane 2: control sample after induction; lanes 3 to 7: control samples taken during cells lysis and protein isolation; lanes 8 to 11: fractions of isolated protein, lane 12: positive control.

Membrane after western blot showed presence of mutant p53/A protein. For western blot detection DO-I antibody (primary, 1:1000 dilution) and Anti-mouse IgG peroxidase conjugated antibody (secondary, goat, 1:10 000 dilution) were used (Figure 17). In lanes 8 to 11 mutant p53/A protein fraction of appropriate size was visibly detected with mass of 43 kDa. In lanes 1 and 2 a large difference in mutant protein production in control samples taken before and after protein induction with IPTG can be seen.



Figure 18 : Western blot analysis of mutant p53/B. Lane 1: control sample after induction; lanes 2 to 6: control samples taken during cells lysis and protein isolation; lanes 7 to 10: fractions of isolated protein, lane 11: positive control.

The signal on the membrane after western blot confirms successful isolation of mutant p53/B protein (Figure 18). The procedure of analyzation was the same as with the previous mutant, for western blot detection DO-I antibody (primary, 1:1000 dilution) and Anti-mouse IgG peroxidase conjugated antibody (secondary, goat, 1:10 000 dilution) was used.

Due to low concentration of protein signal from isolated protein was very low, so the time of the exposure was longer. As a result, signal from positive control was overexposed.

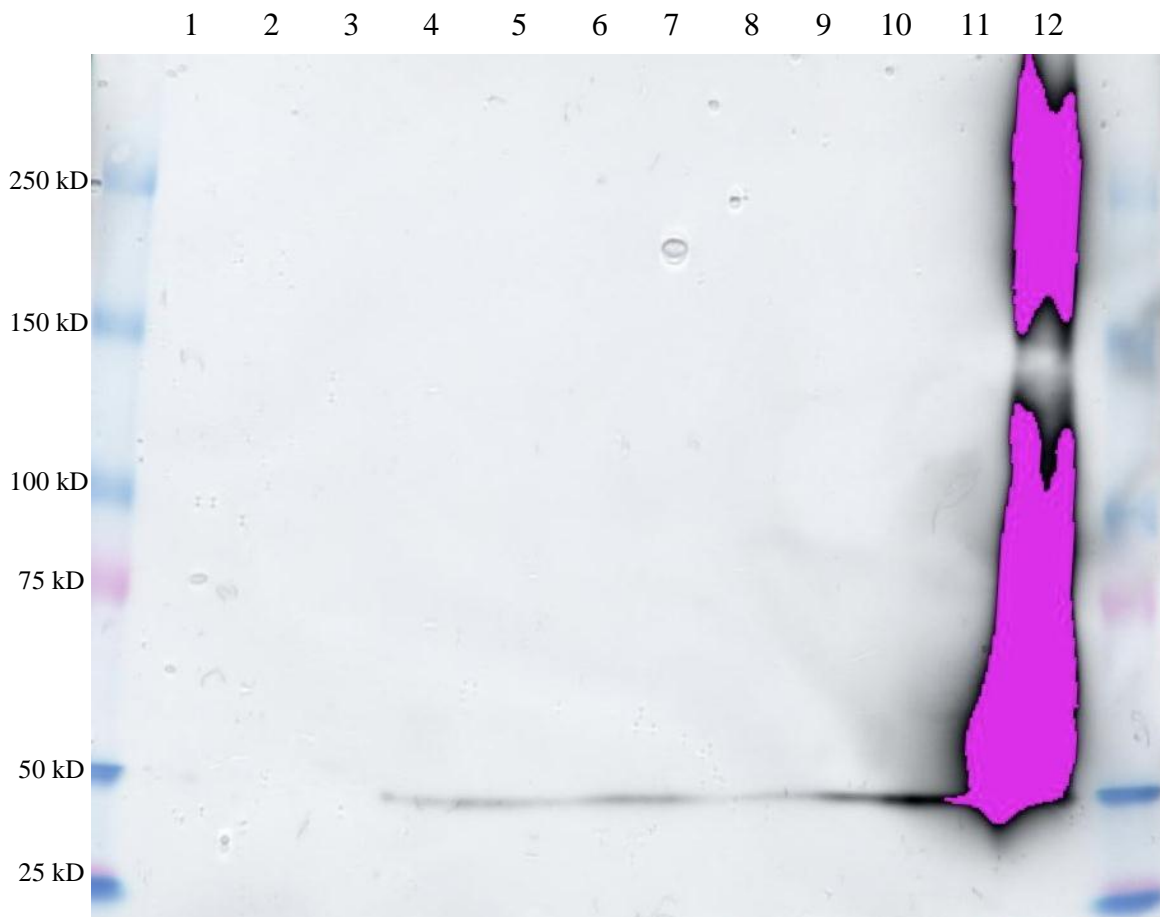


Figure 19 : Western blot analysis of mutant p53/C. Lane 1: control sample before induction; lane 2: control sample after induction; lanes 3 to 7: control samples taken during cells lysis and protein isolation; lanes 8 to 11: fractions of isolated protein, lane 12: positive control.

The signal on the membrane after western blot confirms successful isolation of mutant p53/C protein (Figure 19). The procedure of analyzation was the same as the previous mutant, for western blot was detected by DO-I antibody (primary, 1:1000 dilution) and Anti-mouse IgG peroxidase (secondary, goat, 1:10 000 dilution).

Due to low concentration of protein signal from isolated protein was very low, so the time of the exposure was longer. As a result, signal from positive control was overexposed.

