DNA topology influences p53 sequence-specific DNA binding through structural transitions within the target sites

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DNA TOPOLOGY INFLUENCES P53 SEQUENCE-SPECIFIC DNA BINDING THROUGH STRUCTURAL TRANSITIONS WITHIN THE TARGET SITES

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Abbreviations: scDNA, supercoiled DNA; lin, linear DNA; o, oligodeoxynucleotide; SK, plasmid pBluescript SK-; fl, full length; wt, wild-type;

Keywords: p53 protein / DNA binding / protein-DNA complex

Short title: DNA topology affects p53 binding

ABSTRACT

Tumor suppressor protein p53 is one of the most important factors regulating cell proliferation, differentiation and programmed cell death in response to a variety of cellular stress signals. The p53 is a nuclear phosphoprotein, and its biochemical function is closely associated with its ability to bind DNA in a sequence-specific manner and operate as a transcription factor. Using a competition assay, we investigated the effect of DNA topology on the DNA binding of human wild type p53 protein. We prepared sets of topoisomers of plasmid DNA with and without p53 target sequences differing in their internal symmetry. Binding of p53 to any DNA increased with increasing negative superhelix density ($-\sigma$). At $-\sigma \leq 0.03$, the relative effect of DNA supercoiling on protein-DNA binding was similar for DNAs bearing both symmetrical and non-symmetrical target sites. On the other hand, at higher $-\sigma$ target sites with a perfect inverted repeat sequence exhibited a more significant enhancement of p53 binding due to increasing levels of negative DNA supercoiling. For $-\sigma = 0.07$, about a three-fold additional increase of binding was observed for a symmetrical target site, compared to a non-symmetrical target. The p53 target sequences possessing the inverted–repeat symmetry were shown to form cruciform structure in sufficiently negative scDNA. We show that formation of the cruciforms in DNA topoisomers at $-\sigma \geq 0.05$ correlates with the extra enhancement of p53-DNA binding.

INTRODUCTION

The p53 tumor suppressor protein is an important transcription factor that induces cell cycle arrest or apoptosis in response to oncogenic transformation and DNA damage. Point mutations and deletions in the p53 gene are the most frequently observed alterations in human cancers [1]. The central role of p53 as a tumor suppressor protein has led several studies to explore the possibility of restoring p53 function for therapeutic benefit [2-4]. Interaction with DNA in a sequence-specific manner is crucial for its function. The consensus sequence for p53 binding sites consists of two copies of the motif 5`-RRRC(A/T)(T/A)GYYY-3`, separated by 0 to 13 bp [5]. Surprisingly, some sequences perfectly matching the p53 consensus were found not to bind p53. A comprehensive list of known p53 targets can be found in [6, 7]. While the basic features of p53-DNA binding are known, it is still poorly understood which parameters determine the selectivity of these interactions. Wild-type p53 possesses the ability to linearly diffuse along DNA to find the target sequence [8, 9]. It was proposed that non sequence-specific binding of the p53 protein plays an important role in activation of sequence-specific binding. A family of specific structural elements recognized by p53 has been established, including base mismatches [10], Holliday junction and cruciform structures [11], bent DNA [12, 13], and structurally flexible MAR/SAR DNA [14]. The p53 protein binds preferentially to supercoiled (sc) DNA even in the absence of the target sequence in the scDNA molecule [15-17]. Many p53 target sequences display an internal symmetry, being capable of assuming non-B DNA conformation [18]. These structures are known to be stabilized by negative DNA supercoiling [19]. Contemporary analysis of the symmetry of natural p53 target sequences showed a strong palindrome coupling between the p53 half-sites [20]. The target sites can be very often described as two inverted repeats and the two half-sites are highly repetitive. The symmetric nature of the p53 target sequence corresponds to the symmetric binding of the p53 tetramer [21]. Recent data provide strong evidence that the DNA binding activity of p53 is remarkably dependent on structural features within the target. In addition to DNA bending [13], formation of stem-loop or cruciform structure can enhance p53 DNA binding [22, 23]. Importantly, p53 had a dramatically greater affinity for a DNA microcircle bearing a target site than for the same but linear DNA [12]. The p53 protein has also been shown to co-operate in DNA binding with other proteins

recognizing and/or modulating the DNA structure, such as the cruciform binding protein HMGB1, topoisomerases and various proteins of chromatin remodeling complexes [24, 25]. HMGB1 selectively recognizes cruciform DNA [26] and is capable of interacting with DNA in a non-sequence-specific manner, causing a significant bending of the DNA double helix and acting as an activator of p53 sequence specific DNA binding [24]. The role of DNA superhelicity-induced cruciform extrusion as an event stimulating p53 sequence-specific DNA binding has been proposed [27] but has not been clearly demonstrated at the level of structural transitions of a particular target sequence upon changes of the DNA topology.

Here, we studied p53 binding to topologically constrained DNAs containing internally symmetrical or non-symmetrical target sites. Using a competition assay, we demonstrate a considerable correlation between the change of negative superhelix density, cruciform extrusion within the p53 target sites, and a remarkable extra enhancement of p53 DNA binding. The p53 targets having adopted cruciform structures always exhibited stronger p53 binding than p53 target sites not forming the cruciform or a cruciform formed within a p53 non-target sequence.

MATERIALS AND METHODS

DNA

Duplex oligonucleotides oCFNO (CATGATGTGATCACATCATG), oPGM2 (AGACATGCCTAGGCATGTCT), oPGM3 (AGGCATGCCTAGGCATGCCT), oPGM4 (AAACATGTTTAAACATGTTT), oPEV (AGGCTAGTCTGAGCATGTTC) with *Hind*III adapters were cut by *Hind*III and inserted into the *Hind*III site of SK. The ligated DNA was then used to transform electrocompetent cells of the Top10 strain of *E.coli* (Invitrogen). DNA from single colonies selected for *Amp* resistance were digested with *Pvu*II and electrophoresed. Clones with a *Pvu*II fragment longer than 448 bp were subjected to amplification with M13-20 primer, (5'GTAAAACGACGGCCAGT3') and with the oligo strand of the duplex oCFNO, oPGM2-4, oPEV. Constructs with defined length (84 bp) and orientation were selected. Constructs with cruciform without consensus sequence were denoted as pCFNO, synthetic sequences with target sequence with inverted repeat as pPGM2-4, synthetic sequences with target sequence without inverted repeat as pPEV. pPGM1 was prepared as described [15].

p53 protein

Wt human full-length (fl) p53 protein expressed in insect cells was prepared and characterized as described [15]. A molecular weight of 53kD for this postranslationally modified protein was used in all calculations.

