Two Wavelength High Intensity Irradiation for the Effective Crosslinking of DNA to Protein

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ABSTRACT

Protein-DNA crosslinking is an important method to study protein-DNA interactions. Crosslinking by short pulsed UV lasers is a potentially powerful tool that results in efficient crosslinking, apparently by a two photon process. However, the major problem in using UV laser crosslinking is that the conditions which lead to high crosslinking efficiency also result in high DNA damage.

Previously, it has been shown that a combination of femtosecond laser pulses at two different wavelengths, in the UV (266 nm) and the visible range (400 nm), increases the effective crosslinking yield (i.e. higher crosslinking yields with reduced DNA damage). This new strategy has the advantage that the intensity of the UV pulse for the first excitation step can be kept low, leading to lower UV-induced DNA damage and the second pulse at a visible wavelength can provide enough energy for the UV excited bases to cross their ionization threshold without damaging the DNA .

The objective of this thesis project was to develop a novel UV laser cross-linking technique that would permit higher effective crosslinking yields with the commonly used pulses in the nanosecond (ns)

range. To serve this purpose we tried to extend the two-wavelength femto second laser irradiation approach to longer duration pulses.

We chose MBP-PIF3 protein and its target G-box DNA motif as a model system. Before ultraviolet irradiation of the protein-DNA complexes in vitro, the specific binding interaction of purified MBP-PIF3 protein with the G-box DNA motif was studied by Electrophoretic Mobility Shift Assay (EMSA).

We irradiated the PIF3/DNA complexes with different laser systems (i.e. Nd:YAG and Dye lasers) and their combinations. We were expecting to see that the combination of UV laser pulses (260nm) with longer wavelength dye laser pulses (480nm) will produce higher effective crosslink yields relative to the yield from the UV pulses alone.

However we could not detect any crosslinked MBP-PIF3/DNA bands by denaturing SDS-PAGE after irradiation of protein-DNA complex with UV laser pulses (266 nm, 5 ns, and ~5 mJ/pulse) alone or with UV (266 nm, 5 ns, and ~5 mJ/pulse) and blue laser pulses (480 nm, 800ns, and 60 mJ/pulse) together.

It may be necessary in the future to experimentally determine the optimum range of photon flux for the PIF3/DNA complex to obtain maximum amount of crosslinking. Alternatively, this new approach could be tested on a different DNA-binding protein that has a greater propensity to undergo UV-promoted DNA crosslinking.

INTRODUCTION

PHYTOCHROME SIGNALING and PIF3

Plants have different photoreceptors which transduce informational light signals received from the environment to photoresponsive genes. The first group of photoreceptors is cryptochromes (Cashmore et al., 1999) and other blue/UV light absorbing receptors (Briggs et al., 2000). The second and best characterized group is phytochromes which are red (R) and far-red (FR) light absorbing receptors (Neff et al., 2000).

Phytochrome molecule is a soluble, dimeric chromoprotein consists of two ~125K subunits with a single covalently linked tetrapyrrole chromophore and it has an important regulatory property of photoreversible conversion between its R-absorbing biologically inactive Pr form and its FR-absorbing biologically active Pfr form (Quail, 1997 and Smith, 2000).

In Arabidopsis, the phytochrome family has five members, phytochromes (phy) A-E and each member has differential but sometimes overlapping functions. PhyA and phyB have different photosensory specificities: continuous monochromatic FR (FRc) is detected by phyA whereas continuous monochromatic R (Rc) is detected by phyB. It has

also been shown that phyC, phyD and phyE have different functions to transduce light signals than phyA and phyB (Yamaguchi et al., 1999 and Smith, 2000).

There was little information about the initial step of the phytochrome signal transduction pathway until Quail and his coworkers identified a phytochrome-interacting factor, PIF3, by using a yeast two-hybrid screen (Ni et al., 1998). PIF3 is likely a single-copy gene. It was mapped to chromosome 1 near PHYA using an arrayed YAC library. PIF3 protein contains PAS, Per-Arnt-Sim-like domain; NLS, bipartite nuclear localization signal motif and bHLH, basic helix-loop-helix domain**.** It was localized to the nucleus in transient transfection assays, indicating an important role in regulation of gene expression (Ni et al., 1998). PIF3 was showed to function in both phyA and phyB signal transduction pathways as a result of its binding to C-terminal domains of both phyA and phyB. Therefore, PIF3 has been assigned as an important player of direct signaling pathway from the phytochromes to the photoresponsive genes (Zhu et al., 2000).

Sakamoto and Nagatani, in 1996, proposed that phyB is translocated from cytoplasm to the nucleus after the exposion of Arabidopsis seedlings to red. In 1999 Yamaguchi and coworkers showed that treatment of the transgenic Arabidopsis seedlings with continuous red

light induces accumulation of phyB-GFP fusion in the nucleus (Sakamoto and Nagatani, 1996, Yamaguchi et al., 1999).

As a conclusion of this information one of the strongest proposed mechanisms explains the PIF3 and phytochromes interaction such that phytochrome is cytoplasmic and PIF3 is nuclear in the dark, upon light absorption Pfr formation induces the translocation of phytochrome from cytoplasm to the nucleus where it can interact with its signaling partner PIF3. The PAS domain of PIF3 protein might be the part of protein-protein interactions to form homo- or heterodimerization and PIF3 and phytochromes may also interact through their PAS domains (Yamaguchi et al., 1999, Ni et al., 1999, and Zhu et al., 2000).

It was shown that full-length phyB binds PIF3 in vitro only in its active form and dissociates after conversion to its inactive form (Zhu et al., 2000). It has been demonstrated that the binding of full-length phytochrome B to PIF3 is photoregulated, occurring in its biologically active Pfr form. PhyB can bind to PIF3 in darkness after photoconversion to its active form, without the need for continuous photoactivation. Once bound reconversion to its inactive Pr form causes the rapid dissociation of phyB from PIF3. The rapid and reversible binding mechanism of phyB to PIF3 by means of photoinduced switching between the active and inactive forms of the phyB might be important in regulation of signal flow to PIF3,

which as a transcriptional regulator regulates photoresponsive target genes (Ni et al., 1999).

In the literature it has been stated that the majority of the bHLH proteins bind as dimers to the E box motif of the DNA which has the core sequence CANNTG and the palindromic G box motif CACGTG as a specific member of the E box family is found in many plant gene promoters (Ni et al., 1998). G-box motif binds not only bHLH proteins, but also members of the bZIP family of the DNA-binding proteins such as HY5. PIF3, with an arginine residue at position 13 in the bHLH domain, is the member of Group B subclass of the bHLH family known to bind the G-box motif (Quail, 2000).

According to the research done by Martinez-Garcia and coworkers in 2000, PIF3 as a transcription factor binds specifically to a G-box motif found in light-regulated gene promoters and photoactivated phyB binds to G-box-bound PIF3. PIF3 might bind its specific DNA sequence as a homodimer according to the previously studied structure of DNA-bHLH protein complexes. Moreover it was shown that the bHLH domain of PIF3 by itself is sufficient for sequence-specific binding to the G-box motif and phyB binds specifically to DNA-bound PIF3 only after R light-driven conversion to its active Pfr form (PfrB). However phyB does not bind to the truncated fragment of PIF3 containing only the bHLH domain when it

is in contact with its target sequence which indicates that some different factors other than bHLH domain of PIF3 are also required in phyB-PIF3 interaction. Upon exposure to FR pulses binding of phyB to DNA-bound PIF3 rapidly reversed by conversion of phyB to its inactive Pr form which proves that the biologically active form of phyB is required in its interaction with G-box bound PIF3 (Martinez-Garcia et al., 2000).

PIF3 exhibited interaction with G-box containing sequences from the promoters of a variety of light-regulated genes and did not interact with any other photoresponsive genes without its specific sequence motif which means some phytochrome-responsive genes may have different pathways independent of PIF3. It can be concluded that PIF3 functions to direct phyB specifically to the promoters of several phytochrome responsive genes in early direct signaling pathways of phytochromes (Martinez-Garcia et al., 2000).

According to the data from the studies of Zhu and coworkers, phyA also binds selectively and reversibly to PIF3 upon photoconversion to its active conformer Pfr, but the apparent affinity of phyA for PIF3 is 10-fold lower than of phyB which suggests that PIF3 has a dominant role in phyB signaling, but may have only minor role in phyA signaling. PhyB stoichiometrically binds to PIF3 at an equimolar ratio which makes a complex with one dimeric partner molecule of each partner. In the

recognition process of phyB by PIF3 in addition to the PAS domain of PIF3, a second domain in the C-terminal domain of PIF3 is also required (Zhu et al., 2000).

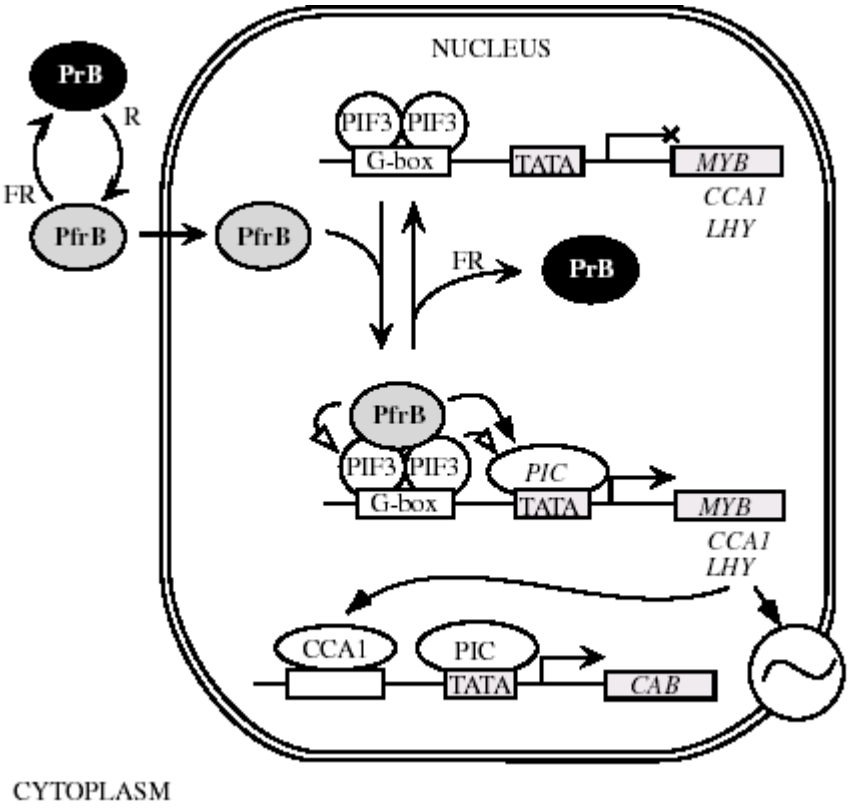


Fig. 1: Proposed mechanism of phyB regulation of gene expression (Quail, 2000).

METHODS FOR STUDYING PROTEIN-DNA INTERACTIONS

Protein-nucleic acid interactions play a major role in basic cellular processes such as DNA replication, recombination, repair, and regulation of gene expression as well as RNA processing transport, and translation (Ho et al., 1994).