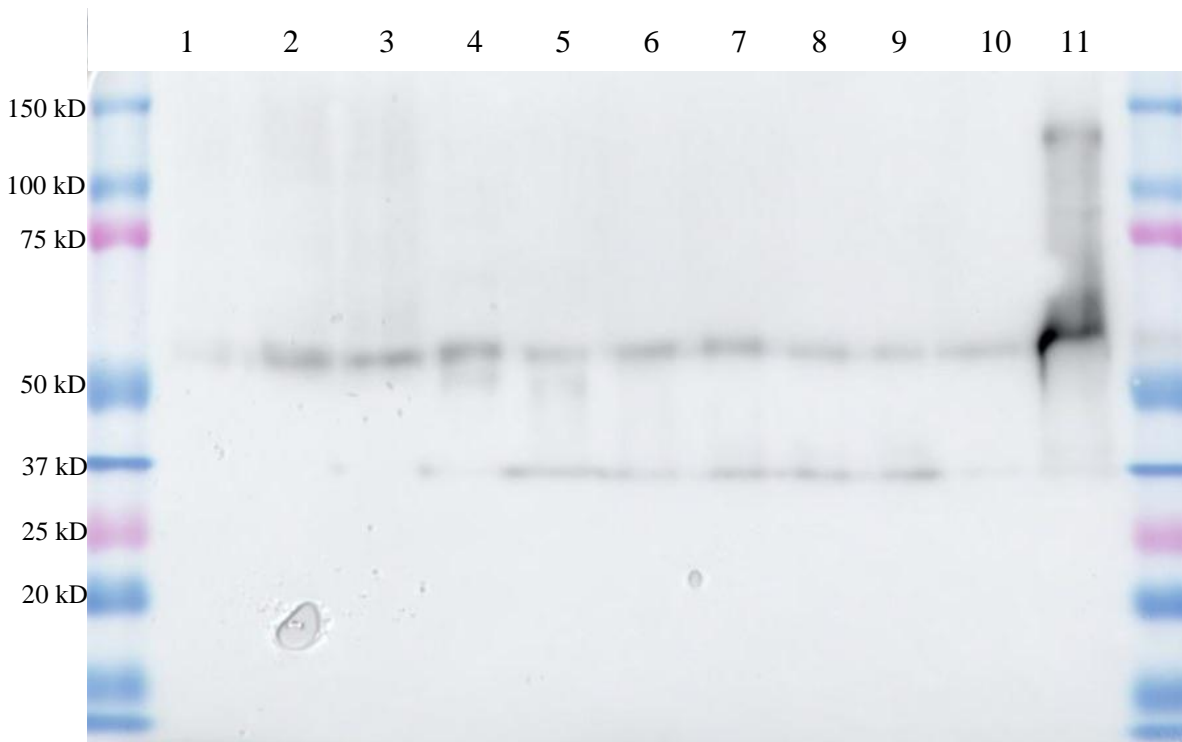


Figure 20 : Western blot analysis of mutant p53/D. Lane 1: control sample after induction; lanes 2 to 6: control samples taken during cells lysis and protein isolation; lanes 7 to 10: fractions of isolated protein, lane 11: positive control.

Western blot of mutant p53/D protein showed a successful isolation of protein of appropriate size, 43 kDa (Figure 20). Procedure was the same as with previous samples. As primary antibody for immunoblotting DO-I (1:1000 dilution) and as secondary Anti-mouse IgG peroxidase (produced in goat, 1:10 000 dilution) were used. Increasing of chemiluminescence signal was detected in the control sample taken after protein induction with IPTG (lane 2) comparing to control sample taken before protein induction (lane 1).

5.6 EMSA

For study proteins binding properties with plasmid DNA 0,5x TBE agarose gel that allows migration of the formed complexes was prepared. The selection molar ratios for the interaction were: 1:1, 1:2, 1:4, 1:8, 1:16 and as a negative control one sample without protein. To analyse the DNA binding properties of mutant and wild-type p53 proteins two types of DNA: superhelical and its digested form were examined. The digested DNA was used to determine specific sequence binding.

5.7 EMSA with Superhelical DNA

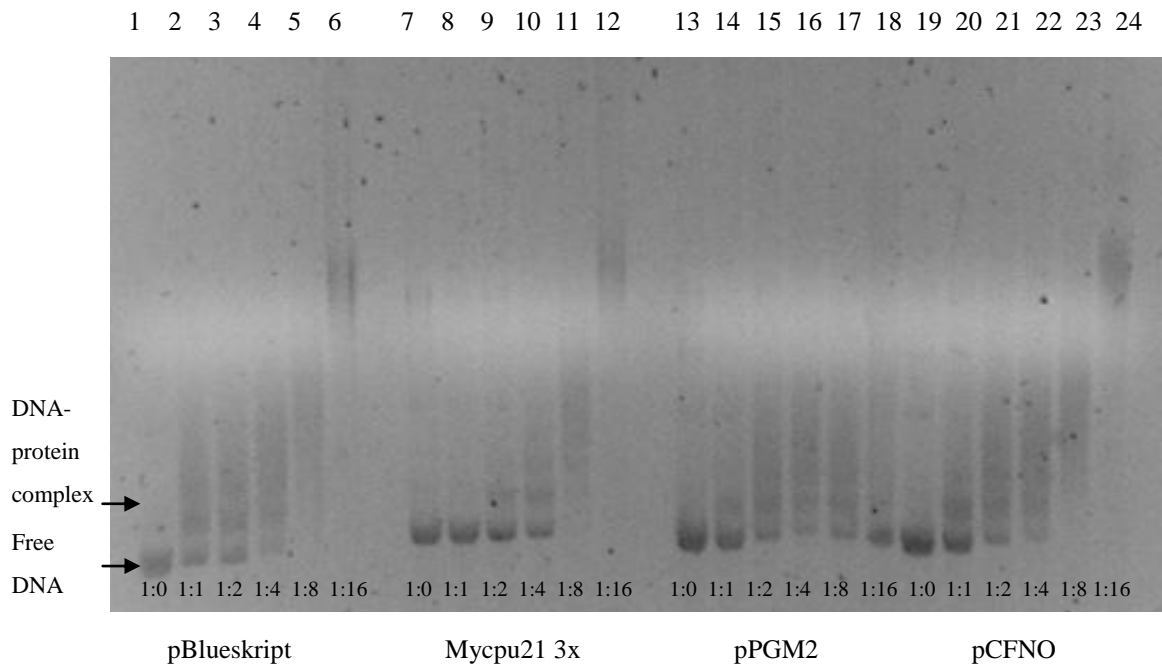


Figure 21 : EMSA of wild-type p53 protein with superhelical DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lanes 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