Competition experiments

0.6 μg of the competitor, 0.4 μg of the indicator pPGM1/*Pvu*II fragments and p53 protein were mixed at 1/5 molar ratio (the ratio was calculated from the concentrations of DNA and p53 tetramers) in 20 μl of DNA binding buffer (5 mM Tris-HCl, pH 7.0, 1 mM EDTA, 50 mM KCl and 0,01% Triton X-100). The samples were incubated for 30 min on ice and loaded on a 1% agarose gel containing 0.33x TBE buffer, pH 8.0. Electrophoresis was performed for 4h at 100V (usually 4 V/cm) at low temperature (~6°C). The gels were stained with ethidium bromide, photographed, scanned and rendered digitally. The band intensities were quantified by Image QuaNT software. Standard deviations of these determinations, obtained with different competitors were calculated from 6 determinations of one competitor.

Proof of the cruciform structure by S1 nuclease cleavage

For the cruciform detection, 2 μg of plasmid were digested by S1 nuclease (2 U/μg DNA) for 2h at 37°C in the nuclease S1 buffer (30 mM sodium acetate pH 4.6, 280 mM NaCl, 1 mM $ZnSO_4$). After the cleavage, samples were precipitated by ethanol, dissolved in water and digested by the restriction endonuclease *Sca*I for 90 min.

Proof of the cruciform structure by modification by Osmium tetroxide 2,2' bipyridine

For detection of the cruciform in the p53 target of the pPGM2, we took advantage of the fact that the CTAG tetranucleotide in the center of the insert is recognized and cleaved by *Xsp*I restriction endonuclease. If osmium tetroxide complexes modify pyrimidines in the tetranucleotide, the cleavage is inhibited and a new band on the agarose gel occurs. Because two other restriction sites located in the vicinity of the CON sequence could give false results, we mutated A to G and G to A corresponding to 728 and 735 positions in the SK plasmid by means of a site-directed mutagenesis kit (Stratagene). For the modification reaction, 2 μg of plasmid were incubated with 2 mM Os,bipy complex in the p53-binding buffer at 37°C for 30min in the volume of 50ul. After the modification, the samples were precipitated by ethanol, dissolved in the buffer and treated with *Xsp*I endonuclease for 90min at 37°C.

Proof of the cruciform structure by 2D electrophoresis

For the cruciform analysis by means of two-dimensional electrophoresis [28], prepared topoisomers [29] were mixed, loaded on the 1.2% TAE-agarose gel and run in the first dimension at 40 V for 20 hours. After electrophoresis, the gel was soaked in the TAE buffer containing chloroquine at the concentration 2.5 μg/ml for 8 hours. For the second dimension, the gel was turned by 90° and electrophoresis was run upright to the first dimension in the TAE-chloroquine (2,5 μg/ml) buffer at 40 V for another 20 hours. During electrophoresis, the buffer was circulated between the anodic and cathodic spaces.

Preparation of plasmid DNA topoisomers

Topoisomeric samples were prepared according to [29]. Briefly, 40 μg of DNA per ml was incubated in a solution containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1

mM EDTA, 1 mM DTT and 20% glycerol (Promega). Appropriate concentrations of ethidium bromide (-σ=0.01 - 1.69μM, -σ=0.03 - 3.8μM, -σ=0.05 - 6.13μM, -σ=0.07 - 9.1μM) and DNA topoisomerase I (Promega) were added and samples were incubated at 37°C for 3 h. After complete relaxation, ethidium bromide and topoisomerase were removed by two-phenol extraction followed by three chloroform extractions and ethanol precipitation. The relaxed (covalently closed circular) DNA was prepared in the same way without ethidium bromide addition.

Determination of DNA superhelix density

Superhelix densities (-σ) of the plasmid DNA samples were determined by electrophoresis on agarose gels containing different concentrations of chloroquine (0, 1, 5, 10 μg per ml) [29, 30]. The electrophoresis was performed for 6 h at 100 V. The gels were stained with ethidium bromide, photographed, scanned and evaluated digitally. The mean superhelix densities of topoisomers were determined from the strongest band in the distribution.

RESULTS

As shown previously, the p53 protein binds preferentially to scDNA regardless of the presence or absence of the target sequence [15] and DNA supercoiling can enhance the p53 binding to certain target sites [27]. The positive effect of negative DNA supercoiling on the sequence-specific DNA binding was well pronounced, especially with targets exhibiting internal symmetry, being able to adopt cruciform structure under the negative superhelical stress (such as in pPGM1) but not those incapable of forming such structure due to the lack of inverted repeat symmetry (such as in pRGC). Here we focused on the effects connected with changes of the DNA negative superhelix density (the driving force of structural transition giving rise to the cruciform structures in inverted repeat DNA segments [19, 29]) on p53 binding to various plasmid DNAs.

We prepared plasmid DNA constructs involving (a) synthetic p53 target sequences with a perfect inverted repeat symmetry (pPGM2-4); (b) a perfect inverted-repeat but p53 non-target sequence (pCFNO) (c) an asymmetrical p53 target sequence (pPEV); (d) pBluescriptSK- (SK) vector as control. In agreement with previous data, all of these scDNAs bound p53 protein, forming in agarose gel ladders of bands due to p53-scDNA complexes migrating more slowly than DNA alone (Figure 1A). No significant differences between DNAs containing (Figure 1A, lanes 3-7) or not containing p53 target sequence (Figure 1A, lanes 1-2) or between DNAs containing internally symmetrical (Figure 1A, lanes 1, 4-7) or non-symmetrical (Figure 1A, lanes 2-3) target sites were observed using this simple, non-competitive gel shift assay, showing efficient p53 binding to any scDNA. With *Pvu*II digests of the same plasmid DNAs we observed specific p53 binding to the shorter (474-bp) fragments provided that these fragments involved a p53 target site (Figure 1B, lanes 1-4). No retardation of analogous fragments of SK and CFNO was detected in agreement with absence of the p53 target site.

To probe differences in relative p53 binding affinity to various scDNAs we used a competition assay proposed previously [27]. Briefly, the 474-bp fragment resulting from *Pvu*II cleavage of the pPGM1 DNA, containing a p53 target site, was used as an indicator substrate in the competition experiments. Binding of the protein to this fragment yielded a well resolved retarded band R (Figure 2, lane 2). Intensity

of this band was affected by the additions of the tested scDNAs. The scDNAs represented the competitors (Figure 2, lanes 3-9). Decrease of the R band intensity relative to the intensity detected in the absence of the competitors reflected relative affinity of p53 to the given competitor. Complete absence of the band R was taken as 100%.