Different methods have been employed to study protein-DNA interactions in vitro and in vivo. Among these methods; the electrophoretic mobility shit assay (EMSA) has been commonly applied to nucleoprotein complexes formed in vitro to analyze binding of specific proteins to specific DNA sequences but it is not applicable to vivo studies (Lejnine et al., 1999). Although EMSA is a useful technique it has some limitations: (i) some protein-DNA interactions are not stable enough to survive the electrophoretic separation, (ii) it does not give information about the size of the protein in the complex, and (iii) it is not useful for the determination of binding kinetics parameters (Ho et al., 1994, and Grishko et al., 1999).

Immunoprecipitation of native nucleoprotein complexes is another widely used technique that can be applied to in vivo systems to isolate the native proteins bound to specific nucleic acid sequences. However, as seen

in EMSA, immunoprecipitation assays might also give some artificial results, i.e. redistribution of the nucleoprotein complexes can not be prevented because there is no formation of covalent bonding between protein and DNA in these techniques (Lejnine et al., 1999).

As another promising method, chemical crosslinking with formaldehyde has been shown to have the potential to 'freeze' DNA-protein interactions by inducing chemical bonds between protein and DNA in vivo. Although chemical crosslinking with formaldehyde has been widely used to study the distribution of histones and other proteins along DNA, it affects the native equilibria by changing protein charges, creating protein-protein crosslinks, and introducing chemical bridges between DNA and protein. Moreover, it is not clear to what extent chemical cross-linking is a quantitative measure of DNA binding, because protein-protein crosslinks lead to detection of proteins which are not directly in contact with DNA but in contact with other DNA binding proteins, and its long incubation times perturb the complex under investigation (Walter and Biggin, 1997, Russmann et al., 1998, Murtin et al., 1998, Gravel et al., 1998 and Lejnine et al., 1999).

Ultraviolet crosslinking has been proved to overcome these limitations. UV light is a "zero-length" cross-linker since UV photolysis does not require any cross-linking reagents in contrast to the spacers used

between the protein and the nucleic acid in chemical cross-linking techniques. UV-light induced crosslinking is a powerful alternative to formaldehyde that produces covalent bond between contact points of nucleic acid and protein within a microsecond with minimum damage of their native equilibria. Therefore, photochemical crosslinking is proposed to be restricted to the amino acid residues that are directly interacting with the nucleic acids within the original complex eliminating the possibility of non-specific cross-linking (Merrill et al., 1984, Williams et al., 1991, and Walter and Biggin, 1997).

In vivo ultraviolet cross-linking provides a method to examine protein-DNA interactions as they occur under physiological conditions. It has been used to study nucleic acid interactions of a variety of proteins, including RNA polymerase II (Gilmour and Lis, 1986), topoisomerase I, and sequence-specific transcription factors such as Eve, Zeste (Walter et al., 1994), and GAGA (O'Brien et al., 1995). In many cases, the pattern of DNA binding discovered differs greatly from that predicted by earlier indirect approaches. It has also been demonstrated that UV cross-linking yields a highly accurate measure of DNA binding. Therefore, UV cross-linking can be used to study quantitatively the DNA binding of sequence-specific transcription factors in vivo (Boyd and Farnham, 1997, and Walter and Biggin, 1997).

Some measurements of in vivo DNA binding by sequence-specific transcription factors in Drosophila embryos and tissue culture cells have been performed using UV crosslinking (Walter and Biggin, 1997). According to their results which were supported by their previous research (Walter et al., 1994), they have stated that in order to use UV cross-linking to study protein-DNA interaction the protein must cross-link to DNA with reasonable efficiency.

Another requirement for the efficient cross-linking is that the binding site of the protein must contain thymidine residues since thymidine residues cross-link to protein much more efficiently than other nucleotides. However, a precise geometry between protein and nucleic acid residues is also required for efficient cross-linking to occur, as the presence of thymidine residues in the binding sequence does not guarantee efficient cross-linking. Because of these reasons they advised that the efficiency of UV cross-linking of a protein to a binding site should be examined in vitro before in vivo experiments (Walter and Biggin, 1997).

In the literature, two different techniques have been used to irradiate samples with UV light of wavelength near 260nm: Low intensity UV light and high intensity UV light (Budowsky et al., 1986). For low intensity UV, the ultraviolet radiation of standard light sources has been widely used to generate polynucleotide-protein crosslinks in

nucleoproteins for different purposes such as to identify amino acid residues that have been crosslinked to nucleic acids (i.e. identification of the site of the cross-linking) (Merrill et al., 1984), to determine the orientation of a DNA binding motif in a protein-DNA complex (Pendergrast et al., 1992), to study DNA binding specifity of two homeodomain proteins in vitro and in Drosophila embryos (Walter and Biggin, 1996), to quantitatively measure in vivo DNA binding by sequence-specific transcription factors (Walter and Biggin, 1997), to study the conformational flexibility of the DNA-binding domains on DNA target (Cleary et al., 1997), to locate a binding site of hRPA70 on the damaged DNA strand (Schweizer et al., 1999), to study crosslinking of the complementary strands of DNA by UV light (Nejedly et al., 2001), to identify the part of the gene which binds to the multi liver-specific factors to activate gene transcription in liver cells (Handa et al., 2002) , to study in vivo footprinting with UV irradiation (Pfeifer and Tornaletti, 1997), to study the effects of Myc, Max and USF proteins on the chromosomal cad promoter (Boyd and Farnham, 1997).

Although it is superior than most of the other methods, photochemical crosslinking with conventional UV light sources; (i) may require prolonged irradiation ranging from minutes to several hours to obtain reasonable amount of crosslinking that causes extensive photodamage to DNA especially by introducing thymidine dimers, creates

conditions for artifactual crosslinking of UV damaged molecules, and is not appropriate for the study of rapid binding kinetics, (ii) it can produce protein-protein crosslinking due to its broad wavelength region, and (iii) generates heat which can also perturb the protein-DNA interactions (Ho et al., 1994, Moss et al., 1997 and Russmann et al., 1998).

As a powerful UV light source high powered UV lasers have been shown to eliminate some of these problems such as it induces high efficiency of crosslinking in a single or small number of pulses by delivering photons in time intervals on the order of nano- or even pico and femtoseconds (Moss et al., 1997, Budowsky et al., and Russmann et al., 1997) and it damages DNA less than conventional UV irradiation (Lejnine et al., 1999). Furthermore, 266nm laser light does not produce protein-protein crosslinks (Angelov et al., 1988). It increases the efficiency of crosslinking sometimes with a single pulse (Ho et al., 1994).

From the results of different in vivo and in vitro experiments high powered UV lasers have been reported to increase crosslinking efficiencies compared to non-coherent UV lamps by two orders of magnitude i.e., histones were crosslinked at an efficiency of 15-20% to DNA in reconstituted histone-DNA complexes (Angelov et al., 1988) whereas this yield is ~0.1% with a conventional lamp (Hockensmith et al., 1986)., high protein-DNA crosslinking yields up to 30% were obtained for

the progesterone receptor (Russmann et al., 1997) and up to 47% of the oligodeoxynucleotides were crosslinked to recombinant heat-shock transcription factor in vitro (Zhang et al., 2001).

Budowsky and coworkers have compared RNA-protein cross-linking yields using low- and high-intensity irradiations. They found out that the cross-linking yield with high intensity irradiation was 20 to 100 times larger than those yields achieved with low intensity UV irradiation (Budowsky et al., 1986).

Zhang and coworkers laser irradiated Arabidopsis cell-culture tissue at 266 nm for 1 min with total 1800 mJ of energy and demonstrated that UV laser light can be used as a crosslinker for transcription factors in vivo. However, in contrast to UV laser light, using 15 W UV bulbs did not induce any protein-DNA crosslinking (Zhang et al., 2001).

The reason for the high crosslinking efficiency of UV lasers compared to conventional UV lamps is described as the function of high power of UV laser pulses (Angelov et al., 1988). Using conventional UV light, DNA bases excited by absorption of single photon undergo low efficiency crosslinking reaction whereas the high power UV laser pulses increase the probability for the absorption of a second photon by an already excited DNA base. Absorption of the second photon leads to a

high yield of cationic radicals which results in a high efficiency of protein-DNA crosslinking (Zhang et al., 2001).

UV laser crosslinking is the most advantageous contemporary method to study protein-DNA interactions both in vivo and in vitro and easily applicable to many different systems (Moss et al., 1997, and Zhang et al., 2001).

MECHANISM OF PHOTOCROSS-LINKING OF NONSUBSTITUTED NUCLEIC ACIDS BY LOW INTENSITY IRRADIATION

The polynucleotide-protein cross-links, induced by the ultraviolet (250-270 nm) irradiation of nucleoproteins, are formed mainly as a result of reactions of highly reactive nucleic acid bases with protein amino acid residues (Budowsky et al., 1986). The absorption spectra of nucleic acid bases in aqueous solution with λmax between 250 and 270nm is due to $\pi\pi^*$ electronic transitions of pyrimidine and purine rings (Nikogosyan, 1990). According to one of the defined mechanisms of photocross-linking; low intensity UV irradiation leads to single photon excitation mechanism where a nucleic acid base is excited to the first excited singlet state (S_1 life time = 10ps) by absorption of a single photon between 250 and 270nm which can either react, relax back to the ground state, or intersystem cross

to the first excited triplet state (T_1 lifetime = 1 microsecond) as shown in Fig**. 2.**

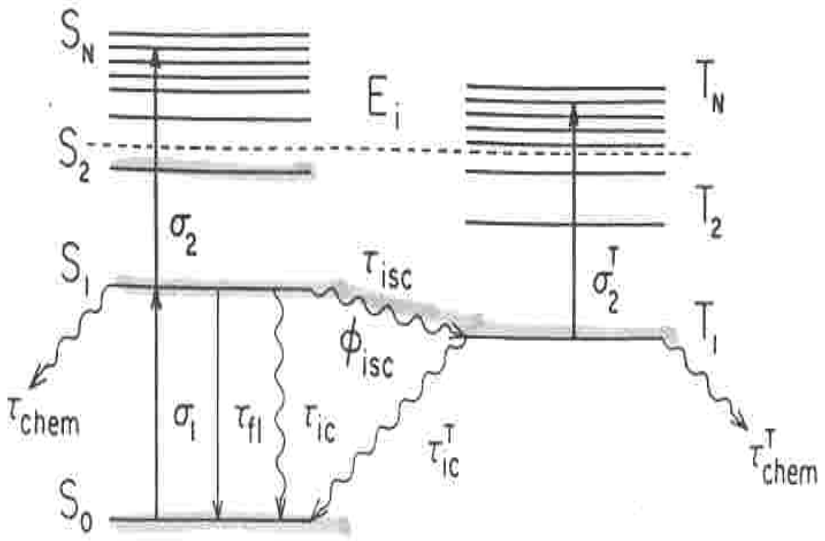


Fig. 2: Simplified scheme of singlet and triplet excited electronic states of nucleic acid bases in aqueous solution and routes of deactivation of electronic excitation energy (Nikogosyan, 1990).

Either S_1 or T_1 excited states can be involved in photocross-linking between the nucleic acid bases and amino acid residues which must be in direct contact at the time of excitation to be able to involve short lived S_1 or T_1 states in the cross-linking mechanism. Therefore, this method should not detect cross-linking of proteins that do not contact DNA directly (Meisenheimer and Koch, 1997, Walter and Biggin, 1997).