This assay shows binding reaction between wild-type p53 protein and pBlueskript, pPGM2 and pCFNO plasmids at lowest concentration 1:1 (Figure 21: lanes 2, 14 and 20). The protein-DNA complex with Mycpu21 3x plasmid was observed only at higher concentration of protein (molar ratio 1:2) (Figure 21: lane 9). At concentration rate 1:4 almost all DNA was bound with wild-type p53 protein and amount of free DNA was minimal. With increasing concentration of protein, more specifically at 1: 8 and 1:16 molar ratios, the wild-type p53 was bound to the plasmid in greater amounts, the resulting signal was blurred and protein-DNA complexes of higher molecular weight were formed.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

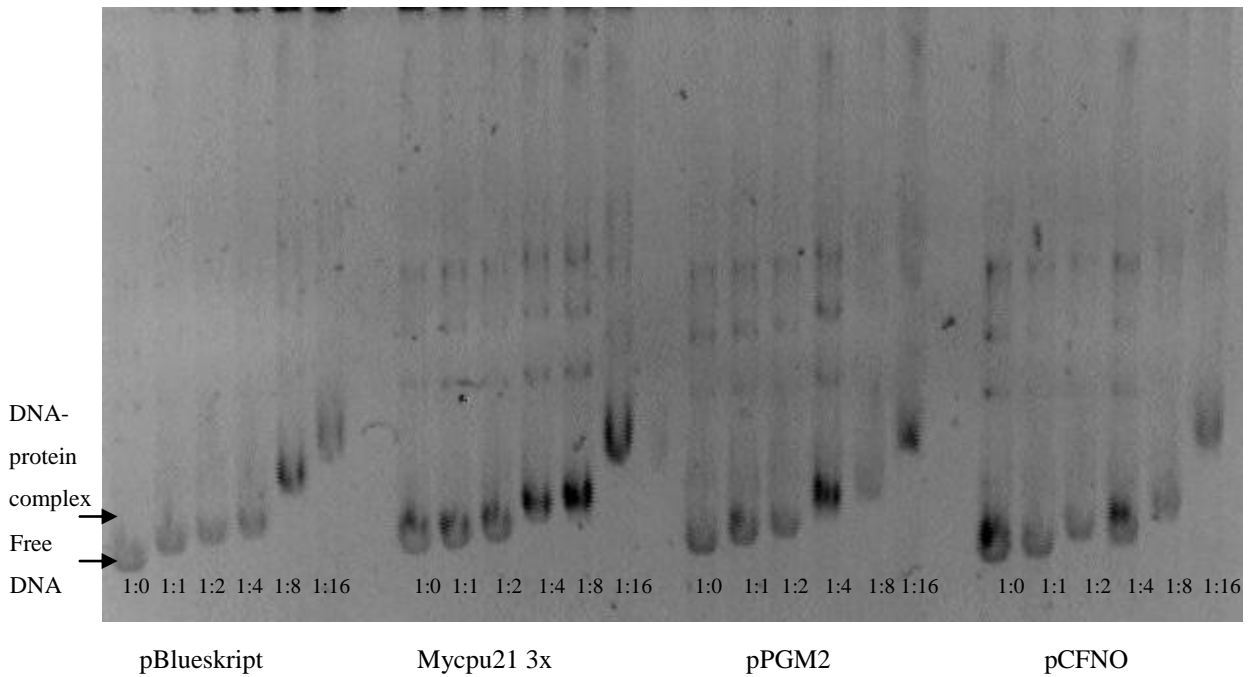


Figure 22 : EMSA of mutant p53/A protein with superhelical DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lanes 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

Binding experiment with mutant form of p53/A protein showed forming of DNA-protein complex at lowest concentration of protein at 1:1 ration with pBlueskript, pPGM2 and pCFNO plasmids (Figure 22: lanes 2, 14 and 20). The mutant protein lanned to bind to the Mycpu21 3x plasmid DNA at higher concentration (1:2 ration, Figure 22: lane 8) and significant signal of formed complex was at 1:4 molar ration (Figure 22: lane 9). All plasmid DNA was bound fully with mutant protein at molar ration 1:8 and 1:16 (Figure 22: lanes 5, 11, 17, 23 and 6, 12, 18, 24).