The intensity of the indicator R band decreased by about 70-85% on addition of scDNA (of native $-\sigma$) with p53 target sequences possessing inverted repeat symmetry (pPGM1, pPGM2, pPGM3 and pPGM4), but only by approximately 50% on addition of a scDNA involving a p53 non-target inverted repeat (pCFNO). There was also a clear difference between DNAs lacking the inverted repeats. The intensity of the indicator R band was decreased by about 62% (pPEV, containing a non-symmetrical target site) and about 40% (SK containing neither target site nor a perfect inverted repeat) respectively. Taken together, the p53 target sequences with the inverted repeat symmetry were the best competitors, followed by a non-symmetrical p53-target and by DNA without any p53 target site with an inverted repeat.

Effect of DNA superhelix density variations on the p53 DNA binding

To study effects of the DNA topological state on p53 binding to various DNA substrates, we prepared sets of topoisomers of the plasmids SK, CFNO, pPEV and pPGM2 and employed them in the competitor assay (Figure 3). All DNAs exhibited increasing competitiveness towards the indicator pPGM1 fragment but courses of the dependences of p53 affinity to individual plasmid DNAs on $-\sigma$ differed remarkably. Relaxed ($-\sigma = 0$) circular duplex DNAs SK or CFNO (both not containing the p53 target site) bound p53 with similar affinities, causing about a 30 % decrease of the indicator band intensity (Figure 3). Apparent affinities of p53 to relaxed DNA's containing a target site (both pPGM2 and pPEV) were higher that those observed for the non-target plasmids but also similar to one another (50-55 % competition). Binding of p53 to SK increased slightly with increasing $-\sigma$, following an almost linear dependence and reaching 40 % competition for $-\sigma = 0.07$. The pPEV plasmid also displayed a linearly increasing dependence of p53 binding on the $-\sigma$, showing 60-65 % competition at $-\sigma = 0.07$. On the other hand, the behavior of the pPGM2 DNA was

different. While between -σ = 0 and 0.03 affinity of p53 to this plasmid followed practically the same trend as observed for pPEV, at more negative superhelix density a considerably steeper increase of binding with -σ was detected. At -σ = 0.05 the pPGM2 DNA caused almost 70 % competition (while pPEV 60 %) and at -σ = 0.07 even 85 % competition (60 – 65 % for pPEV). Interestingly, a break on the apparent binding affinity dependence on -σ was observed also for pCFNO which thus became a better substrate for p53 starting from -σ = 0.05 (but remained a substantially worse competitor than pPEV even at -σ = 0.07). The ability of all plasmids in their linearized forms to compete for p53 (not shown) was similar as observed for the respective DNA's in their relaxed (covalently closed circular) forms (Figure 3A).

Cruciform extrusion in plasmids involving inverted-repeat inserts

The above results showed that differences in p53 binding (competition ability) within the groups of plasmid DNA substrates either containing (pPGM2 or pPEV) or lacking (SK or pCFNO) the p53 target sites were not statistically significant (Figure 3A) when the DNAs were relaxed or slightly negatively supercoiled (-σ ≤ 0.03). However, after the negative superhelix density reached a certain critical value (-0.05), the pPGM2 plasmid (with the internally symmetrical target site) became a significantly "better" competitor than pPEV (with non-symmetrical target site). Such behavior suggests that formation of the cruciform structure within the symmetrical p53 target site of pPGM2 DNA at sufficient –σ may be responsible for the observed extra enhancement of p53 binding. To test this assumption we performed an analysis of structural transitions in the pPGM2 based on S1 nuclease cleavage (Figure 4A), on 2D agarose gel electrophoresis (Figure 4B) [19], and by modification by Osmium tetroxide 2,2' bipyridine. The S1 nuclease cleaves selectively single-stranded DNA and base-unpaired sited in dsDNA (such as loop parts of hairpins and cruciforms). Cleavage of sc pPGM2 (at native –σ) by S1 followed by restrictase *Sca*I produced two specific DNA fragments (1720bp, 1267bp), indicating presence of the cruciform structure within the p53 target site of this scDNA (see scheme in Fig 4C). Upon the same treatment of relaxed pPGM2 we obtained only full-length linear DNA (2987bp), suggesting absence of the cruciform structure representing the specific cleavage site for the S1 nuclease (Fig 4C, lane 1). The same experiments were performed with

pPGM2 topoisomers differing in the –σ levels. We did not observe any indication of the specific S1 cleavage at -σ=0.01 or 0.03, but we detected the cruciform in sc pPGM2 at superhelical densities –0.05 or –0.07 (Figure 4A, lanes 4,5). A comparison of the band intensities resulting from the S1 cleavage suggested that the cruciform content in pPGM2 increased strongly from -σ=0,05 to 0.07. (The pBluescript vector, used for construction of all plasmid substrates in this work, contains a few imperfect inverted repeats [31, 32] that may undergo local structural transitions in the scDNA. The weaker bands in lanes 4-6, 8-11) of Figure 4, corresponding to about 2211 and 776 bp DNA fragments, may have arisen from S1 cleavage close to replication origin where the major intrinsic inverted repeat is located). We obtained the same results using a single-strand selective chemical probe osmium tetroxide, 2,2' bipyridine [19, 33] combined with a restriction cleavage inhibition assay (not shown). We detected extrusion of the cruciform structure in pPGM2 also by DNA 2D gel electrophoresis. Presence of the supercoil-stabilized cruciform was indicated by the shift of the DNA bands curve [28] approximately at -σ=0.05 (Figure 4B, arrow), thus confirming the S1 cleavage experiments. The superhelix density level critical for the cruciform appearance (-σ=0.05) correlated well with the -σ value at which the pPGM2 (and also pCFNO) displayed the break on the superhelix density-relative p53 binding dependences (Figure 3A).

Similarly, we detected the negative superhelicity-dependent structural transitions in the scDNAs involving the (p53 non-target) inverted repeat (pCFNO Fig 4A, lane 10) as well as in plasmids containing other symmetrical p53 target sites (pPGM3-4, not shown). S1 cleavage occurred in all cases at sites of anticipated cruciform extrusion (i.e., within the inserted inverted repeats). On the other hand, no indication of a distinct supercoil-dependent structural transition was detected under the same conditions with SK and pPEV (Figure 4, lane 11, 9) plasmids not containing sequence motifs prerequisite for such transitions (Figure 3B).