The main role of amino acid residues in proposed photo chemical cross-linking mechanisms has been defined differently as hydrogen atom donors, electron donors, or nucleophiles (Meisenheimer and Koch, 1997). One of the most common mechanisms of the UV light induced crosslinking reaction is a free radical mechanism where the photoexcited nucleic acid base abstracts a hydrogen atom from a neighboring amino acid residue to produce a pyrimidinly radical along with a radical on the side chain of amino acid which functions as a hydrogen atom donor and the covalent bond formation occurs by combination of the radicals as shown in Fig. 4 (Meisenheimer and Koch, 1997, and Williams et al., 1991). Theoretically crosslinking can occur between any amino acids and nucleic acid bases and because of this property UV crosslinking method can be considered as widely applicable to any systems (Williams et al., 1991, and Zhang, 2001).

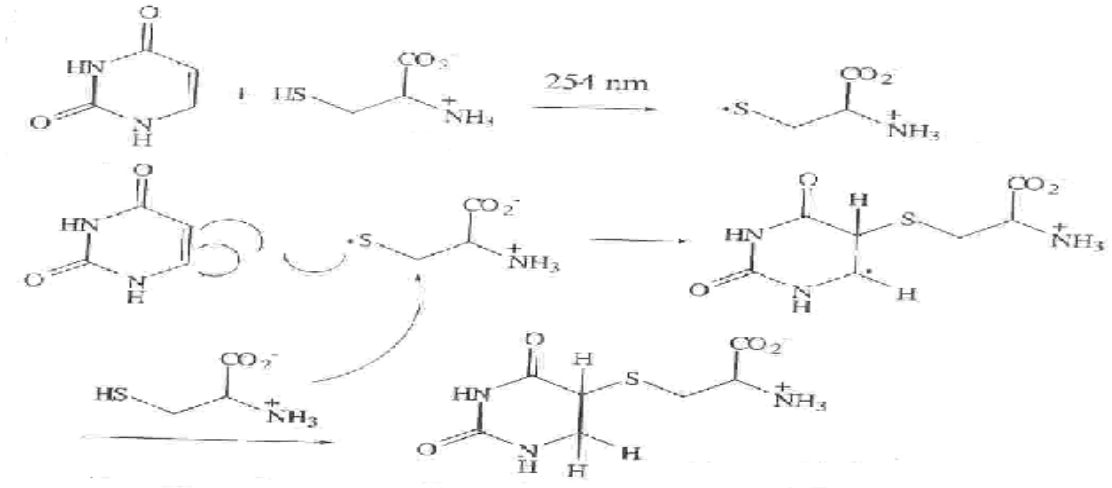


Fig. 3: Proposed mechanism for the photoaddition of L-cysteine to uracil.

Some model experiments were performed in 1984 to measure the quantum yields of the photoaddition of 20 amino acids to calf thymus DNA (Shetlar et al., 1984). As a result of these studies: Cys, Lys, Phe, Trp, and Tyr were found to be the most reactive amino acids whereas His, Glu, and Asp showed moderate reactivity and Arg, Leu were defined as the least reactive amino acids in photocross-linking to DNA. However, it was also concluded that any amino acid residue can participate in photocross-linking mechanism (Meisenheimer and Koch, 1997).

According to some other previous studies related to the photoactivity of amino acid residues in cross-linking mechanism; serine, isoleucine, threonine, tyrosine and cysteine have been shown to be effective in the formation of covalent bonds in different protein and nucleic acid systems. It has also been stated that amino acids responsible for the cross-linking reactions were found to be in close contact to the nucleic acid bases in those cases where three dimensional structures of the proteins are available (Merrill et al., 1984).

Although any nucleic acid bases can be effective in generation of polynucleotide-protein crosslinks in nucleoproteins, the pyrimidines are photochemically more reactive than the purines. Among the pyrimidines thymine is the most photoreactive deoxynucleotide and uridine is the ribonucleotide base yielding the most efficient photochemical cross-linking to proteins. In one of their studies the Hockensmith group ranked

the nucleotide residues in order of decreasing photoreactivity as: dT>>dC>rU>rC, dA, dG (Hockensmith et al., 1986, Williams et al., 1991, and Russmann et al., 1998).

Some of the main advantages of nonsubstituted nucleic acid-protein photocross-linking by single photon excitation mechanism with low-intensity UV irradiation are after excitation of the nucleic bases, a reaction can take place with numerous amino acid residues and reported yields of cross-linking have been anywhere from ~5 to 20% with some yields reported as high as 85%. On the other hand irradiation at wavelengths below 300nm induces absorption by other chromophores in the complex which can lower the crosslinking yield and/or can make the characterization of the cross-linking reaction complicated. Long irradiation times needed to obtain a reasonable crosslinking yield might be other drawbacks of low-intensity UV irradiation (Meisenheimer and Koch, 1997).

PHOTOCROSSLINKING OF SUBSTITUTED NUCLEIC ACIDS BY SINGLE PHOTON EXCITATION

Photocross-linking can be accomplished either by irradiating the wild-type complex or by irradiating a photoactivatable group incorporated into the nucleic acid or amino acid residue (Meisenheimer and Koch, 1997).

Substituted nucleic acids with some advantages over non-substituted nucleic acids have been used in some photocross-linking experiments. For example in order to study site-specific photocross-linking of human replication protein (hRPA) to ssDNA, thymidine-analog 5-iodo-2'-deoxyuridin (5-IdU) was used as a zero-length crosslinking chromophore. Homolysis of carbon-iodine bond of 5-IdU upon UV-irradiation forms uridyl radical with an extremely short lifetime and short lived radical allows crosslinking only to amino acids which are in close distance. This advantageous property of 5-IdU has been used to find out close contact points in protein-nucleic acid complexes.

The higher excitation wavelength of 5-IdU-chromophore above 300nm is also another advantage of 5-IdU-chromophore which minimizes photodamaging of protein and nucleic acid and reduces nonspecific photocross-linking.

Several studies have shown that for ssDNA-binding proteins; π-stacking interactions between the unpaired photoreactive nucleobases and hydrophobic, mainly aromatic, amino acid side chains facilitate excitation and result in efficient cross-linking. In this specific study it has also been stated that high cross-linking yield of protein, up to 60%, might be the result of interactions of aromatic amino acid residues within its ssDNA binding domain with 5-IdU substituted DNA (Schweizer et al., 1999).

PHOTOLESIONS IN NUCLEIC ACID BIOPOLYMERS UNDER LOW-INTENSITY UV IRRADITION

Some specific photolesions have formed in nucleic acid biopolymers if they exposed to low-intensity UV irradiation. Purines are known to be much more photostable, i.e. less photoreactive, than pyrimidines and because of this purine photoproducts do not play a dominant role in UV photolysis of nucleic acids (Nikogosyan, 1990 and Russmann et al., 1998).

There are two main types of photoproducts forming with low-intensity UV irradiation of pyrimidines in aqueous solution: cyclobutyl dimers, best known photoproduct in DNA upon UV irradiation, and photohydrates.

Cyclobutane-type pyrimidine dimers such as thymine-thymine, thymine-cytosine and uracil-uracil dimers are the most important UV induced photolesions observed in DNA and/or RNA with the highest quantum yields ranges from 1 to 3 percent and cyclobutane dimers are mostly seen at 5'-TpT sites (Nikogosyan, 1990, and Pfeifer and Tornaletti, 1997).

Pyrimidine photodimerization involves the breaking of the C_5-C_6 double bond in two adjacent pyrimidine bases and the formation of a cyclobutane ring between these bases as shown in Fig. 4a (Nikogosyan, 1990). Cyclobutane pyrimidine dimers have cis-syn, cis-anti, trans-syn and trans-anti isomers and the cis-syn dimer is dominant in UV irradiation of DNA.

It was stated that in a dilute aqueous solution ($< 10^{-3}$ M) the excitation of the triplet state (T_1) of the nucleic acid base leads to the dimerization reaction under low intensity UV irradiation. As a result of this data it can be stated that increasing the quantum yield of intersystem crossing makes the dimerization quantum yield higher (Nikogosyan, 1990, and Lejnine et al., 1999).

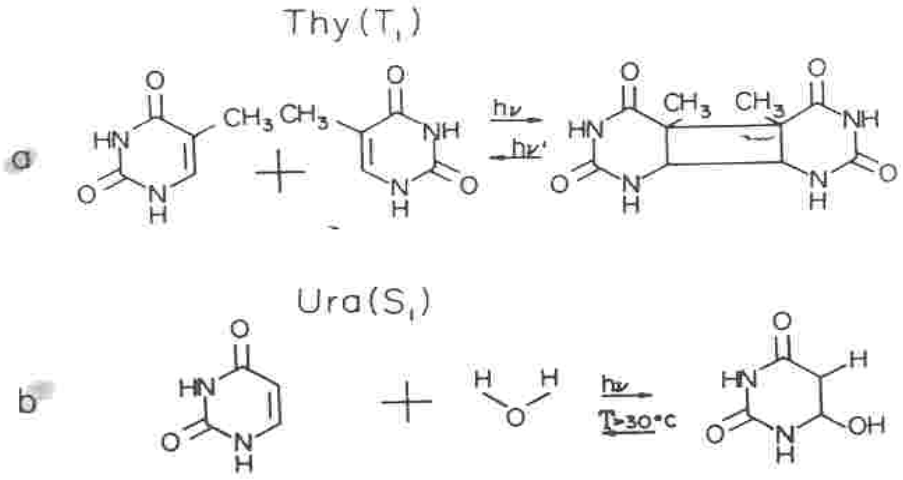


Fig. 4: Photochemical reactions of pyrimidine bases: dimerization of thymine (a) and hydration of uracil (b) (Nikogosyan, 1990).

It has been shown that the UV absorption spectra of dimers are shifted to shorter wavelengths than the monomers. Irradiation with UV light in the spectral region of $\lambda \sim 280$nm where dimers do not absorb results in accumulation of dimers whereas shorter-wave UV irradiation $\lambda \sim 240$nm causes the photosplitting of the cyclobutadipyrimidines (Nikogosyan, 1990).

It has been proposed that formation of the intrastrand thymine dimer also depends on the conformational change of DNA from the canonical geometry for the neighboring thymines to adopt a mutual geometry required for crosslinking (Nejedly et al., 2001).

Pyrimidine hydration, as the second most common photolesion under low intensity UV irradiation, occurs as a result of the hydration of C_5-C_6 double bond and is particularly dominant in uracil and cytosine as shown in Fig. 3b (Nikogosyan, 1990). Pyrimidine hydrates such as uracil and cytosine hydrates, quantum yield about 1 per cent, are common mainly in single-stranded nucleic acids i.e. RNA and denatured DNA upon low-intensity UV irradiation (Nikogosyan, 1990). Pyrimidine hydrates are believed to be formed from the excited S1 level or from the vibrationally excited ground S_o* state. The photohydration quantum yield is independent of irradiation wavelength (Nikogosyan, 1990).

None-cyclobutane-type dimers, also known as pyrimidine (6-4) pyrimidine photoproducts, involve a single covalent bond between positions 6 and 4 of two adjacent pyrimidines (Pfeifer and Tornaletti, 1997). (6-4) photoproducts are formed as a result of excitation of cytosine-thymine and thymine-thymine nucleotide pairs under low-intensity UV irradiation with a relatively low quantum yield. (6-4) photoproducts are most frequently detected at 5'-TpC and 5'-CpC sequences (Pfeifer and Tornaletti, 1997).