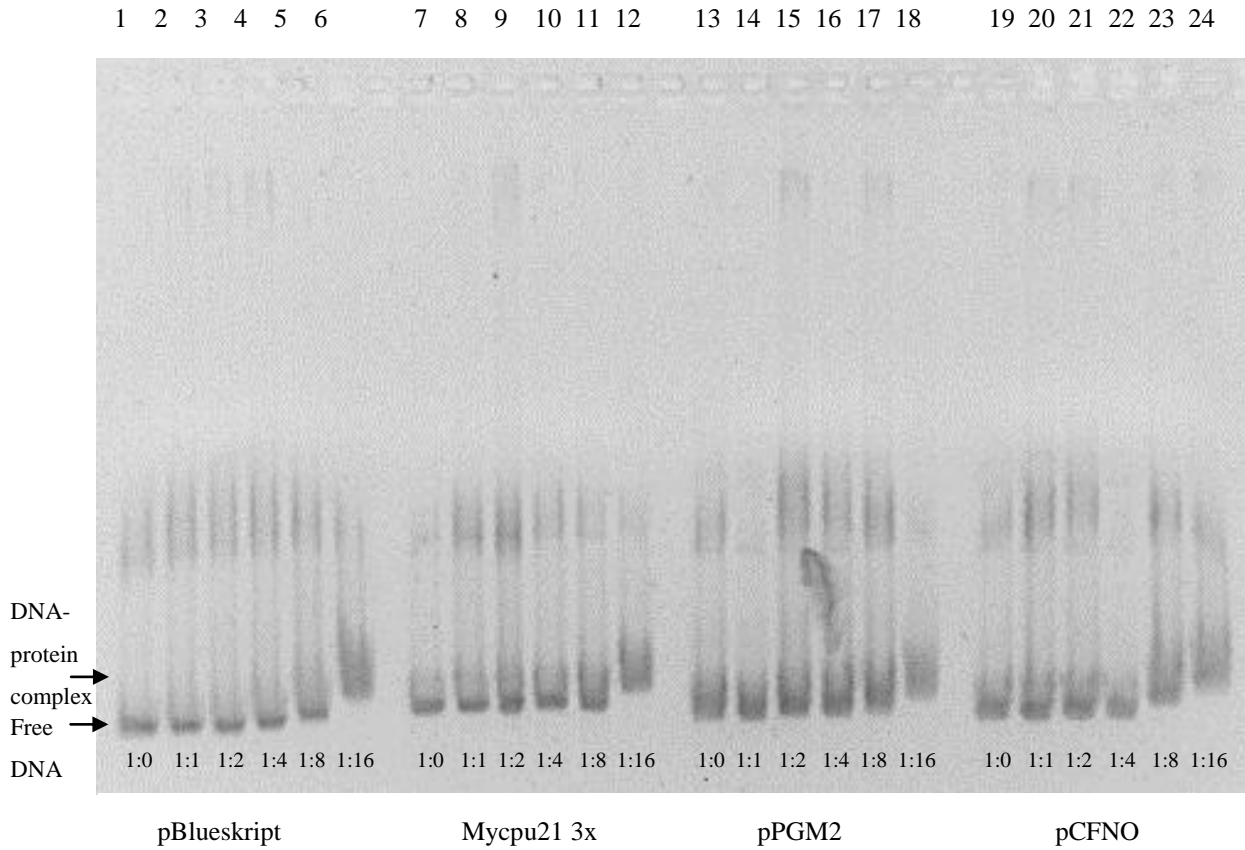


Figure 23 : EMSA of mutant p53/B protein with superhelical DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lanes 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

Mutant p53/B interacted with pBlueskript, Mycpu21 3x and pPGM2 plasmid DNA at highest concentration of protein (1:16 ration, Figure 23: lanes 6, 12 and 18). In the case of pCFNO plasmid DNA, p53/B protein lanned to bind to DNA at 1:8 molar ration (Figure 23: lane 23).

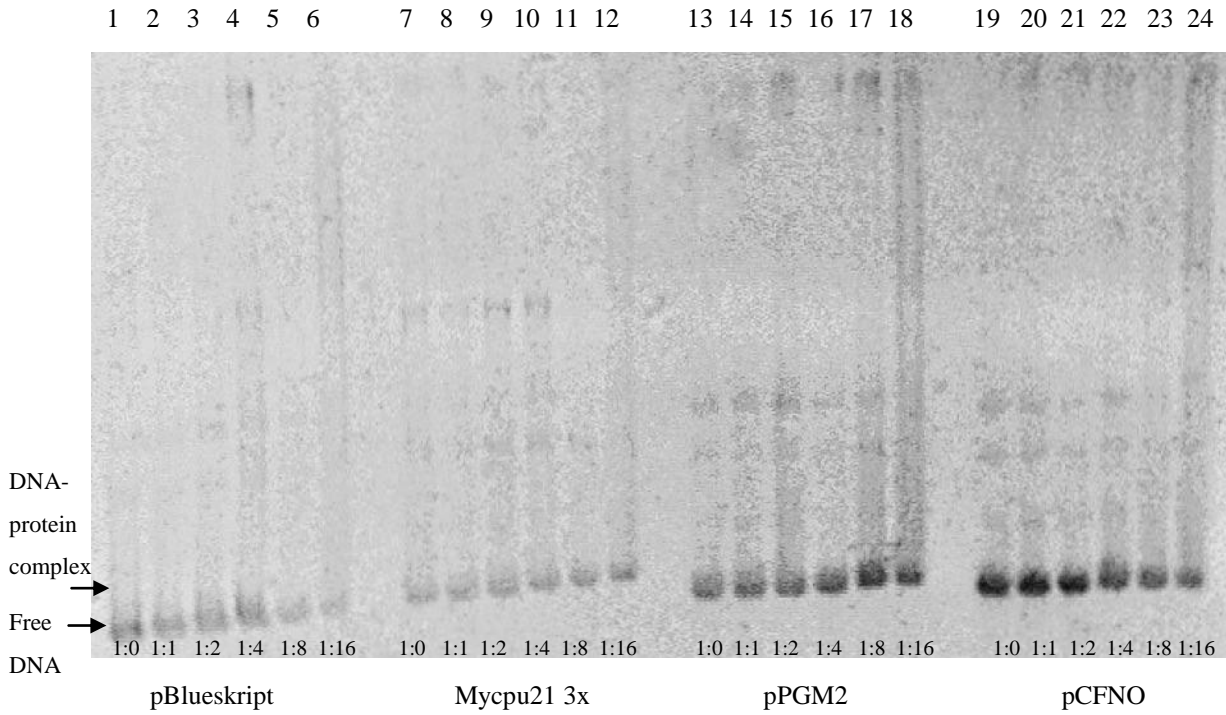


Figure 24 : EMSA of mutant p53/C protein with superhelical DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lanes 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

Marginal changes in all plasmid's DNA were detected at highest concentration of mutant p53/C (1:16 ration, Figure 24: lanes 6, 12, 18 and 24).