DISCUSSION

Presence of cruciform structure in p53 target sequences is an important attribute in p53-DNA binding

Numerous recent data have provided evidence that DNA binding of p53 is strongly dependent on structural features of the target DNA [13, 17, 21, 34]. It has been shown that full length (fl) p53 was able to bind sequence-specifically to target oligonucleotides adopting stem-loop structures in the absence of "activating" antibodies such as PAb421 [18]. Later Prives et al. [12] showed that a p53 target site within a topologically constrained DNA minicircle was bound by the fl p53 with a higher affinity than the same target site in non-constrained linear DNA. Our observations [27] revealed enhanced binding of the fl p53 protein to certain target sites within large (~3 kB) plasmid DNAs, compared to the same target sites in 20-mer oligonucleotides or in the same but linearized plasmids. Strikingly, effects of negative DNA superhelicity were especially well pronounced for symmetrical target sites able to adopt cruciform structures under topological constraint in the negatively scDNA. Hence, it has been tentatively concluded that cruciform extrusion was the cause for the enhanced sequence-specific p53-DNA binding.

In this paper we demonstrate for the first time that relative affinity of the p53 protein to the same (internally symmetrical) p53 target site is remarkably changed upon extrusion of a cruciform structure within the inverted repeat featuring the target site. Thus, the structural transition in the binding site upon topological stress represents a switch between two apparently distinct states differing in the affinity of p53-DNA recognition (Figure 3B). Change from the low-affinity to the high-affinity binding state correlated well with the jump of the topoisomer spots in the 2D agarose gel electrophoresis and the cruciform extrusion starting at $-\sigma$=0.05 (Figure 4). On the other hand, relative binding of p53 to the pPEV scDNA (in which the p53 target site was non-symmetrical and thus unable of forming the cruciform structure, Figure 3B) had a monophasic, approximately linear dependence on $-\sigma$ (Figure 3A). This trend reflected the known ability of fl p53 to bind selectively scDNA with native superhelix density regardless of the presence or absence of a target site. The SK vector, containing neither a p53 target site nor a perfect palindromic DNA segment, exhibited

a dependence that was parallel to that observed for the pPEV but was shifted to lower relative binding affinities (Figure 3). Hence, in the pPEV the overall p53 binding affinity appears to be a simple sum of the sequence-specific binding to the target site (not undergoing a distinct structural transition upon changes of $-\sigma$), and the supercoil-selective (but sequence non-specific) p53 DNA binding. Interestingly, the pCFNO displayed the same behavior as the SK for $-\sigma \leq 0.03$ but exhibited certain extra enhancement of p53 binding at superhelix densities sufficient for the cruciform extrusion. This suggests that the cruciform structure itself (lacking features of the sequence-specific p53 binding site) can be bound by the protein with certain selectivity, in agreement with results obtained previously by scanning force microscopy [11]. The difference in competition abilities of plasmids pCFNO and SK at $-\sigma=0.07$ was about 10%, in contrast to at least 20 % difference between plasmids pPGM2 (or pPGM3-4) and pPEV. The best substrate for p53 protein binding is therefore a p53 target sequence with the extruded cruciform structure. Natural p53 responsive elements often possess more or less perfect inverted repeat symmetry [18], and our preliminary experiments with some of them (such as mdm2 or gaad45) revealed similar effects of negative DNA superhelicity as reported here for the synthetic p53 targets (not shown). A combination of the structural and sequence features of the DNA substrate can thus be important for fine tuning the protein DNA interaction.

The binding of p53 to its target DNA presents an apparent steric problem: p53 target sites may but need not involve insertions of variable DNA segments between the half-site. The question arises as to how p53 is able to bind such a broad spectrum of targets differing in both nucleotide sequence and conformation. It is unclear just how p53 can recognize specific DNA sequences, and at the same time how it can recognize different geometries with sequence variability as cruciform structure. Cruciform extrusion itself might solve the possible problem with the intervening sequences that, in the cruciform structure, are displaced into the single-stranded loops while the target half-sites are located in the hairpin stems. It simultaneously renders the binding site a considerable flexibility at the four-way junction. For target sites without the intervening stretches, central parts of the binding sequence are inherently located within the cruciform apical loops, which may cause another

problem for the p53-DNA recognition. Nevertheless, Kim et al. have demonstrated [18] sequence-specific p53 binding to various hairpin oligonucleotide substrates, including those mimicking the p53 target (half) sites having adopted the cruciform (stem-loop) structure.

In more complex DNA, such as scDNA or DNA in the chromatin environment, specific structural profiles may be influenced by DNA supercoiling and/or by chromatin remodeling activities maintaining the structural fluidity of the chromatin [35].It seems that flexibility of the p53 target sequences in long DNA targets can be one of the crucial factors in p53-DNA binding. Determination of the p53 protein bound to DNA by X-ray crystallography showed that the p53 protein bends the DNA by about 20° [13, 36]. Our results are in good agreement with the new view of p53 regulatory tasks in the context of chromatin assembly in eukaryotic cells. In this view, the p53 protein will not bind simultaneously to all p53 targets sequences, but only to those p53 targets that exhibit a structural architecture that is compatible with p53-preferred DNA structure. Cruciform target sites stabilized by DNA supercoiling can be one of the favorite structures for effective p53/DNA binding *in vivo*. The correlation between p53 binding affinity and the presence of cruciform structures in topologically constricted DNAs reflects the complexity of protein-DNA interaction. Transient supercoils occurring in the eukaryotic genome during DNA replication, transcription (processes that involve a local separation of DNA strands [37, 38]) as well as in result of proteins binding [39, 40] may facilitate formation of secondary structures in DNA. Moreover, active chromatin remodeling is a typical feature for many promotors and is essential for gene transcription [41]. The strong impact of topological conformation of DNA on p53 sequence specific binding *in vitro* predicts that the ability of p53 to bind its target sites will be strongly influenced by factors capable of modifying chromatin structure in a living cell. Changes in DNA supercoiling and stabilization of the non-B DNA structures may thus up- and down- regulates p53-DNA binding abilities.

ACKNOWLEDGEMENTS

We thank M. Kotlyar for critical reading of the manuscript. This work was supported by grants of GACR 301/07/P160 and 301/05/0416, GA ASCR IAA500040701, IAA500040513 and B500040502, IGS UO 1074 and by the Academy of Sciences of the Czech Republic, grants AV0Z50040507, AV0Z50040702.