Single-strand breaks (ssb) of sugar-phosphate chains in double-stranded DNA and breaks of the sugar-phosphate chain in single-stranded DNA and RNA are also important changes in the secondary structure of

nucleic acids induced by low-intensity UV radiation. Such lesions form as a result of the interaction between the hydroxyl radical $OH^{\cdot}$, product of photodissociation of water molecule, and nucleic acids (Nikogosyan, 1990).

Formation of covalent crosslinks between two complementary DNA chains is another type of UV-induced photolesions which also affects nucleic acid secondary structure. Studies with short DNA fragments have shown that thymine is not required for the DNA strands to be crosslinked upon low intensity UV irradiation. It has been demonstrated that complementary DNA strands of human chromosomes are crosslinked at very low doses of UV irradiation and crosslinking of the complementary strands increases with the increasing (A+T) content of DNA. Interstrand crosslinks formation may also require that two bases should adopt a favorable mutual orientation as seen in intrastrand thymine dimer formation (Nikogosyan, 1990, and Nejedly et al., 2001).

Formation of DNA-protein and RNA-protein crosslinks with low-intensity UV irradiation (λ=254nm) are specific type of photolesions which is widely used for studying the relative spatial arrangement of nucleic acids and proteins in UV-induced complexes and for understanding the mechanism of complex formation (Nikogosyan, 1990 and Williams and Konigsberg, 1991). Buck and Cannon published a

simple procedure for visualizing protein-nucleic acid complexes by photochemical crosslinking in 1994. In their research it has been stated that protein crosslinking to DNA is effective at suppressing the UV light reactivity of certain residues in the promoter target DNA due to inhibition of pyrimidine dimer formation (Buck and Cannon, 1994).

FACTORS AFFECTING THE EXTENT OF PHOTOCHEMICAL CROSS-LINKING UNDER LOW INTENSITY UV LIGHT

To be able to study any protein-DNA interactions by UV cross-linking, the yield of formation of covalent complex upon low intensity UV irradiation should be sufficient enough to make detailed chemical characterization. There are some important factors affecting the rate and extent of photochemical cross-linking under low-intensity UV light such that to obtain a reasonable amount of cross-linking in a specific protein-nucleic acid system one should consider both the experimental conditions as well as the photochemical nature of the nucleic acid, protein and the three-dimensional structure of the complex (Williams and Konigsberg, 1991).

The very first factor for efficient photocross-linking is the extent of the protein in the reaction mixture that is actually complexed with its specific nucleic acid sequence. To serve this purpose preliminary band-

shift experiments are performed in order to make sure that experimental conditions used for crosslinking experiments support higher yield of protein-DNA complex formation. Before UV irradiation; the optimum amount of protein and nucleic acid, and the salt concentration of the binding buffer for higher yield of complex formation should be determined (Williams and Konigsberg, 1991, Russmann et al., 1997 and Ho et al., 1994). Merrill and his coworkers have been demonstrated that the decline in the amount of complex formation due to disruptive electrostatic forces provided by high NaCl concentration is directly proportional to the decrease in the amount of photo cross-linked complexes. Moreover when they carried out the binding reaction in 2M NaCl no cross-linking was observed (Merrill et al., 1984).

Another important variable to obtain maximum amount of cross-linking is the experimental determination of the optimum dose of photon flux for the system under study. It has been stated in the literature that the amount of photon fluxes in the range from 0.2 to 2 J/cm^2 can provide maximum extent of photo cross-linking.

Exposure to UV light denatures proteins and there is a variation in the photosensitivity of proteins. Some proteins have not shown any detectable photodenaturation at all. To unambiguously identify cross-linking sites only the native protein should be cross-linked to the nucleic

acid. Once an optimum dose of UV light has been found the extent of photoinactivation of the protein at this dose as well as effect of denatured protein on cross-linking should be determined. Only the native (non-denatured) protein should take place in cross-linking to nucleic acid (Lejnine et al., 1999, Williams and Konigsberg, 1991 and Meisenheimer and Koch, 1997).

In the literature Merrill group has shown that the extent of crosslinking with previously irradiated protein sample resulted in 3.8% decrease in cross-linking compared to the identical sample of unirradiated protein, which suggests that as protein is exposed to UV light it loses its ability to bind DNA and only the native form of protein plays a role in cross-linking to nucleic acids (Merrill et al., 1984).

One of the likely requirements for efficient cross-linking is that the binding site of the protein must contain thymidine residues as they are more efficient in cross-linking than the other nucleotides (Hockensmith et al., 1991). However, a matching geometry between protein and nucleic acid residues is also another important requirement for efficient cross-linking, since the presence of thymidine residues in the recognition sequence does not always guarantee higher yield of cross-linking (Walter and Biggin, 1997).

Topology is an important factor for amino acids to cross-link to nucleic acids and it has been shown that for some systems a specific topological arrangement between an amino acid residue and a nucleotide base must be obtained to have successful photochemical crosslinking (Williams and Konigsberg, 1991). Many transcription factors bend or kink the DNA at or near their recognition site, and these structural alterations in the DNA double helix may favor or disfavor the formation of UV photoproducts at specific sequences (Pfeifer and Tornaletti, 1997).

MECHANISM OF PHOTOCROSS-LINKING OF NONSUBSTITUTED NUCLEIC ACIDS BY HIGH INTENSITY IRRADIATION

The mechanism of UV laser crosslinking has been understood as a two photon process (Russmann et al., 1997). The parallel increase in power and quantum yield confirms a two-photon mechanism (Nikogosyan, 1990).

As it was described in photocross-linking of nucleic acids by single photon excitation mechanism; single-photon excitation promotes a nucleic acid base from S_o to S_1 and intersystem crossing from S_1 to T_1 can follow. With high intensity pulsed lasers emitting 10^9 W/m2 with nanosecond pulses or 10^{12} W/m2 with picosecond pulses; an excited nucleic base can

absorb a second quantum of light (Meisenheimer and Koch, 1997, Douki et al., 2001) if the rate of its further excitation from S_1 or T_1 levels to the high-lying S_N or T_N levels is higher than the rate of deactivation of the intermediate S_1 and T_1 levels (Nikogosyan et al., 1990).

Two-step excitation causes an increase in the population of high-lying electronic states with energies ~6-10 eV, whereas the gas phase ionization potentials of the nucleic bases are > 8 eV. It has been shown that under high-intensity picosecond UV irradiation nucleotides, oligonucleotides, polynucleotides, DNA and RNA exceed their ionization thresholds by 3.5-4.5 eV due to successive absorption of two photons. As a result, two-photon absorption can cause ionization of the nucleic base to form cation radicals (Nikogosyan et al., 1990 and Russmann et al., 1998).

As biphotonic excitation of a nucleic acid base can be via the intermediate state S_1 (the singlet channel of two-step excitation) or the intermediate state T_1 (the triplet channel of two-step excitation), there are two possible pathways which could cause crosslinking as shown in Fig. 4 (Russmann et al., 1997, and Russmann et al., 1998).

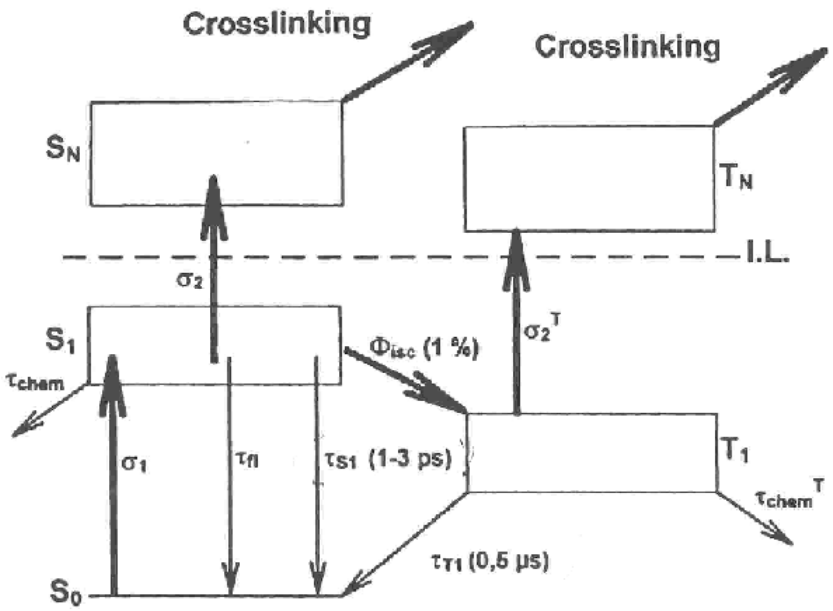


Fig. 5: Singlet and triplet pathways of two-step excitation of nucleic acid bases in water solution (Russmann et al., 1997).

Laser pulse duration determines which pathway will be dominant in two-step photochemical reaction. Singlet-mediated crosslinking dominates if the laser pulse is shorter than the intersystem crossing rate constant (average for the S_1-T_1 transition is $10^9 s^{-1}$), if the laser pulse is longer crosslinking follows the triplet route (Russmann et al., 1997).

It has been shown that by picosecond UV irradiation of nucleic acid components in aqueous solution (λ=266nm, τ_p =23-29ps) the singlet channel is followed but in the case of nanosecond UV irradiation (λ=266nm, τ_p =10ns or λ=249, τ_p =20ns) the triplet channel is predominant

(Nikogosyan et al., 1990). Russmann and coworkers also have shown by calculations that in the case of 100 ps pulses, there will be a negligible population in the T_1 state during the pulse whereas in the case of longer pulse length (i.e. 5 ns) a substantial amount of the excited molecules will reach triplet state T_1 during the pulse length (Russmann et al., 1997).

It was stated that the efficiency of two-step photochemical reaction via the $S_1 \rightarrow S_N$ transition may be much higher than $T_1 \rightarrow T_N$ transition (Nikogosyan et al., 1990). When Russmann et al. (1997) were studying the influence of pulse length and pulse intensity on crosslinking efficiency they compared crosslinking efficiency for the same pulse intensities and the same total amount of energy but of different pulse lengths. From this study they found out that the singlet path is more efficient compared to triplet-mediated crosslinking (Russmann at al., 1997).

It was proposed that the photolysis reactions following picosecond and nanosecond UV irradiation are qualitatively identical with the same photoproducts in both cases: The primary products of two-step photolysis of nucleic acid bases are cation-radical R^+ and hydrated electron e_{aq}^- (Nikogosyan et al., 1990).

The quantum yield of both single photon and two photon excitation is defined as the ratio of the amount of photoproducts to the number of absorbed photons. The quantum yield is a nonlinear quantity because it depends on irradiation intensity (Nikogosyan et al., 1990).

PHOTOLESIONS IN NUCLEIC ACID BIOPOLYMERS UNDER HIGH-INTENSITY UV IRRADITION

The ionization of nucleic acid bases in DNA or RNA during high intensity laser irradiation causes some photolesions which are different than photolesions formed under low intensity UV irradiation.

Irradiation with conventional UV lamps produces mainly pyrimidine dimers which are related to the excitation of the triplet state of the nucleic acid base .With high intensity picosecond or femtosecond pulses pyrimidine dimers formation decreases more than 10-fold depending on irradiation intensity because of the decrease in population of triplet states of the bases with shorter pulses (Nikogosyan, 1990, and Lejnine et al., 1999).