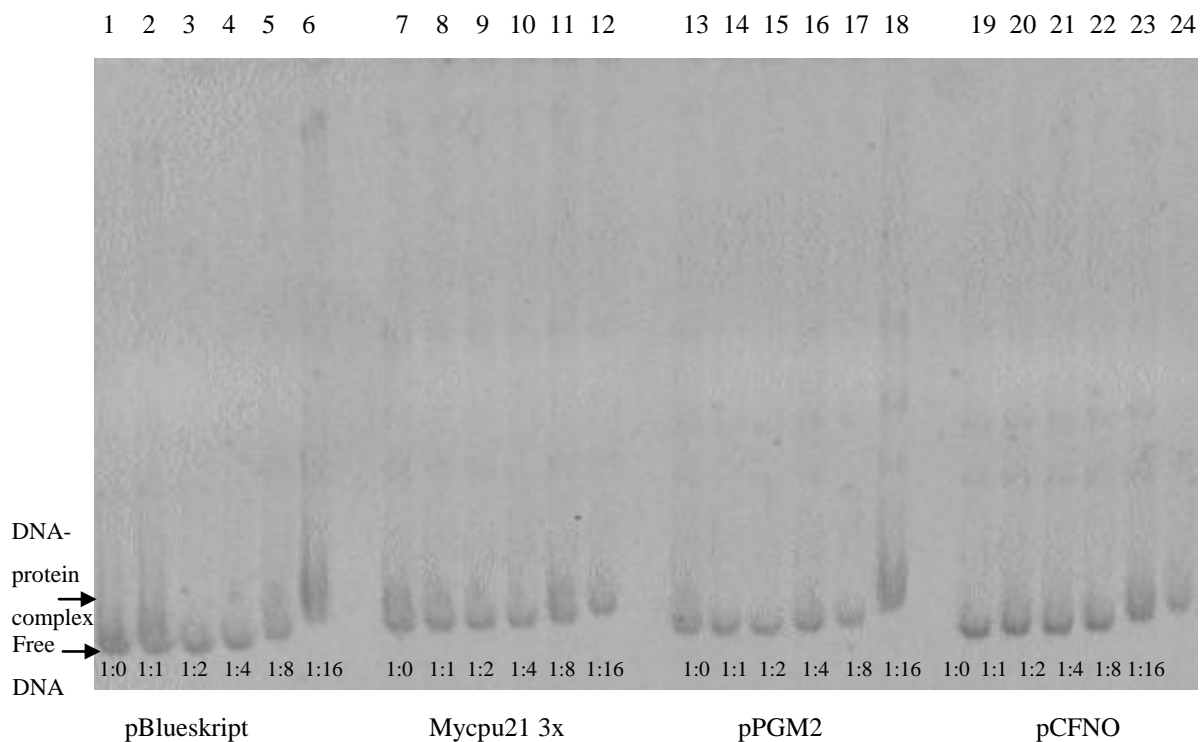


Figure 25 : EMSA of matant p53/D protein with superhelical DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lanes 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

All tested DNA plasmids binds with mutant p53/D at highest concentration. First changes in mobility of all plasmids was noticeable at 1:8 ration (Figure 25: lanes 5, 11, 17 and 23) that can lead to the fact of first interaction with protein. With higher concentration of protein, mobility of plasmid DNA in the gel was significantly changed.

5.8 EMSA with Digested DNA

EMSA with digested DNA plasmids was carried with four forms of mutant p53 protein and wild-type p53 protein. The 0,5x TBE gel was prepared to study DNA-protein interactions. The selections of molar ratios for the interaction were: 1:1, 1:2, 1:4, 1:8, 1:16 and as a negative control one sample without protein was prepared.

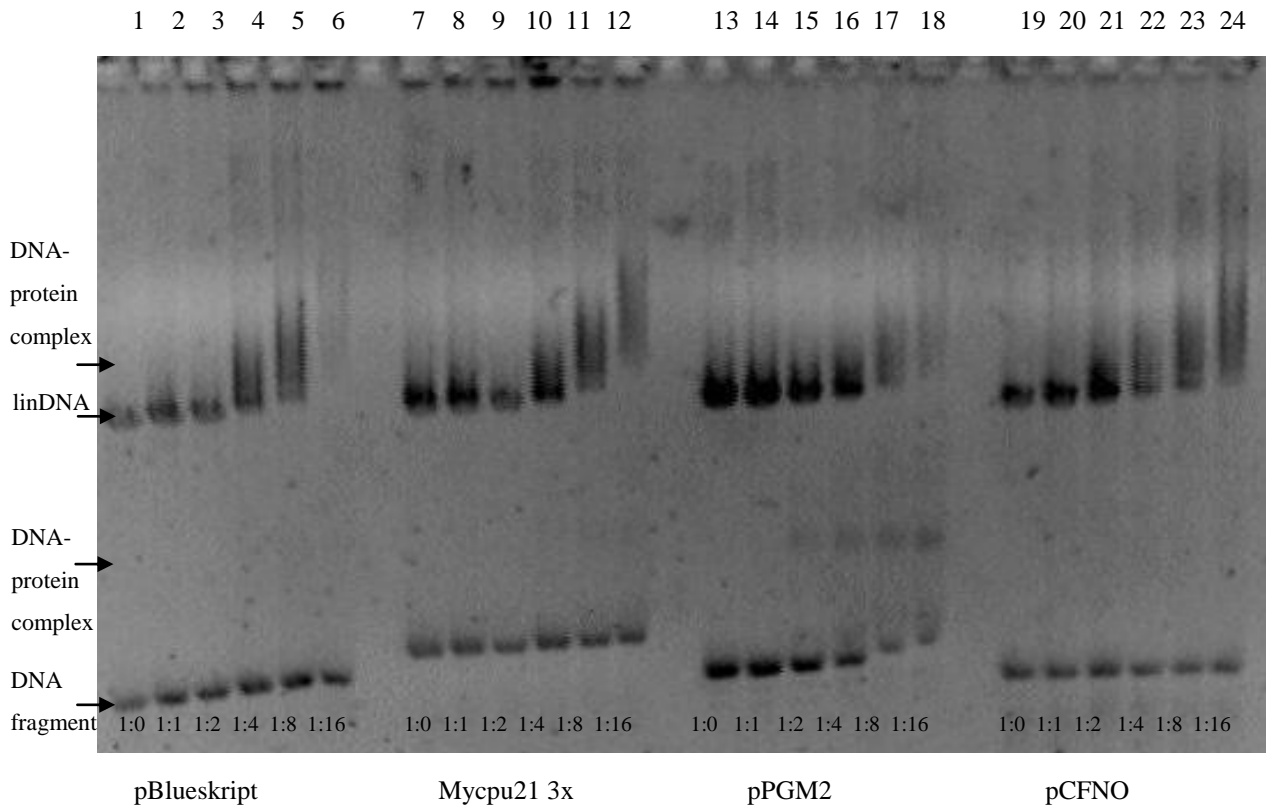


Figure 26 : EMSA of wild-type p53 protein with digested DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lane 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