REFERENCES

1 Hainaut, P., Hernandez, T., Robinson, A., Rodriguez-Tome, P., Flores, T., Hollstein, M., Harris, C. C. and Montesano, R. (1998) IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. Nucleic Acids Res. **26**, 205-13

2 Hupp, T. R., Lane, D. P. and Ball, K. L. (2000) Strategies for manipulating the p53 pathway in the treatment of human cancer. Biochem. J. **352 Pt 1**, 1-17

3 Baroni, T. E., Wang, T., Qian, H., Dearth, L. R., Truong, L. N., Zeng, J., Denes, A. E., Chen, S. W. and Brachmann, R. K. (2004) A global suppressor motif for p53 cancer mutants. Proc. Natl. Acad. Sci. U S A **101**, 4930-5

4 Brazda, V., Muller, P., Brozkova, K. and Vojtesek, B. (2006) Restoring wild-type conformation and DNA-binding activity of mutant p53 is insufficient for restoration of transcriptional activity. Biochem. Biophys. Res. Commun. **351**, 499-506

5 el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W. and Vogelstein, B. (1992) Definition of a consensus binding site for p53. Nat. Genet. **1**, 45-9

6 Qian, H., Wang, T., Naumovski, L., Lopez, C. D. and Brachmann, R. K. (2002) Groups of p53 target genes involved in specific p53 downstream effects cluster into different classes of DNA binding sites. Oncogene **21**, 7901-11

7 Wei, C. L., Wu, Q., Vega, V. B., Chiu, K. P., Ng, P., Zhang, T., Shahab, A., Yong, H. C., Fu, Y., Weng, Z., Liu, J., Zhao, X. D., Chew, J. L., Lee, Y. L., Kuznetsov, V. A., Sung, W. K., Miller, L. D., Lim, B., Liu, E. T., Yu, Q., Ng, H. H. and Ruan, Y. (2006) A global map of p53 transcription-factor binding sites in the human genome. Cell **124**, 207-19

8 McKinney, K., Mattia, M., Gottifredi, V. and Prives, C. (2004) p53 linear diffusion along DNA requires its C terminus. Mol. Cell **16**, 413-24

9 Brazda, V., Jagelska, E. B., Fojta, M. and Palecek, E. (2006) Searching for target sequences by p53 protein is influenced by DNA length. Biochem. Biophys. Res. Commun. **341**, 470-7

10 Degtyareva, N., Subramanian, D. and Griffith, J. D. (2001) Analysis of the binding of p53 to DNAs containing mismatched and bulged bases. J. Biol. Chem. **276**, 8778-84

11 Jett, S. D., Cherny, D. I., Subramaniam, V. and Jovin, T. M. (2000) Scanning force microscopy of the complexes of p53 core domain with supercoiled DNA. J. Mol. Biol. **299**, 585-92

12 McKinney, K. and Prives, C. (2002) Efficient Specific DNA Binding by p53 Requires both Its Central and C-Terminal Domains as Revealed by Studies with High-Mobility Group 1 Protein. Mol. Cell. Biol. **22**, 6797-808.

13 Nagaich, A. K., Appella, E. and Harrington, R. E. (1997) DNA bending is essential for the site-specific recognition of DNA response elements by the DNA binding domain of the tumor suppressor protein p53. J. Biol. Chem. **272**, 14842-9

14 Deppert, W. (1996) Binding of MAR-DNA elements by mutant p53: possible implications for its oncogenic functions. J. Cell. Biochem. **62**, 172-80

15 Palecek, E., Vlk, D., Stankova, V., Brazda, V., Vojtesek, B., Hupp, T. R., Schaper, A. and Jovin, T. M. (1997) Tumor suppressor protein p53 binds preferentially to supercoiled DNA. Oncogene **15**, 2201-9.

16 Palecek, E., Brazdova, M., Brazda, V., Palecek, J., Billova, S., Subramaniam, V. and Jovin, T. M. (2001) Binding of p53 and its core domain to supercoiled DNA. Eur. J. Biochem. **268**, 573-81.

17 Brazda, V., Palecek, J., Pospisilova, S., Vojtesek, B. and Palecek, E. (2000) Specific modulation of p53 binding to consensus sequence within supercoiled DNA by monoclonal antibodies. Biochem. Biophys. Res. Commun. **267**, 934-9.

18 Kim, E., Albrechtsen, N. and Deppert, W. (1997) DNA-conformation is an important determinant of sequence-specific DNA binding by tumor suppressor p53. Oncogene **15**, 857-69.

19 Palecek, E. (1991) Local supercoil-stabilized DNA structures. Crit. Rev. Biochem. Mol. Biol. **26**, 151-226

20 Ma, B., Pan, Y., Zheng, J., Levine, A. J. and Nussinov, R. (2007) Sequence analysis of p53 response-elements suggests multiple binding modes of the p53 tetramer to DNA targets. Nucleic Acids Res. **35**, 2986-3001

21 Kitayner, M., Rozenberg, H., Kessler, N., Rabinovich, D., Shaulov, L., Haran, T. E. and Shakked, Z. (2006) Structural basis of DNA recognition by p53 tetramers. Mol. Cell **22**, 741-53

22 Gohler, T., Reimann, M., Cherny, D., Walter, K., Warnecke, G., Kim, E. and Deppert, W. (2002) Specific interaction of p53 with target binding sites is determined by DNA

conformation and is regulated by the C-terminal domain. J. Biol. Chem. **277**, 41192-203

23 Zlatanova, J. and van Holde, K. (1998) Binding to four-way junction DNA: a common property of architectural proteins? Faseb J. **12**, 421-31.

24 Jayaraman, L., Moorthy, N. C., Murthy, K. G., Manley, J. L., Bustin, M. and Prives, C. (1998) High mobility group protein-1 (HMG-1) is a unique activator of p53. Genes. Dev. **12**, 462-72

25 Lee, D., Kim, J. W., Seo, T., Hwang, S. G., Choi, E. J. and Choe, J. (2002) SWI/SNF complex interacts with tumor suppressor p53 and is necessary for the activation of p53-mediated transcription. J. Biol. Chem. **277**, 22330-7

26 Bianchi, M. E., Beltrame, M. and Paonessa, G. (1989) Specific recognition of cruciform DNA by nuclear protein HMG1. Science **243**, 1056-9

27 Palecek, E., Brazda, V., Jagelska, E., Pecinka, P., Karlovska, L. and Brazdova, M. (2004) Enhancement of p53 sequence-specific binding by DNA supercoiling. Oncogene **23**, 2119-27

28 Bowater, R., Aboul-ela, F. and Lilley, D. M. J. (1992) in Methods in Enzymology, vol. 212 (Lilley, D. M. J. and Dahlberg, J. E., eds.), pp. 105-120, Academic Press, Inc., London

29 Singleton, C. K. and Wells, R. D. (1982) Relationship between superhelical density and cruciform formation in plasmid pVH51. J. Biol. Chem. **257**, 6292-5.