The studies about the photochemistry of uridine with high-intensity picosecond UV irradiation have been shown that with increasing intensity the quantum yields of single-quantum products (i.e. hydrates formed from the S_0^* state and dimers formed from the T_1 state), decreased. Another

researchers have stated the similar result that with increasing intensity of picosecond UV irradiation, the quantum yield of dimerization in dTpdT and poly(dT) is reduced by half, and in DNA reduced by two orders of magnitude (reviewed in Nikogosyan, 1990).

The Douki group (2001) has also shown that the quantum yield of TT and TC cyclobutane dimers and (6-4) photoproducts was found to decrease with increasing laser pulse intensity (i.e., from 100 mJ/cm^2 to 300 mJ/cm^2) and in contrast to pyrimidine dimeric photolesions, the quantum yield of oxidized bases increased with increasing intensity of the laser pulse (Douki et al., 2001).

One of the main photolesions of high intensity laser irradiation is single-strand breaks of the nucleic acid (Panyutin et al., 1989). It has been shown that the increase in the number of single-strand breaks (ssb) with increasing intensity of picosecond UV irradiation at constant dose pointed to the two-quantum mechanism of formation of ssb in double-stranded DNA and the quantum yield of ssb formation in plasmid DNA with picosecond UV irradiation of $I > 10^{12}$ Wm^{-2} was 40-50 times higher than with low-intensity continuous UV irradiation (Nikogosyan, 1990).

Because the purines have the lowest ionization potentials (i.e. guanine is at 8.24 eV), most single-strand breaks occur at the 3' phosphodiester bond of the nucleotide possessing the purine (Meisenheimer and Koch, 1997).

Other types of photolesions which are increased by high intensity irradiation ($I > 10^{11} Wm^{-2}$) are: sugar-phosphate chain breaks, double strand breaks and interstrand crosslinks in DNA (Nikogosyan, 1990).

The yield of different damages may change with wavelength, intensity, and pulse length of the light as well as with physical conditions of the experiment. As an example, using Tris-containing buffer lowers the amount of double-strand breaks 4 times more than water (Lejnine et al., 1999). Kovalsky and co-workers (1990) have also suggested that single-strand breaks can be affected by buffer conditions and ligands (Kovalsky et al., 1990).

Analysis of nucleoproteins often involves restriction enzyme digestion and size separation by electrophoresis. Lejnine and coworkers have demonstrated that irradiation of intact human nuclei using 60 femtosecond, 266nm laser pulses for 30 min did not produce any noticeable double-strand breaks but caused significant amount of thymidine dimers formation at the AT rich sequence of DNA which prevented restriction enzyme digestion (Lejnine et al., 1999).

Although UV laser irradiation has the potential to damage DNA, Zhang and coworkers, as a result of their in vitro studies, stated that UV laser irradiated DNA is functional in hybridization and by PCR techniques (Zhang et al., 2001).

The Russmann group has demonstrated an important example for the effect of pulse duration on DNA damage, such that using shorter pulses increased the ratio of crosslinked to the damaged DNA. The ratio is improved by a factor of more than 5 when using femtosecond laser instead of nanosecond laser (Russmann et al., 1997).

In order to reduce the UV-induced DNA damage the combination of UV and blue pulses have been applied. This novel two-wavelength approach has been shown to increase effective crosslink yield by 10% compared to UV laser pulses alone (Russmann et al., 1998).

IN VIVO AND IN VITRO APPLICATIONS OF UV LASER CROSSLINKING

Laser irradiation is monochromatic, coherent, and intense. Consequently, UV laser crosslinking allows the study of many important transient DNA-protein interactions either in vitro or in vivo (Grishko et al., 1999).

In vivo laser footprinting is a rapid and non-perturbing method of monitoring protein-DNA complexes. A footprint is derived by a comparison between laser-irradiated whole cells with or without the DNA binding protein, and identifies the positions in the immediate environment of the DNA that have changed due to the cross-links between the DNA and the protein, exclusion of solvent molecules from the vicinity of the DNA or conformational changes of DNA structure (i.e. bending of DNA) (Murtin et al., 1998).

In UV laser footprinting the nucleoprotein complexes are irradiated with a UV laser pulse and photomodified bases in the DNA are identified by primer extension using DNA polymerase, which stops synthesis when it subjects to a modified base. Using this method, which provides information about contact points between the protein and DNA, it was shown that the interactions between E. coli integration host factor (IHF)

and its specific binding sites are identical in vitro and in vivo (Engelhorn et al., 1995).

Irradiation of cells or intact nuclei with UV laser pulses crosslinks nucleoproteins in their native state and it has been shown that 6-12% of proteins crosslinked in nuclei with a 266nm nanosecond laser (Mutskov et al., 1997) and in another experiment 266nm picosecond laser gave sufficient crosslinking of specific histones to genomic sequences in nuclei (Angelov et al., 1988).

Single-pulse UV crosslinking is a simple method that can be used for the estimation of mass of crosslinked DNA binding proteins, purification of DNA-binding proteins, and the study of protein-DNA interactions both in vitro and in vivo (Grishko et al., 1999).

The fourth harmonic of a pulsed Nd:YAG laser was used for crosslinking the transcriptional regulator RAP1 to DNA in vitro. It was shown that a single 50 mJ pulse can crosslink DNA-binding proteins in crude or purified preparations. The stable protein-DNA complexes generated was detected by SDS-PAGE and the molecular mass of the protein was estimated (Ho et al., 1994).

One of the most promising approaches to attain higher crosslinking yields with low DNA damage (i.e. higher effective crosslinking) is two-wavelength femtosecond (TWF) laser irradiation. This strategy has the advantage that the intensity of the femtosecond UV pulse in the first step can be kept low, thus reducing DNA damage caused by the UV photons. However, higher crosslinking yields can still be attained by applying the second femtosecond pulse with a very short time delay and at a visible wavelength too long to excite DNA bases from the ground state. The second pulse can not damage DNA, but it can provide additional energy for the UV excited bases to pass their ionization level, leading to crosslinking (Russmann et al., 1998).

FACTORS AFFECTING THE RATE AND THE EXTENT OF UV LASER CROSSLINKING

While performing a UV laser crosslinking experiment different factors that may affect the rate and the extent of crosslinking should be considered very carefully.

Efficiency of UV crosslinking of proteins to DNA changes with protein type and DNA sequence. The yields of cross-linked product obtained vary greatly between various nucleotide residues, and also with the wavelengths of light used. It has been showed that 266nm laser pulses

might be the best for cross-linking through cytosine or adenosine, whereas 204.1nm radiation might be effective for guanosine residues (Hockensmith et al., 1986).

In the presence of a specifically bound protein, some amino acid residues in close contact with DNA may interfere with the electron-migrating process along the DNA helix by trapping the positive charge in place of a guanine residue (i.e. oxidized guanosine can be reduced by tyrosine and tryptophan in solution) (Stemp and Barton, 2000, Angelov et al., 2003). The energy transfer between nucleotides (i.e. the energy in nucleic acids is transferred within a distance of 2.6 nucleotides in single-stranded or denatured DNA and 2.7 nucleotides in RNA) (Nikogosyan, 1990) may also affect the efficiency of UV crosslinking.

Single-stranded DNA has shown higher crosslinking efficiency than double-stranded DNA (Angelov et al., 1988) and its crosslinking efficiency increases with shorter pulses such that the crosslinking efficiency of progesterone receptor to single-stranded DNA has been found to be five times higher using a 200 femtosecond laser than 100 picosecond laser with the same total energy (Russmann et al., 1997).

In terms of effect of salt concentration on crosslinking yield, Hung et al., have determined that increasing NaCl concentration from 50 mM to 100 mM reduced the formation of crosslinked complexes significantly. As increasing salt concentration affects the binding equilibrium of nucleoprotein complex (Hung et al., 1996).

DTT can act as a radical scavenger and quench the crosslinking reaction (Ho et al., 1994). It was stated that 5mM DTT in solutions to be irradiated reduce the efficiency of crosslinking by about 50-60% (Moss et al., 1997). Hockensmith and coworkers had found elevated levels of cross-linked complex formation in the absence of reducing agent and had carried out their cross-linking experiments in the presence of 5 mM β-mercaptoethanol; substitution of dithiothreitol for mercaptoethanol had no effect (Hockensmith et al., 1986).

Degradation of protein by high intensity UV irradiation may also alter the crosslinking yield. Although irradiation of reconstituted chromatin with picosecond laser in vitro and femtosecond irradiation of intact human nuclei did not produce any histone-histone crosslinking or protein damage (Angelov et al., 1988, Lejnine et al., 1999), irradiation of Rap1-DNA complex in vitro with a nanosecond laser showed 25% damage of the Rap1 protein (Ho et al., 1994).

Studies about the cross-linking of gene 32 protein to $(dT)_{10}$ at various temperatures (0^o, 12^o, 23^o, and 37 ^oC) were performed. There was no detectable change in the final yield of crosslinked products. In addition to this result incubation at either 0^o or 37 ^oC for 30 min after irradiation did not increase or decrease the amount of crosslinked product when compared to an irradiated sample that had been denatured immediately in SDS sample buffer by boiling after cross-linking (Hockensmith et al., 1986).

METHODS AND MATERIALS

PLASMID CONSTRUCTION FOR T7-MEDIATED EXPRESSION OF MBP-PIF3

To subclone PIF3 coding sequence into a T7-promoter/MalE vector; T7-GADPIF3 and T7-MBP plasmids were digested by Bam HI (NEB) and Hind III (Promega) restriction enzymes, respectively. Both digested plasmids were blunted with T4 DNA Polymerase (NEB) and cleaned-up by QIAquick columns (QIAGEN). Purified DNAs were digested by EcoRI restriction enzyme (NEB); ~ 1.6 kb PIF3 fragment and ~3.9 kb MBP cloning vector were purified from a 1% agarose gel by QIAquick gel extraction kit (QIAGEN), ethanol precipitated and suspended in 1X TE. Ligation of PIF3 fragment into dephosphorylated T7-MBP vector was done in a reaction containing 0.3 µl of T4 DNA ligase (NEB), 1 µl T7-MBP vector and 3 µl PIF3 insert. Reaction was incubated at 16^{o}C for 16 hours. Ligated DNA was transformed into electrocompetent DH5α cells prepared by Josh Black in 2002. Cells (100 µl) were plated on Luria Broth (5 g yeast extract, 10 g NaCl, 10 g tryptone and 15 g agar, per liter water) supplemented with 100 µg/ml of ampicillin. Plates were incubated at 37^{o}C overnight.

PLASMID ANALYSIS

Several colonies were chosen and inoculated into 5 ml of LB with 100 µg/ml of ampicillin. Minicultures were grown overnight in a 37^{o}C shaker at 250 rpm and pelleted bacterial cells from these cultures were used to purify the T7-MBP-PIF3 plasmid by a QIAprep Spin Miniprep kit (QIAGEN). Concentration of plasmid DNA was detected as 200 ng/µl by UV Spectrophotometer at 260nm. Plasmid DNA was cut with EcoRI and double cut with Kpn I & Pst I enzymes to determine if the correct MBP-PIF3 junction, orientation and length for insert was produced.