Binding experiment with wild-type p53 protein showed a forming of DNA-protein complex with pPGM2 plasmid fragment. This interaction is explained by presence of consensus sequence sequence, which provides sequence specific binding properties. The complex with pPGM2 plasmid fragment was noticed at 1:2 molar ration of protein concentration (Figure 26: lane 15). The wild-type p53 had a significant binding property with linear DNA, which was produced after cleavage of the plasmid DNA fragment, at a molar ration of 1:4 (Figure 26: lanes 4, 10, 16 and 22). With increasing of protein concentration, interactions with linear DNA were higher due to changes in DNA mobility.

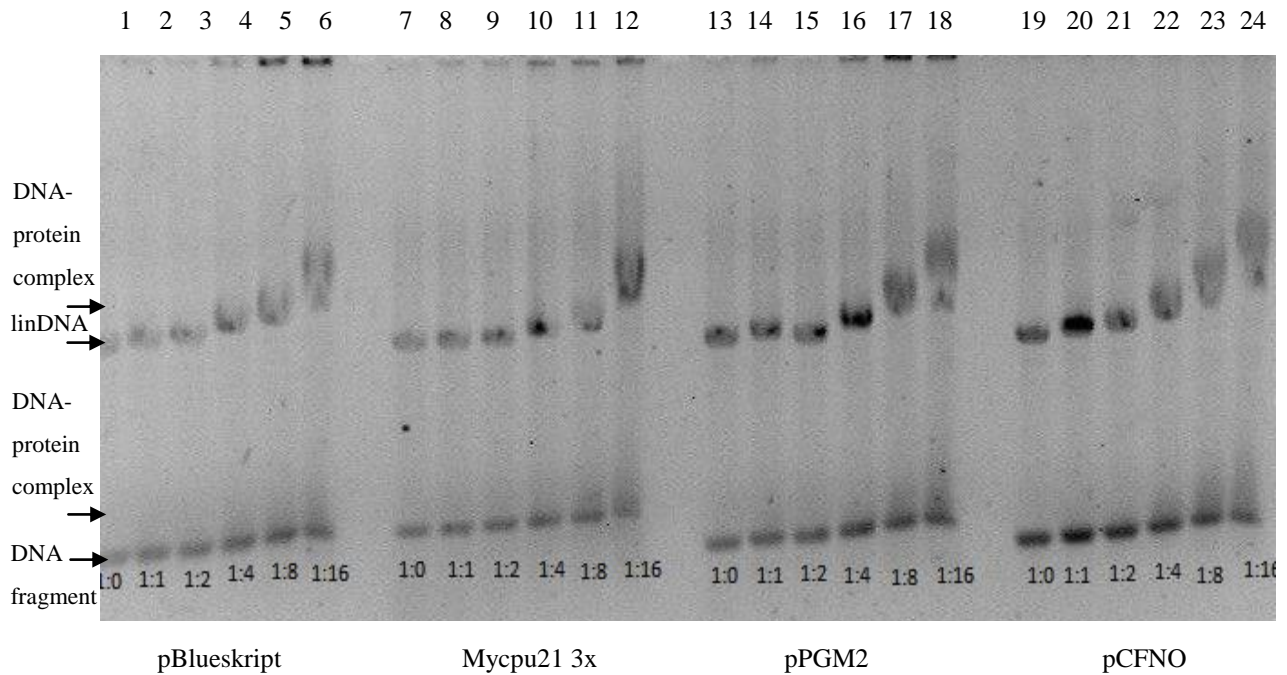


Figure 27 : EMSA of mutant p53/A protein with digested DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lane 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

At the performed EMSA agarose gel binding between smaller fragments of digested plasmid DNA and mutant p53/A was not evident at low concentration. No significant change in mobility of all DNA fragments was noticed at 1:8 molar ration (Figure 27: lanes 5, 11, 17 and 23). With increasing protein concentration DNA-protein interactions were weak, amount of free DNA was at high level. Mutant p53/A showed interaction with higher linear DNA at low concentration, DNA-protein complexes were formed at a molar ration of 1:4 (Figure 27: lanes 4, 10, 16 and 22). Increasing amounts of mutant protein was observed to greatly enhance DNA-protein binding.