30 Bates, A. D. and Maxwell, A. (2005) DNA Topology, Oxford University Press, Oxford

31 Short, J. M., Fernandez, J. M., Sorge, J. A. and Huse, W. D. (1988) Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res. **16**, 7583-600

32 Lin, C. T., Lyu, Y. L. and Liu, L. F. (1997) A cruciform-dumbbell model for inverted dimer formation mediated by inverted repeats. Nucleic Acids Res. **25**, 3009-16

33 Palecek, E. (1992) Probing of DNA structure with osmium tetroxide complexes in vitro. Meth. Enzymol. **212**, 139-155

34 Kim, E. and Deppert, W. (2003) The complex interactions of p53 with target DNA: we learn as we go. Biochem. Cell. Biol. **81**, 141-50

35 Kingston, R. E. and Narlikar, G. J. (1999) ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes. Dev. **13**, 2339-52

36 Ho, W. C., Fitzgerald, M. X. and Marmorstein, R. (2006) Structure of the p53 core domain dimer bound to DNA. J. Biol. Chem. **281**, 20494-502

37 Pearson, C. E., Zannis-Hadjopoulos, M., Price, G. B. and Zorbas, H. (1995) A novel type of interaction between cruciform DNA and a cruciform binding protein from HeLa cells. Embo J. **14**, 1571-80

38 Pearson, C. E., Zorbas, H., Price, G. B. and Zannis-Hadjopoulos, M. (1996) Inverted repeats, stem-loops, and cruciforms: significance for initiation of DNA replication. J. Cell. Biochem. **63**, 1-22

39 Peter, B. J., Ullsperger, C., Hiasa, H., Marians, K. J. and Cozzarelli, N. R. (1998) The structure of supercoiled intermediates in DNA replication. Cell **94**, 819-27.

40 van Holde, K. and Zlatanova, J. (1994) Unusual DNA structures, chromatin and transcription. Bioessays **16**, 59-68.

41 Brettingham-Moore, K. H., Rao, S., Juelich, T., Shannon, M. F. and Holloway, A. F. (2005) GM-CSF promoter chromatin remodelling and gene transcription display distinct signal and transcription factor requirements. Nucleic Acids Res. **33**, 225-34

FIGURE LEGENDS

Figure 1

(A) Binding of the p53 protein to supercoiled DNAs.

0.4 μg sc pCFNO (lane 1), sc SK (lane 2), sc pPEV (lane 3), sc pPGM1 (lane 4), sc pPGM2 (lane 5), sc pPGM3 (lane 6), sc pPGM4 (lane 7) DNAs were incubated with 0.35 μg of wt p53 (lanes 1-9) in 50 mM KCl, 5 mM Tris-HCl (pH 7.6) and 0.01% Triton X-100 on ice for 30 min. Samples were electrophoresed on 1% agarose gel at 100 V and 4°C for 3-4 h. Lane 8 contains DNAs of sc SK without p53 protein. (B) Binding of the p53 protein to DNA fragments. Plasmids pPEV (lane 1), pPGM1 (lane 2), pPGM2 (lanes 3, 7), pPGM3 (lane 4), pCFNO (lane 5), SK (lane6) were cleaved by *Pvu*II into two fragments; shorter one (474bp) contains the p53 target site except SK and pCFNO (lanes 5 and 6). The fragments were incubated with 0.35 μg of wt p53 (lanes 1-6) in 50mM KCl, 5 mM Tris-HCl (pH 7.6) and 0.01% Triton X-100 on ice for 30 min. There is a control without p53 protein in lane 7.

Figure 2

(A) Competition of superhelical DNAs for p53 SSDB to p53 target in lin 474bp fragment.

0.4 μg of indicator pPGM1/*Pvu*II (lanes 1-9), with 0.6 μg of sc DNA of SK (lane 3), pPGM1 (lane 4), pPGM2 (lane 5), pPGM3 (lane 6), pPGM4 (lane 7), pPEV (lane 8) and pCFNO (lane 0) were incubated with 0.55 μg of wt p53 (lanes 2-9) in 50mM KCl, 5 mM Tris-HCl (pH 7.6) and 0.01% Triton X-100 on ice for 30 min. (B) Bar graph of increasing p53 binding to competitor DNAs expressed as the inverted percentage of band R without competitor DNAs (lane 2).

Figure 3

(A) Competition of linear DNA, relaxed DNA and topoisomers for p53 SSDB to p53 target in lin 474bp fragment.

0.4 μg of indicator pPGM1/*Pvu*II with 0.6 μg of competitor DNAs were incubated with 0.55 μg of wt p53 in 50mM KCl, 5 mM Tris-HCl (pH 7.6) and 0.01% Triton X-100 on ice for 30 min. The graph of increasing p53 binding to competitor DNAs expressed as the inverted percentage of band R without competitor DNAs (pPGM2 – full, black,

diamonds, pPEV – short dashed, square, pCFNO – full, triangle, SK – long dashed, square). (B) Scheme of the DNA structure of the pPGM2, pPEV, pCFNO, and SK in linear (relaxed) and superhelical state. The grey color denotes p53 responsible sequence, the bold arrow denotes inverted repeats in DNA sequence.

Figure 4

Evidence of cruciform structure in topoisomers of pPGM2

(A) Evidence of cruciform in topoisomers of pPGM2 by S1 nuclease cleavage. The plasmids: pPGM2 relaxed (lane 1), pPGM2 -σ=0.01 (lane 2), pPGM2 -σ=0.03 (lane 3), pPGM2 -σ=0.05 (lane 4), pPGM2 -σ=0.07 (lane 5), sc pPGM2 (lane 6), sc pCFNO (lane 8), sc pPEV (lane 9), sc pPGM2 (lane 10), sc SK (lane 11) undergone cleavage by S1 nuclease and then by treatment by *Sca*I restriction endonuclease. SK digested by *Bgl*I (lane 7) was used as a control. Bands resulting from the nuclease S1 cleavage at the cruciform site in pPGM2 are about 1820 and 1167 bp. (B) Evidence of cruciform in topoisomers of pPGM2 by 2D electrophoresis. Arrow shows –σ=0.05 (for details see Materials and Methods). (C) Proof of the cruciform structure by S1 nuclease cleavage – scheme (a) The result of S1/*Sca*I cleavage without presence of cruciform. (b) The result of the S1/*Sca*I cleavage in presence of cruciform. (c) The control cleavage by *Bgl*I restriction enzyme.