In order to obtain larger amount of purified T7-MBP-PIF3 high-copy plasmid HiSpeed Plasmid Midi Kit (QIAGEN) was used. Plasmid DNA concentration was determined as 310.75 ng/µl by UV Spectrophotometer at 260nm and EcoRI restriction digest was done to check the correct length of the plasmid.

EXPRESSION, PURIFICATION and DETECTION OF MBP-PIF3

In order to express protein in E. coli; chemically competent BL21 (DE3) Star strain (Invitrogen) was used. BL21 (DE3) cells were transformed with MBP-PIF3 plasmid purified from HiSpeed Plasmid Midi Kit and then grown overnight in 2xYT (16 g Bacto-Tryptone, 10 g yeast

extract and 5 g salt) rich medium with 100μg/ml ampicillin. An aliquot of an overnight culture were diluted 20-fold into fresh 2xYT medium containing 100μg/ml ampicillin and 4%glucose and grown at 37°C in a shaker at 250rpm until $O.D._{600}$ of 0.6 at which MBP-PIF3 expression was induced by adding IPTG to 0.5mM for 10 h at 15°C. The cells from 400 ml cultures were harvested by centrifugation at 6000g for 15min at 4°C and the pellet was suspended in column buffer (20mM MOPS, pH 7.4, 1M NaCl, 1mM EDTA, 2mM AEBSF, pH 7.4). The lysate was sonicated six times at full power for 30sec to reduce its viscosity and centrifuged at 10,000g for 20 min at 4°C in Oakridge tubes. The supernatant was removed and placed into a new tube.

MBP-PIF3 protein was purified by Affinity Chromatography using amylose-agarose resin (NEB). 1.7 ml of amylose resin pre-swollen in 40% ethanol was poured in a column with 1.7 cm of diameter. The column was washed with 8 column volumes of Column Buffer. The resin has maximum binding capacity of 3 mg/ml. 15 ml of crude extract was loaded at a flow rate of 0.5 ml/min. The optimal lysate:resin ratio was determined experimentally. Then, the column was washed with 12 column volumes of column buffer. MBP-PIF3 fusion protein was eluted by column buffer with 20mM maltose. 10-15 fractions of 1 ml each were collected at 4°C. After purification eluates were frozen by liquid N_2 and stored at -80°C.

The cell lysate, crude extract, pellet, flow through, final wash samples, and all the elution samples were mixed with 5X Sample Buffer (10% SDS, 625 mM Tris, 25 ml glycerol, 25 ml β-mercaptoethanol and 50 mg Bromophenol blue, pH 6.8) and boiled at 100°C for 5 min) before loading onto 10% SDS polyacrylamide gels with 5% stacking gels. After 1-2 hours of electrophoresis at 40mA/gel at room temperature, the amount of protein was monitored by staining with Coomassie blue. Protein concentration determinations were performed using a Bradford protein assay kit (Bio-Rad).

Immunoblotting was performed as described in Molecular Cloning, using Protran nitrocellulose membrane (Schleicher & Schuell) and the submerged transfer apparatus (Idea Scientific). 5% nonfat milk was used in the blocking buffer. Anti-MBP Antiserum (NEB) as primary antibody (1:5000 dilution) and Anti-rabbit horseradish peroxidase conjugate as secondary antibody (1:3000 dilution) were used to detect MBP-PIF3 protein on the membrane. TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) with 0.1% Tween-20 was used to wash the membrane after incubation with each antibody. Detection of secondary antibody was done either with TMB-Blotting reagent (Pierce) or by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

BAND SHIFT ASSAY

PROBE DNA PREPARATION

12N1 plasmid kindly provided by Dr. Peter H. Quail (University of California, Berkeley, USA) which encompasses the PIF3 binding site, i.e. G-box motif, was double digested by XhoI and SpeI restriction enzymes (Promega) yielding a ~66 base-pair fragment. Digested DNA was run on a 12% non-denaturing polyacrylamide gel containing 0.5xTBE at 100V for 1-2 hours. 66 base-pair fragment containing part was excised from the PA gel with a clean, sharp scalpel, chopped into small pieces and mixed with 1x TE buffer. To disrupt the PA gel structure the mixture was subjected to couple of freeze-thaw cycles and to increase the amount of DNA passed into the 1x TE buffer the mixture was rotated at 37°C overnight. The purified DNA was ethanol precipitated and the concentration of the double stranded 66 base-pair fragment was determined by PicoGreen® dsDNA Quantitation Reagent (Molecular Probes, Inc.) which is 2 ng/µl.

In order to radioactively label the DNA, 5' overhangs were filled with labeled dATP: 20 ng of DNA was mixed with 1 µl of 100 mM un1abeled dNTPs (minus dATP), 2 µl of 10 mg/ml bovine serum albumin, 3.8 µl of 10X NEB2 buffer, 1 µl [α-^{32}P]dATP (3000 Ci/mmol) and 0.4 µl T4 DNA Polymerase (Promega). The reaction was incubated for 5 min at

37°C and then 1 µl of 0.5 M EDTA was added to stop the reaction. The probe was purified through a spin column (Princeton Separations, Inc.) in order to remove unincorporated nucleotides and through a Micropure-EZ Enzyme remover (Millipore) in order to remove T4 DNA Polymerase.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The purified MBP-PIF3 fusion protein (0.2mg/ml) and the probe (~ 25fmol) were incubated for 15 min at room temperature in 40 µl binding buffer containing 20mM HEPES-KOH, pH 7.6, 50mM KCl, 10% glycerol, 0.1mg/ml BSA, 0.05% TritonX-100, 3mM $MgCl_2$, 2mM DTT, and 2,5ng/µl poly(dI.dC) as nonspecific competitor DNA molecules. The efficiency of binding was checked by EMSA that was performed at 4°C in freshly poured 4% (acrylamide:bisacrylamide ratio 37.5:1) of polyacrylamide (37.5:1 acrylamide-bis acrylamide) 0.5X Tris-Borate-Ethylene diamine tetraacetic acid (TBE) gels. The gel was pre-electrophoresed for 30 min before loading the samples and 10 µl of incubated samples were run at 95 V for 1-2 hours. The gel was dried on Whatman paper for 1-2 hours on a vacuum gel dryer and dried gel was analyzed with a PhosphorImager (Fujix BAS 1000) or exposed to BioMax MS film (Kodak) at -80°C.

As positive controls MBP-PIF3, HIS-PIF3 and MBP were expressed in TNT Quick Coupled Transcription/Translation System (Promega). 40 µl of TNT T7 Quick Master Mix was mixed with 1 µl of 1mM methionine, proper amount of plasmid DNA and nuclease free water to give final reaction volume of 50 µl. The reaction was incubated at 30°C for 60-90 minutes. TNT expressed protein was mixed with labeled probe in binding buffer and its binding activities were studied by EMSA as described above.

ULTRAVIOLET IRRADIATION

LOW INTENSITY UV CROSS-LINKING ASSAY

A low-pressure mercury lamp was used as a low-intensity light source. Samples from binding reactions were irradiated as 20µl drops on parafilm covered previously chilled metal block at a distance of 3 cm from set of four 8W germicidal bulbs (emission maximum at 254nm) in a UV Crosslinker (FB-UVXL-1000, Fischer Biotech). The metal block was cooled from below by an ice-water bath during the irradiation. Irradiations with intensity of 0.5 J/cm^2 and 0.9 J/cm^2 took 30 min and 60 min, respectively. After UV irradiation, samples were mixed with 1/3 volume SDS sample buffer (200mM Tris-HCl, pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol, 1% 2-mercaptoethanol, 1M urea) for 5 min and were heated to 100°C before separating on 10%

(acrylamide:bisacrylamide ratio 37.5:1) SDS-polyacrylamide gels containing 1 M urea in the absence of a stacking gel. After electrophoresis, the gel was dried and analyzed with PhosphorImager (Fujix BAS 1000).

HIGH INTENSITY UV CROSS-LINKING ASSAY

DNA-protein complexes were irradiated with the fourth harmonics (266 nm) of a 5ns pulsed radiation delivered by a Surelite IV Nd:YAG (neodymium-yttrium-aluminum-garnet) laser (Continuum, Santa Clara, CA) with energy per pulse of ~5 mJ at a repetition frequency of 10 Hz. Twenty microliter of incubated samples from the binding reactions were irradiated in 1.5 ml microcentrifuge tubes (Eppendorf) with an unfocused UV beam (approx 4 mm x 2 mm) with Gaussian spatial distribution. Samples were irradiated with different number of pulses to make comparison.

Immediately after irradiation, samples were mixed with 1/3 vol of 4 × SDS loading buffer (200mM Tris-HCl (pH 6.8), 8% SDS, 0.4% bromophenol blue, 40% glycerol, 1 % β-mercaptoethanol, 1 M urea) and boiled for 5 min. The 10 µl of samples were analyzed by SDS-PAGE in 10% (acrylamide:bisacrylamide ratio 37.5:1) gels containing 1 M urea. After 1-2 hours electrophoresis at 100 V, the gels were dried onto blotting

paper and exposed to Kodak BioMax MS film or analyzed with PhosphorImager (Fujix BAS 1000).

TWO WAVELENGTH LASER INDUCED PHOTO CROSSLINKING

20 µl of samples from the binding reactions were irradiated in 1.5 ml microcentrifuge tubes (Eppendorf) with the fourth harmonics (266 nm) of a 5ns pulsed radiation delivered by a Surelite II Nd: YAG laser (Continuum, Santa Clara, CA) with energy per pulse of ~5 mJ at a repetition frequency of 0.25 Hz along with 480 nm of a ~ 800 ns pulsed radiation delivered by Candela 500-M Flashlap-pumped dye laser (Candela Corporation, Wayland, MA) with energy per pulse of ~ 60 mJ. 480 nm pulses started 300ns before and ended 700 ns after the UV pulses. The irradiated samples were analyzed as described above in the UV cross-linking assays.

ANALYSIS OF THE INTEGRITY OF DNA

12N1 plasmid (200µg/ml, in binding buffer) containing the PIF binding motif was irradiated by UV Crosslinker, Nd-YAG laser or Nd-YAG laser with Candela 500-M Flashlap-pumped dye laser as described above. After UV irradiation 12 NI plasmid was cleaved with restriction enzyme XhoI (Promega) and analyzed by PCR using the [γ-^{32}P] 5'-end-

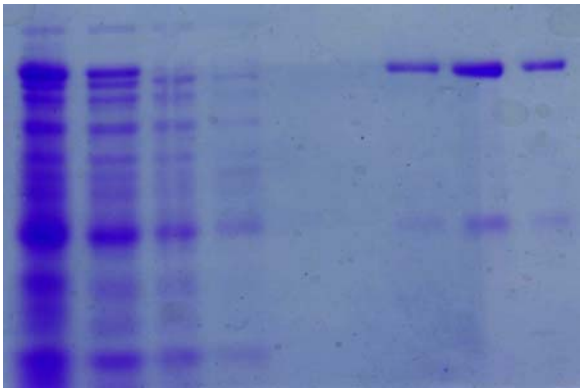
labeled primer 5'-AGT GAG CGC AAC GCA ATT A-3' and HotMaster Taq DNA polymerase (Promega). The primer labeling reaction mixture contained the following: 11.36 µl of [γ-^{32}P]dATP (45000 Ci/mmol), 0.5 µl of primer (50 pmol/µl), 1 µl of T4 Polynucleotide Kinase (Promega) and 12.135 µl of 1X kinase buffer. The PCR machine was programmed according to the user manuals of the polymerase and the melting temperature (T_m) of the primer which is 54.5°C. The thermocycler program was: 94°C for 2 min; 94°C for 20 sec; 50°C for 20 sec; 65°C for 1 min and 65°C for 5 min.