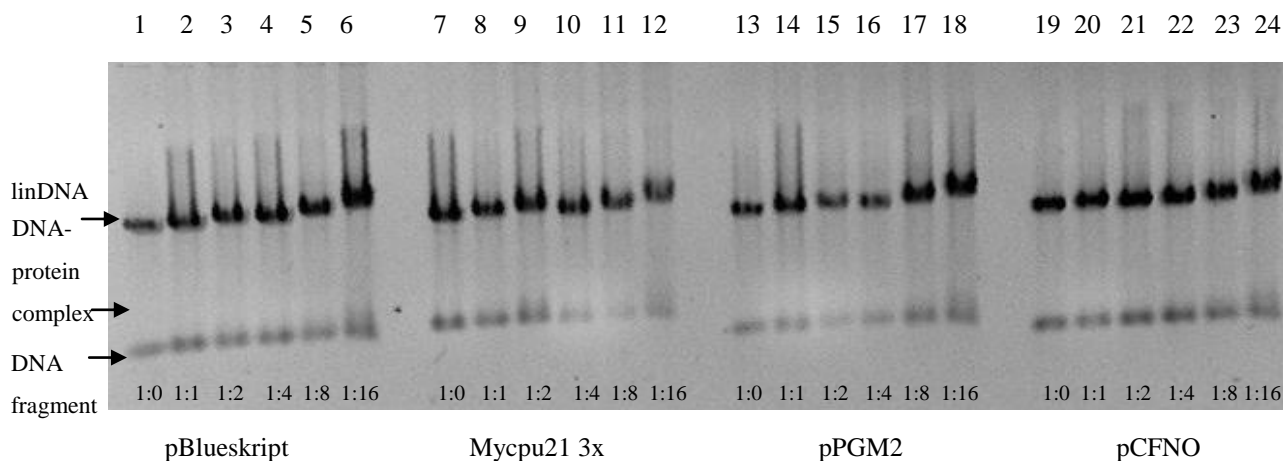


Figure 28 : EMSA of mutant p53/B protein with digested DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lane 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

EMSA with mutant p53/B protein ran at 90 V for 45 minutes. The mutant p53/B protein did not show any binding properties with all of DNA plasmids smaller fragments at lower concentration. Forming of DNA-protein complexes was detected at highest concentration of protein at a molar ration of 1:16 (Figure 28: lanes 6, 12, 18 and 24). Interaction between p53/B and linear DNA was not detected.

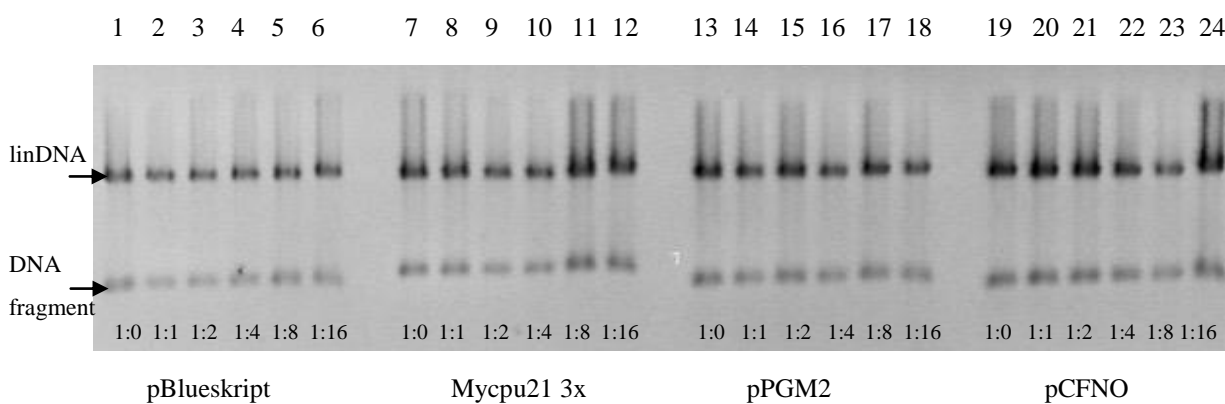


Figure 29 : EMSA of mutant p53/C protein with digested DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lane 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

EMSA with mutant p53/C protein ran at 90 V for 45 minutes. The p53/C protein did not interact with fragments of plasmid DNA. Also, any binding between p35/C and linear DNA was not detected.