Biochemical Journal Immediate Publication. Published on 14 Feb 2008 as manuscript BJ20071648

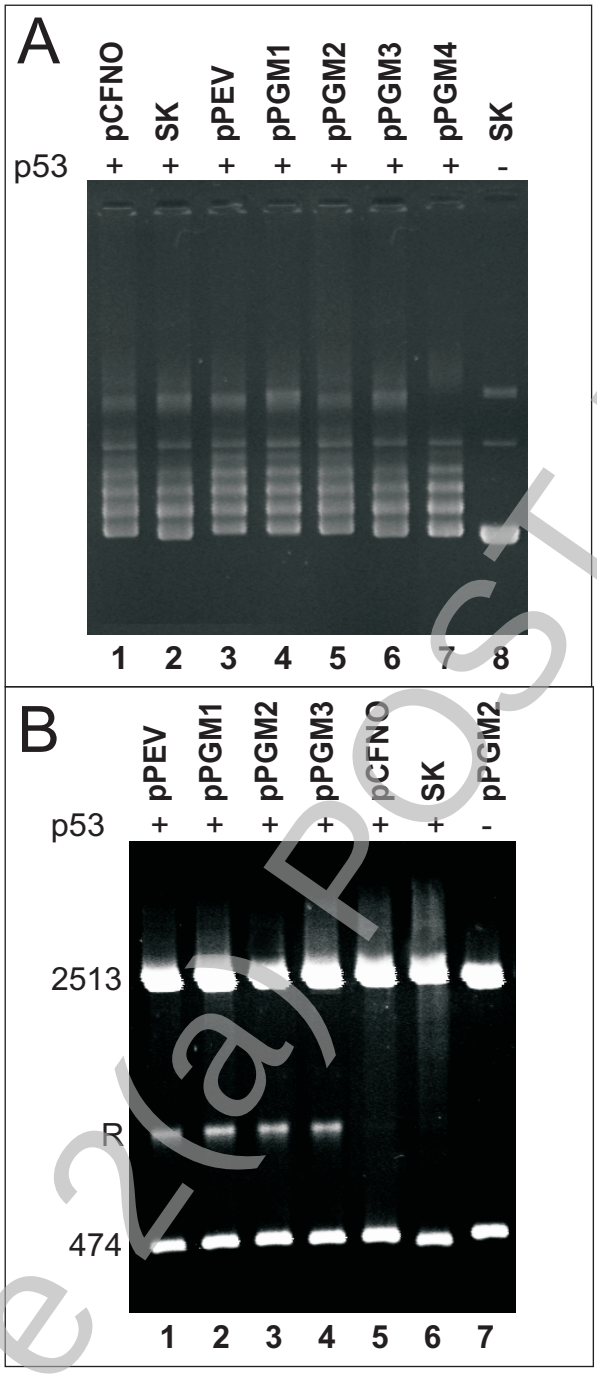

Fig. 1