Reaction products were analyzed by denaturing polyacrylamide gel electrophoresis on a 6% sequencing gel supplemented with urea. After electrophoresis, the gel was dried and analyzed with PhosphorImager (Fujix BAS 1000).

RESULTS

Expression, Purification and Detection of MBP-PIF3

In order to express MBP-PIF3 in E. coli; chemically competent BL21 (DE3) Star strain (Invitrogen) was used. E. coli cell culture was grown at 37°C until $O.D._{600}$ of 0.6 reached. Then MBP-PIF3 expression was induced by adding IPTG to 0.5mM for 10 h at 15°C. We changed the induction temperature from 37°C to 15°C because PIF3 originates from a plant that grows at temperatures below 37°C. Using lower induction temperature allowed us to purify properly folded protein by Affinity Chromatography using amylose-agarose resin as shown in Fig. 6.



CL CE FT W E1 E2 E3 E4 E5

Fig. 6: Total protein was purified by affinity chromatography method using amylose-agarose resin, analyzed by 10% SDS-PAGE and the gel was stained by Coomassie blue. CL: Cell lysate, CE: crude extract, FT: flow through, W: Wash, E1: Eluate 1, E2: Eluate 2, E3: Eluate 3, E4: Eluate 4, and E5: Eluate 5.

Purified MBP-PIF3 protein was monitored by immunoblotting using Anti-MBP Antiserum (NEB) against MBP domain. 2° antibody, Anti-rabbit HRP attached to 1° antibody was detected by 1-Step TMB-Blotting reagent as shown in Fig. 7. MBP-PIF3 has a molecular weight of ~ 121.3 kDa.

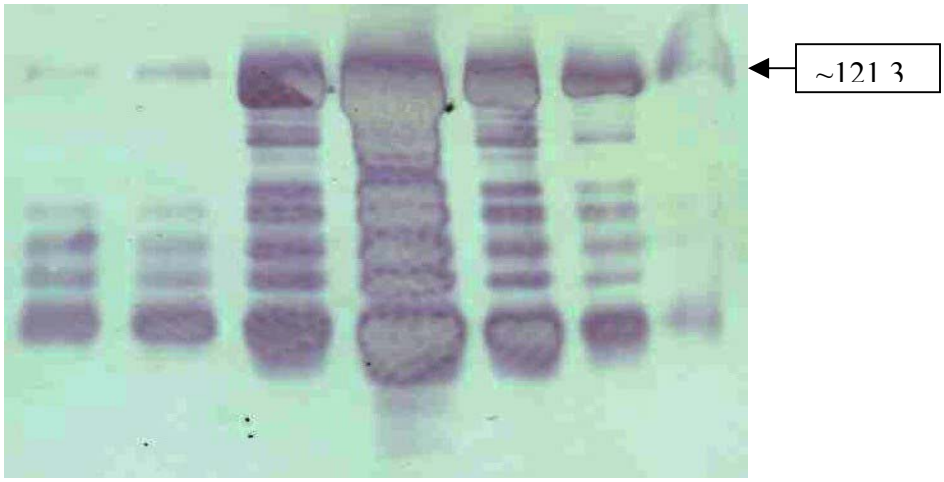


Fig. 7: The western blot of MBP-PIF3.

Electrophoretic mobility shift assay

Our goal was to explore two-wavelength laser crosslinking of protein to DNA. We chose the bHLH protein PIF3 and its conjugate binding site for our crosslinking experiments. In previous studies His6:PIF3 has been shown to specifically bind G-box motifs through its bHLH domain (Martinez-Garcia et al., 2000). Here we showed that

purified MBP-PIF3, which is more soluble than His6:PIF3 can also bind specifically to a 66-mer oligonucleotide containing its specific G-box motif (Fig. 8, lane 2). Binding of MBP-PIF3 to the probe was carried out in binding buffer containing nonspecific competitor, poly[d (I.C)], to prevent nonspecific binding. No complexes were observed when the probe was incubated without MBP-PIF3 (Fig. 8, lane 1).

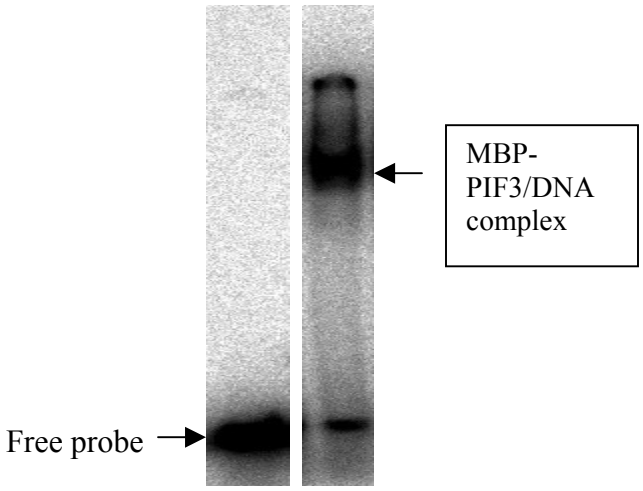


Fig. 8: Electrophoretic Mobility Shift Assay; 25fmol of 32P-labeled probe was incubated without MBP-PIF3 or with 2µg MBP-PIF3 for 15 min at room temperature.

Analysis of DNA crosslinking to purified MBP-PIF3 with low intensity UV irradiation

After incubation of purified protein with its specific probe in binding buffer as for the band-shift assays, samples were irradiated with low intensity UV light at two different intensities (0.5 J/cm^2 and 0.9

J/cm^2). Parallel irradiated and unirradiated samples were analyzed by denaturing SDS-PAGE (Fig. 9). Under denaturing conditions complexes that are not crosslinked dissociate. Our results showed that DNA was damaged extensively with conventional UV light and damage increased with increasing intensity. Although MBP-PIF3 and radioactively labeled probe were showing non-covalent interactions in EMSA (Fig. 8) we could not detect any covalent bonding induced by photocrosslinking in SDS-PAGE.

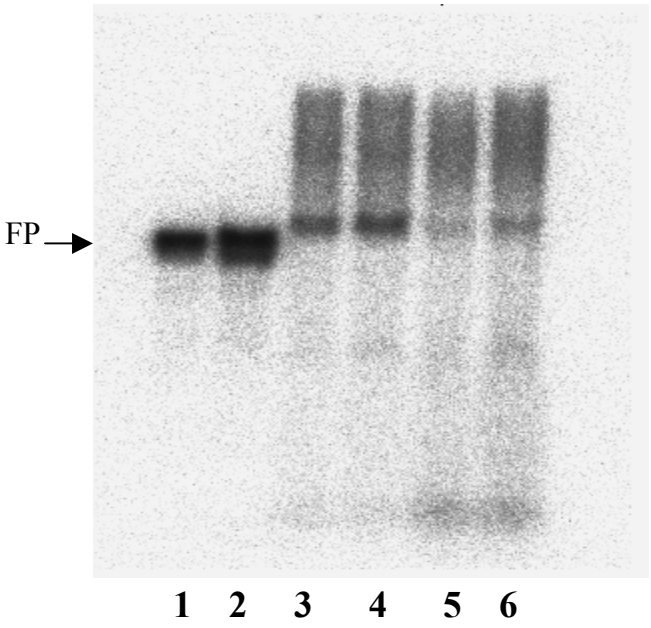


Fig. 9: Radioactive 66-mer oligonucleotide was incubated without MBP-PIF3 (lanes1, 3, 5) or with 2µg MBP-PIF3 (lanes 2, 4, 6) as for the band-shift assays. 20µl of binding sample was used for each irradiation. Lanes 1 and 2: no irradiation. Lanes 3 and 4: irradiation with intensity of 0.5J/cm^2 for 30 minutes. Lanes 5 and 6: irradiation with intensity of 0.9J/cm^2 for 60 minutes. Samples were analyzed by SDS-PAGE. FP: free probe

Analysis of DNA crosslinking to purified MBP-PIF3 with high intensity UV irradiation

The UV laser cross-linking was carried out using purified recombinant MBP-PIF3 (Fig.6) and a 66 bp DNA duplex that contained the specific binding site; G-box motif (CACGTG). MBP-PIF3 formed a specific complex with DNA, which was observed as a single band with a lower electrophoretic mobility (Fig. 10, lanes 5 and 6). The positive effect of 2 mM DTT on the complex formation has been determined in Fig 10 as a comparison between lane 5 and 6. On the other if freshly purified protein was used in EMSA, the effect of DTT on complex formation was negligible (data not shown).

The complex thus formed was then exposed to 5 ns, 266 nm laser pulses. No change on the mobility of the complex was observed on the band-shift gel, indicating that the UV irradiation caused little or no complex disruption (Fig. 10, lanes 7, 8, 9, 10, 11, 12). No complexes were observed when the probe was incubated without MBP-PIF3 and was irradiated (Fig. 10, lanes 3 and 4) or not irradiated (Fig. 10, lanes 1 and 2).

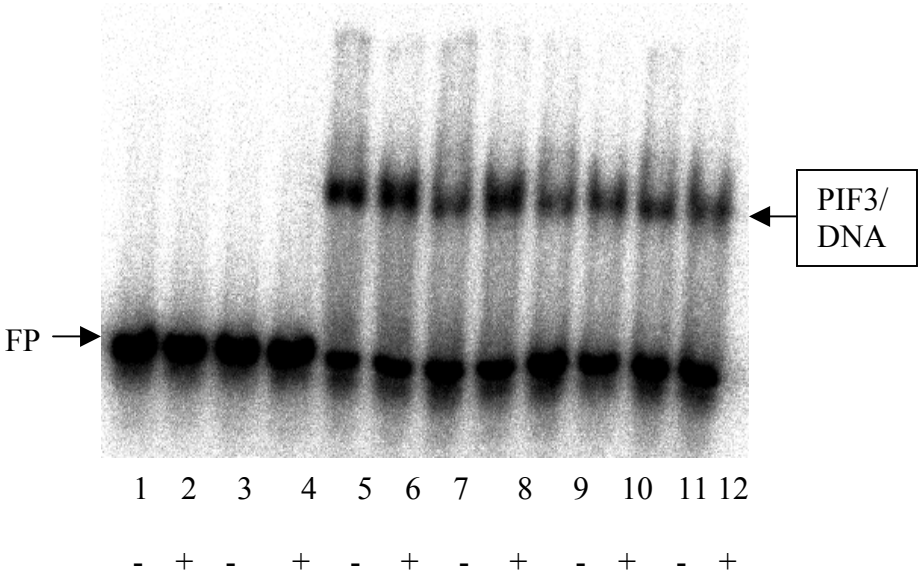


Fig. 10: Crosslinking of recombinant MBP-PIF3 to DNA; 25fmol of 32P-labeled probe was incubated without MBP-PIF3 (lanes1, 2, 3, 4) or with 2µg MBP-PIF3 (lanes 5-12) in the presence of 100ng poly[d (I.C)] for 15 min at room temperature. Binding buffer has DTT (+) in lanes 2, 4, 6, 8, 10, 12 and does not have DTT (-) in lanes 1,3,5,7,9,11. Lanes 1, 2, 5, 6: no irradiation. Irradiations were performed with a quadrupled Nd: YAG laser (5ns, 5.5 mJ/pulse, 266 nm) with different number of pulses. Lanes 3, 4, 11, 12: with 30 pulses. Lanes 7 and 8: with 6 pulses and lanes 9 and 10: with 30 pulses. Samples were analyzed by EMSA.