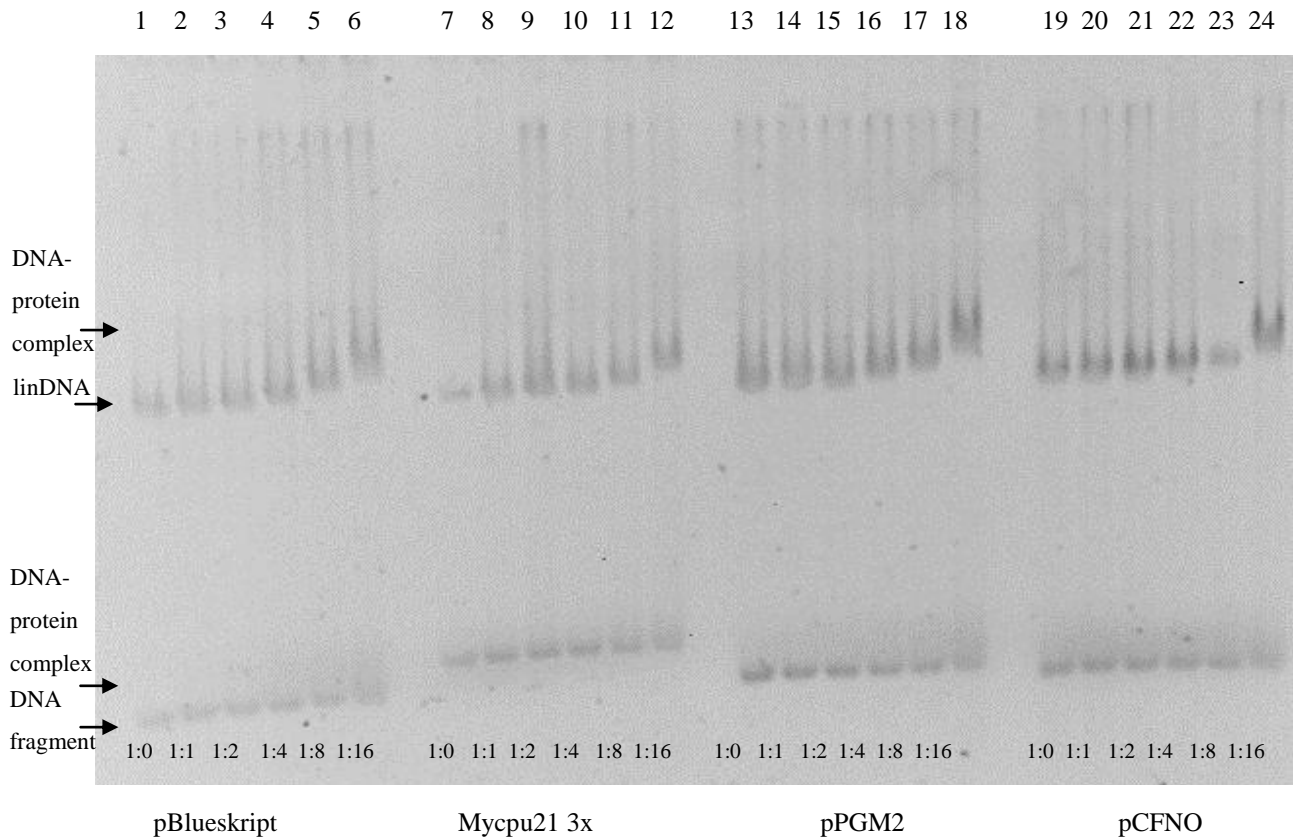


Figure 30 : EMSA of mutant p53/D protein with digested DNA. Lanes 1, 7, 13 and 19 has only DNA without protein; lanes 1 to 6 have pBlueskript plasmid in it; lane 7 to 12 have Mycpu21 3x plasmid; lanes 13 to 18 have pPGM2 plasmid; lanes 19 to 24 have pCFNO plasmid. Samples have increasing ration of protein concentration (1:1, 1:2, 1:4, 1:8, 1:16).

Binding experiment with mutant p53/D protein showed changes in mobility of plasmid DNA fragment at highest protein concentration (Figure 30: lanes 6, 12, 18 and 24). Forming of DNA-protein complexes were detected at a molar ration of 1:8 with linear DNA of pBlueskript, Mycpu21 3x and pPGM2 plasmids (Figure 30 : lanes 5, 11, 17 and 23). With increasing of protein concentration, mobility of linear DNA has changed.

6 DISCUSSION

Theoretical part of this thesis is focused on the description of the structure, function and mechanism of p53 protein activation. Subsequently, secondary structures of DNA, like G-quadruplex and crosslink were characterized. This work deals with mutant p53 protein, so different mutation of p53 protein and its binding properties has been described. Principle of Gateway cloning system and advantages of this technique were explained.

Practical part of this work was focused on the protein production and studying binding properties of wild-type and mutant p53 proteins. In the first step four expression vectors for protein production using Gateway cloning system were prepared. Four entry clones, prepared in Marburg, were used for the LR reaction, sequences from entry clones were inserted by recombination into the destination vector pDEST17, which adds a polyHisTag sequence to expressed protein. The polyHisTag allows affinity protein isolation and is used for immunodetection with specific primary antibody. Successfully four expression clones (pDEST17/A, pDEST17/B, pDEST17/C, pDEST17/D) were prepared.

All four expression clones were used for DNA-protein interaction and, for comparison as a negative control also wild-type p53. All protein was induced and isolated (p53/A, p53/B, p53/C, p53/D, wild-type p53).

Interaction between various plasmid DNA with cloned insert containing non-B DNA local secondary structures and proteins were monitored by gel retardation analysis. Interaction of wild-type p53 protein with superhelical DNA was detected at the lowest concentration ratios. Forming of DNA-protein complexes with mutant p53/A were observed at lowest protein concentration, while p53/B and p53/D proteins bound to DNA only at concentration ratios 1:8 and higher. The mutant p53/C showed interaction with superhelical DNA only at highest concentration.

Interactions between mutant and wild-type p53 proteins and digested plasmids were analysed by the same method. The wild-type p53 complex was formed only with the fragment of pPGM2 plasmid and was clearly visible at a concentration ration of 1:4. Due to the fact, that pPGM2 plasmid has a responsive element of p53 protein and also can form cruciform structure, forming of this complex was expected. The mutant p53/A started to form complexes with plasmids DNA fragments at molar ratio of 1:8 of protein concentration, while complexes with linear DNA were formed at lower concentration (molar ratio of 1:4). Binding to plasmids fragments was detected with p53/B and p53/D proteins at highest concentration (molar ratio of 1:16), also was observed interaction between p53/D and linear DNA of pBlueskript, Mycpu21 3x and pPGM2 plasmids at a molar ratio of 1:8. Mutant p53/C protein did not show any interaction with DNA.

To sum up, in the practical part of the diploma thesis, the preferential binding of the p53 protein to secondary structures and sequences with target site for the p53 protein was observed. Mutant p53/A protein showed strong ability to bind to superhelical and also digested DNA. Specific interaction with any digested fragment was not observed. In the case of p53/B, and p53/D forming of DNA-protein complexes were detected only at the highest protein concentration. Mutant p53/B did not interact with digested DNA at all, that lead to consequence, that this mutant may interact only with DNA in superhelical state. Mutant p53/D protein showed preference of forming nonspecific complexes with linear DNA in

comparison to p53 target DNA sequence. P53/C protein bound to superhelical DNA weakly at the highest molar ratio and did not interact with digested DNA at all.

Based on this research, it can be suggested that p53/A and p53/D proteins, in consequence to mutations, have lost their ability to interact with specific sequence for p53 protein and prefer rather unspecific binding to DNA in superhelical or linear state. These mutants therefore probably lost ability to transactivate target genes of wild-type p53 *in vivo* and may either loss their transactivation potential at all or can transactivate different and yet unknown set of genes. Also, mutant p53/B protein, which bound only nonspecifically to DNA in superhelical state, has lost its tumour suppressor function and may transactivate different set of genes. Different binding pattern from mutant p53/A and p53/D suggest preference for superhelical DNA. Mutant p53/C bound weakly only to superhelical DNA just at highest molar ratio (1:16), therefore probably have lost the function of transcription factor at all. Based on literature, p53/C protein may inhibit function of wild-type p53 through conformational changes or inhibiting the DNA-binding activity of wild-type p53 on target genes and thus contribute to oncogenic transformation in different way as mutants p53/A, p53/B and p53/D [30].

7 CITATIONS

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