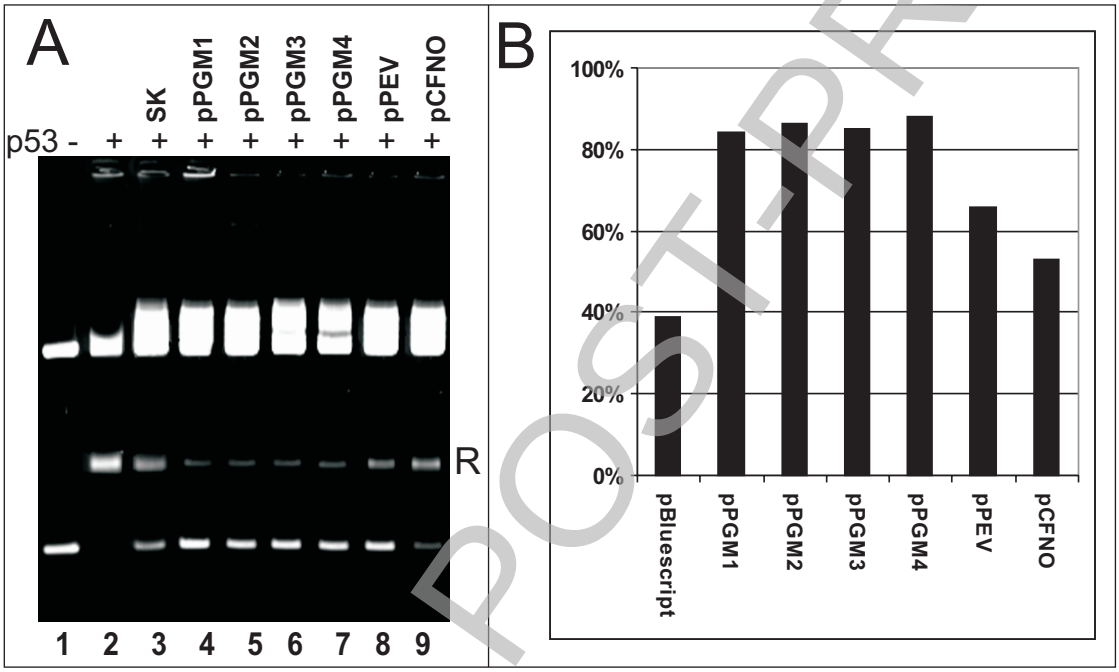

Fig. 2

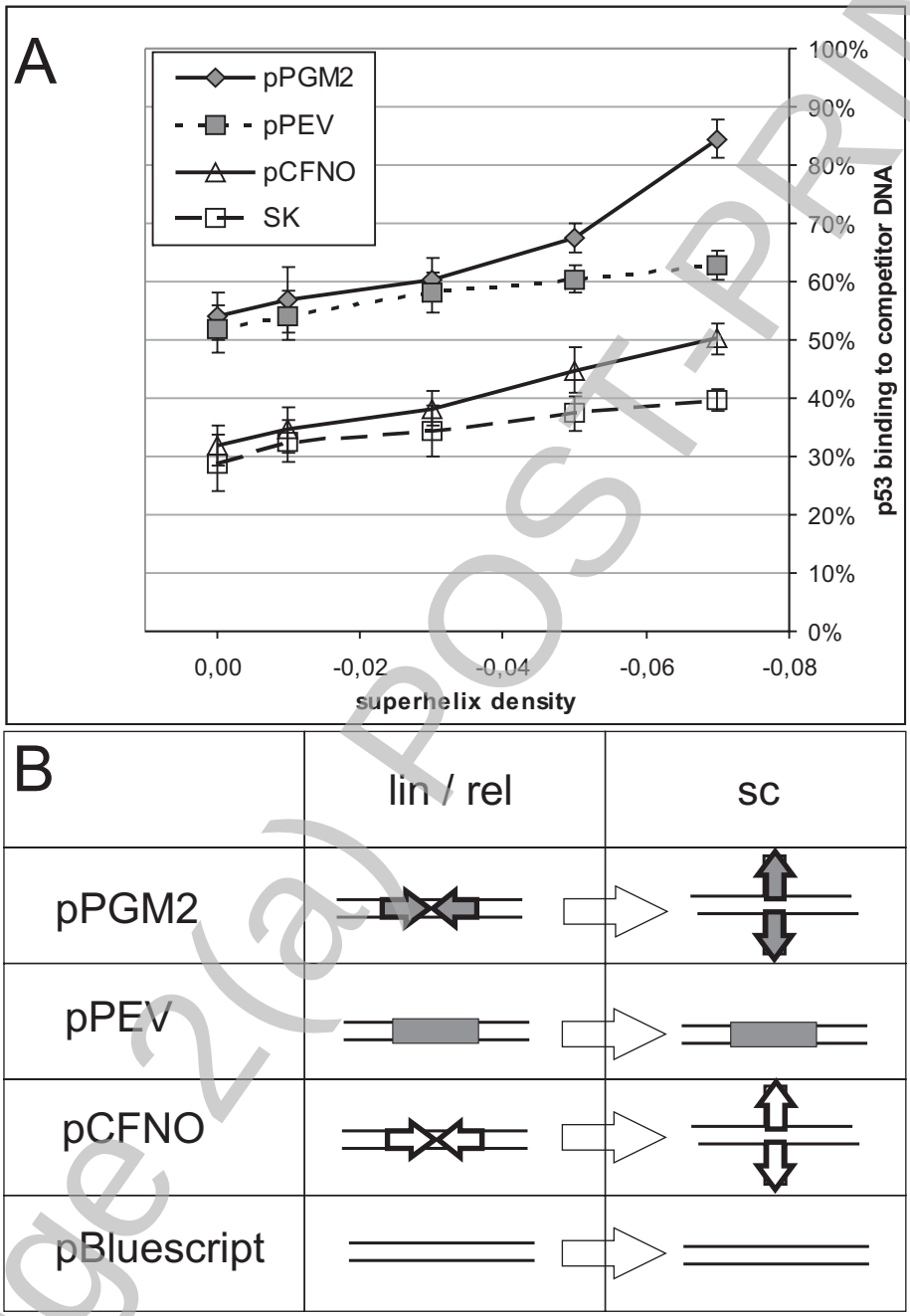

Fig. 3

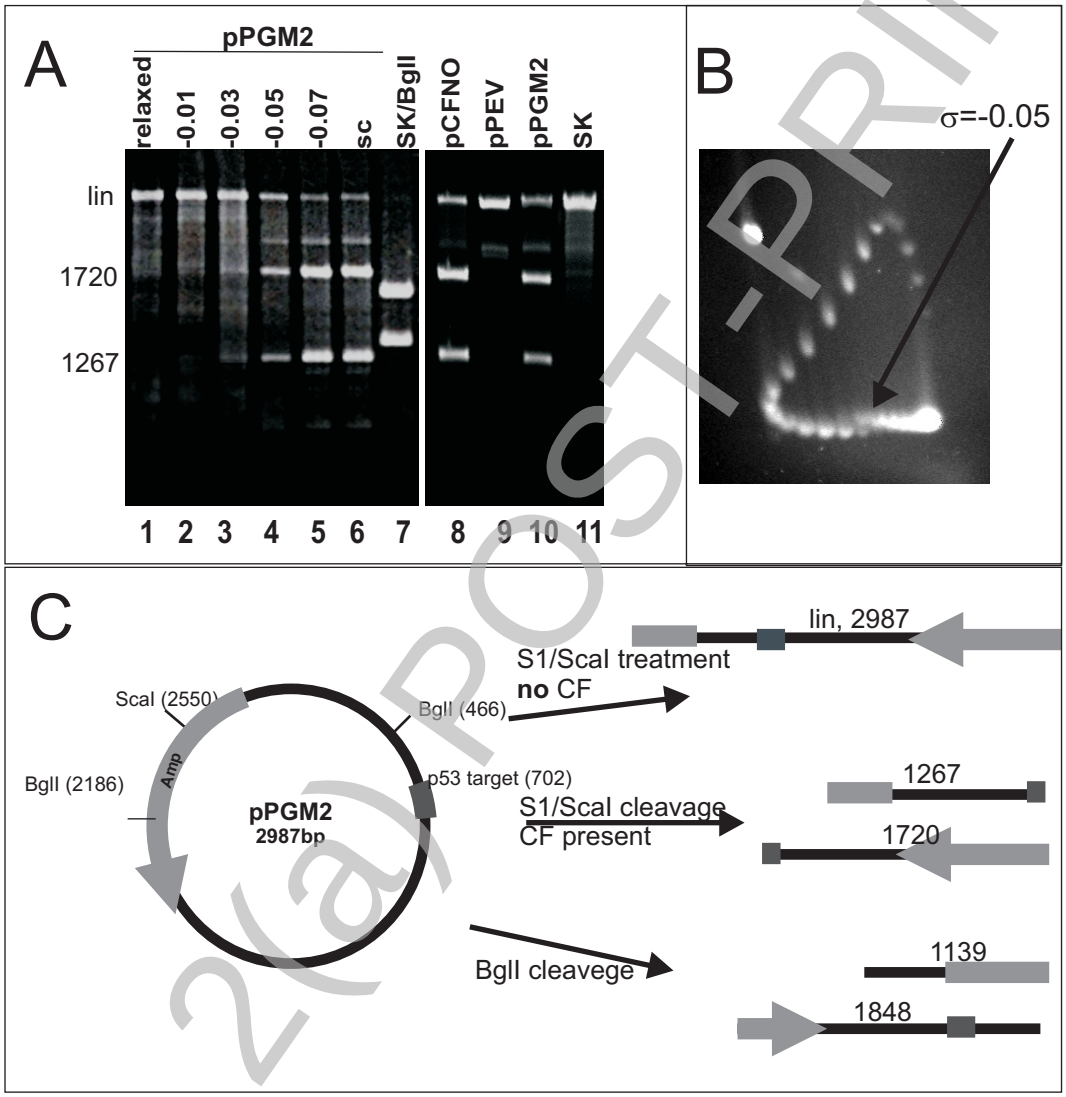

Fig. 4