Parallel irradiated and unirradiated samples were also analyzed by denaturing SDS-PAGE (Fig. 11). Under denaturing conditions complexes that are not crosslinked dissociate and the DNA probe migrates off the gel. No covalent complexes induced on the laser irradiation were observed by SDS electrophoresis (Fig. 11).

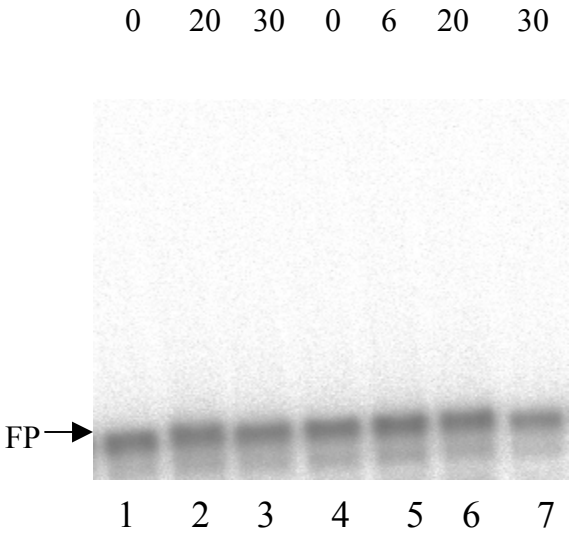


Fig. 11: Radioactive probe was incubated without protein (lane 1) or with 2 μg protein (lanes 2, 3, 4, 5, 6, 7) as for the band-shift assays. Lanes 1 and 4: no irradiation. Samples were irradiated either with UV laser pulses (5ns, ~5mJ/pulse, and 266 nm); lanes 5, 6, 7 or with a combination of UV laser pulses ((5ns, ~5mJ/pulse, and 266 nm) and blue laser pulses (~800ns, 56 mJ/pulse, 480 nm); lanes 2 and 3, at different number of pulses (i.e. 6, 20 and 30). Samples were analyzed by SDS-PAGE. FP: free probe

Analysis of the integrity of DNA upon Low-Intensity UV Irradiation

Samples containing a plasmid with G-box motif were irradiated with a set of four 8 W germicidal bulbs with an incident fluence of 0.5 J/cm^2 or 0.9 J/cm^2, cleaved with the restriction enzyme and analyzed primer extension. The amount of full-length primer extension product is inversely related to the amount of DNA damage. We have demonstrated that after conventional UV light irradiation the full-length product, 300 bp, was not detectable at all, unlike to the unirradiated sample **(Fig. 12).**

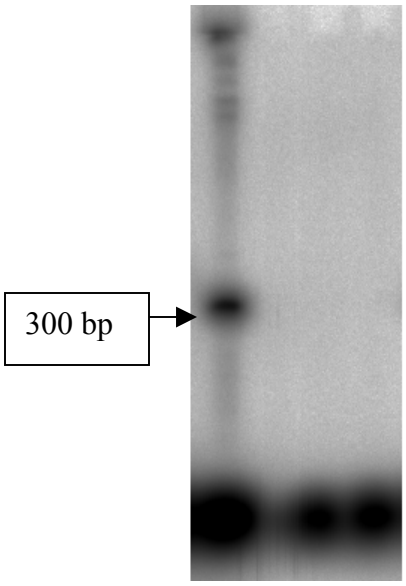


Fig. 12: Samples with 12N1 plasmid were irradiated with conventional UV light (254 nm), cleaved with restriction enzyme XhoI and analyzed by primer extension. The reaction products were separated on a 6% sequencing gel and dried gel was analyzed with a PhosphorImager. Lane 1, unirradiated control; lane 2 and 3, samples were irradiated with 0.5 J/cm^2 and 0.9 J/cm^2, respectively.

The Effects of irradiation with UV laser pulses alone and with a combination of UV laser pulses and blue pulses on DNA integrity

Samples containing a 12N1 plasmid were irradiated with either a quadrupled Nd: YAG laser (5ns, ~5mJ/pulse, and 266nm) or with a combination of UV laser pulses (5ns, ~5mJ/pulse, 266nm) and blue laser pulses (~800ns, 56mJ/pulse, 480nm) at different number of pulses. Irradiated samples were cleaved with the restriction enzyme to linearize

the plasmid DNA. Digested plasmids were analyzed by primer extension to check the DNA integrity.

We have demonstrated that by UV laser irradiation (5ns, ~5mJ/pulse, and 266nm), the amount of full-length extension products, ~300 bp, was decreasing with increasing number of pulses (Fig. 13 and Fig 14). The amount of extendable DNA was shown to be higher (Fig. 14) when the irradiation buffer has 2 mM DTT as compared to without DTT (Fig. 13). The amount of extendable DNA after the two wavelength pulses did not show any enhancement compared to UV pulses alone (Fig.15).

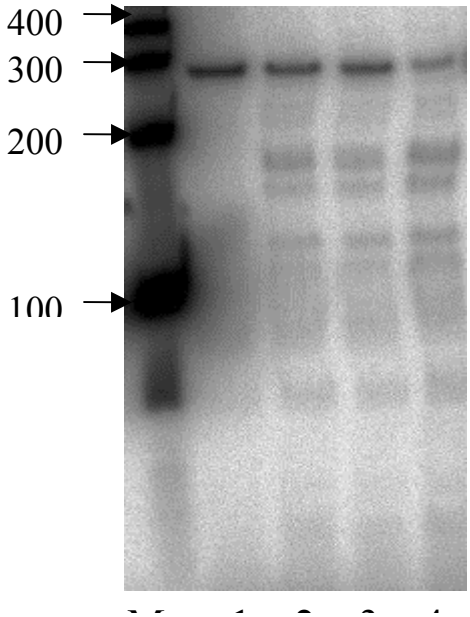


Fig. 13: Analysis of the integrity of DNA upon UV laser irradiation (5ns, ~5mJ/pulse, and 266nm). Samples containing a 12N1 plasmid were irradiated with different number of pulses (Lane 1: unirradiated control; lane 2, 3 and 4, samples irradiated with 6, 20, 30 number of pulses respectively. The sample buffer has no DTT.

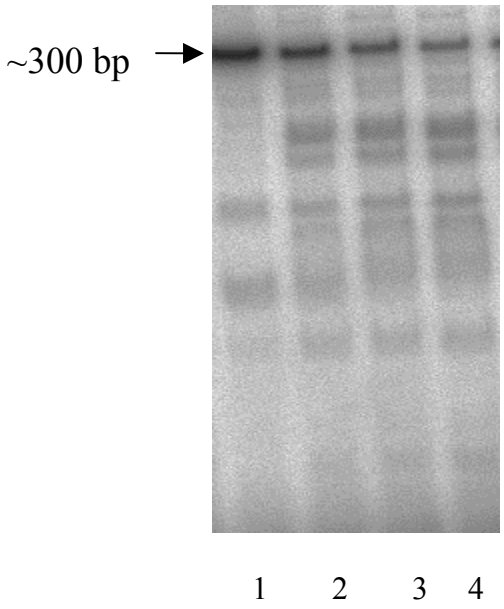


Fig. 14: Analysis of the integrity of DNA upon UV laser irradiation (5ns, ~5mJ/pulse, and 266nm). Samples containing a 12N1 plasmid were irradiated with different number of pulses. Lane 1: unirradiated control; lane 2, 3 and 4, samples irradiated with 6, 20, 30 number of pulses respectively. The sample buffer has 2 mM DTT.

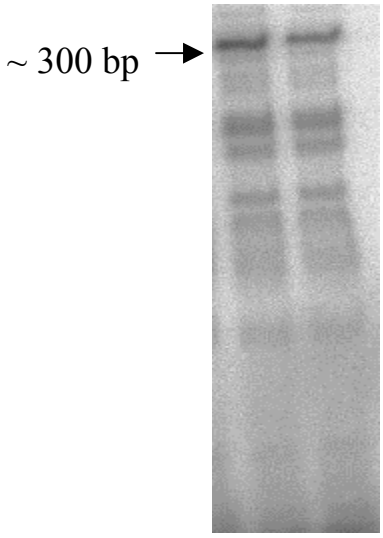


Fig. 15: Analysis of the integrity of DNA after irradiation with a combination of UV laser pulses (5ns, ~5mJ/pulse, 266nm) and blue laser pulses (~800ns, 56mJ/pulse, 480nm). Lane 1, sample irradiated with 20 pulses; lane 2, sample irradiated with 30 pulses. The sample buffer does not have any DTT.

DISCUSSION

Ultraviolet light has been shown to induce crosslinks between DNA and a variety of DNA binding proteins. Conventional crosslinking methods use low-power broad-band germicidal lamps, and to produce sufficient crosslinked product, irradiation times of minutes to hours are required, resulting in severe nucleic acid and protein degradation (Ho et al., 1994). Previous studies have been demonstrated in general, UV laser light increases crosslinking efficiencies compared to conventional UV lamps by two orders of magnitude (Budowsky et al., 1986). However, we could not increase crosslinking efficiency with UV laser light high enough to detect crosslinked MBP-PIF3/DNA bands by denaturing SDS-PAGE.

In this study we have compared DNA damage caused by ultraviolet irradiation of low and high intensity (10 $W/m^{2,}$ 254nm, continuous irradiation, and up to 10^{12} W/m^2, 266nm, pulse duration 5ns). We irradiated same amount of protein-DNA complexes with total of maximum ~10 mJ energy in low-intensity UV experiments and total of maximum ~100 mJ in high-intensity UV experiments. We found that after UV laser irradiation the amount of full-length product was almost 2/3 of that from the unirradiated DNA whereas upon low intensity irradiation we could not detect any amplified DNA.

Pyrimidine dimers have been reported to be the main photolesions caused by conventional UV lamps and the amount of pyrimidine dimers have been shown to be decreasing with high intensity laser irradiation (Nikogosyan, 1990 and Douki et al., 2001). Although UV laser pulses cause DNA lesions such as single-strand nicks (Russmann et al., 1997), our data show that high powered UV laser pulses damage DNA less than conventional UV irradiation as reported previously (Lejnine at al., 1999, Moss et al., 1997, and Budowsky at al., 1986).

As a future work we should experimentally determine the optimum range of photon flux for the system of interest to obtain maximum amount of cross-linking. Alternatively, we might switch to a different DNA-binding protein that has a greater propensity to undergo UV-promoted DNA crosslinking.

Abbreviations: EMSA, Electrophoretic mobility shift assay; SDA-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; AEBSF, (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride); IPTG, isopropylthio-β-D-galactoside; TBE, Tris-Borate-Ethylene diamine tetraacetic acid; TMB, 3,3',5,5'- tetramethyl benzidine; HEPES, N-2-Hydroxyethyl-piperazine-N9-2-Ethanesulfonic acid

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