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Iodouracil-mediated photo-cross-linking of DNA to *Eco*RII restriction endonuclease in catalytic conditions

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ABSTRACT

We use a XeCl eximer laser with 50 ns pulses, a frequency of 0.3 Hz and a wavelength of 308 nm in appropriate conditions for the photocrosslinking of *Eco*RII restriction endonuclease to a 14-mer DNA duplex, containing a 5-iodo-2'-deoxyuridine residue (IdU). IdU replaced the thymidine residue within the *Eco*RII recognition sequence 5'-CCT/AGG. The binding of *Eco*RII endonuclease to IdU-containing DNA duplex was analyzed by gel retardation assay in the presence of Ca^{2+} or Mg^{2+} ions. Photocrosslinking of *Eco*RII to IdU-containing DNA duplex occurred only in a pre-reactive complex formed in the presence of Ca^{2+} ions. Photocrosslinking yields as a function of time and UV-laser light intensity were studied.

INTRODUCTION

Type II restriction–modification systems (RMS) are common in bacteria, protecting cells from foreign DNA. One of the *Escherichia coli* RMS, *Eco*RII, includes endonuclease and DNA methyltransferase which recognize the DNA sequence 5'↓CCA/TGG. Restriction endonuclease *Eco*RII cleaves DNA (cleavage site is indicated with an arrow) in the presence of Mg^{2+} ions and methyltransferase *Eco*RII methylates C5 of the internal cytosine (underlined) in the recognition site. Endonuclease *Eco*RII belongs to the type IIE restriction enzymes, which require binding to another recognition sequence for DNA cleavage [1,2]. Recently, the crystal structure of a type IIE restriction enzyme *Nae*I has been reported, [3] but that of *Eco*RII is unknown. The reaction catalyzed by *Eco*RII shows positive substrate cooperativity [4]. The active *Eco*RII-substrate complex consists of two subunits of endonuclease interacting with two DNA recognition sites [5]. Depending on the substrate concentration, functional cooperativity can occur between two recognition sites on a single molecule (in *cis*) or between two sites from different DNA molecules (in *trans*) [1]. Transmission electron microscopy provided direct evidence that *Eco*RII mediates loop formation [6]. The identification of functional domains of *Eco*RII is at its very beginning. Recently, the sites of enzyme responsible for specific DNA-binding were determined by membrane-bound peptide repertoires [7]. However, *Eco*RII-DNA interface is not characterized, catalytic sites and their location in a dimeric enzyme are unknown. One of techniques for investigating a structure of enzyme-substrate complexes is photo-cross-linking of DNA to the associated protein[8]. A 5-iodo-2'-deoxyuridine residue (IdU), which is "zero length" label is being incorporated into DNA to enhance photosensitivity[8]. A single substitution of IdU for dT does not appreciably disturb the protein-DNA complexes. The incorporation of IdU into nucleic acids and the use of high powered UV-lasers permit to obtain high photo-cross-linking yields providing adequate material for characterization [9]. Photoaffinity labeling of DNA methyltransferases with IdU-DNAs is widely used for determination of functionally important protein regions or/and amino acid residues [10-12]. Methyltransferases are usually photo-crosslinked to their cognate IdU photolabel containing DNAs with high efficiency [10-12]. There are

few reports on photoaffinity labeling of endonucleases by IdU-DNAs. Photocrosslinking of *Sso*II endonuclease to IdU-containing DNA *via* Trp61 by irradiation with helium/cadmium laser (325 nm) has been reported elsewhere [13] Here, we used the light-induced photochemical crosslinking with a XeCl laser emitting at 308 nm to examine the covalent attachment of type IIE endonuclease *Eco*RII to IdUsubstituted DNA. Our long term goal is to determine the architecture of the *Eco*RII–DNA complex. The chemical crosslinking of *Eco*RII to DNA duplexes containing chemically active groups in the sugar-phosphate backbone has been reported elsewhere.[14,15] Moreover, DNA duplex with a photoreactive aryl(trifluoromethyl)diazirine group has been tested for crosslinking to this enzyme.[16] **.**

EXPERIMENTAL

Enzymes and oligonucleotides

The restriction endonuclease *Eco*RII (21 µM/monomer, 200 U/µl) was purified from *Escherichia coli* JM 109 cells carrying the isopropil-β-D-thiogalactopyranoside–inducible overexpression plasmid pQER15 which contained the *ecoRIIR* gene [17]. The *Eco*RII encoded by pQER15 plasmid possessed an terminal His_6 affinity tail, making it possible to purify the enzyme by one–step Ni-chelate affinity column chromatography. One unit of restriction endonuclease activity was defined as the amound of enzyme that completely digested 1 µg of λ DNA at 37ºC in 1 h.

Oligonucleotides containing 5-halopyrimidines were synthesized as described elsewhere [18]. To avoid degradation of the 5-iodo- and 5-bromouracil and during deprotection, special phosphoramidites carrying the t-butylphenoxyacetyl group for the protection of the exocyclic amino group of the natural bases were used. These phosphoramidites allow to run the ammonia deprotection at room temperature where decomposition of 5-halopyrimidines is minimal. DNA duplexes were ^{32}P-labeled by T4 polynucleotide kinase (10 U/µl, Sintol, Russia) and [γ-^{32}P]-ATP (1000 Ci/mol, Izotop, Russia).

Cleavage analysis of DNA duplexes by *Eco*RII was performed as described [14].

Gel mobility shift assay

0.15-0.48 μM *Eco*RII was incubated with 0.5 μM ^{32}P-labeled DNA duplexes I (hereafter concentrations of enzyme and DNA duplexes were calculated per monomer and per duplex, respectively) in 10 μl of 40 mM Tris-HCl buffer (pH 7.6), containing 50 mM NaCl, 7 mM dithiotreitol (DTT), 8% glycerol (buffer A) and 10 mM $CaCl_2$ or 5-10 mM $MgCl_2$ for 10 min at 37° C and 20 min on ice. The reactions were run for 3 h at 100 V on native 7.5% polyacrylamide gel (PAG). The gel was pre-run for 1 h at 100 V. For authoradiography of the electrophoretic pattern, Kodak-XOMAT-S film was exposed at 4˚C overnight.

Photochemical cross-linking

Photo-cross-linking reactions of *Eco*RII (0.5 or 0.7 μM) to DNA duplexes I (0.5 or 0.7 μM) or II (0.7 μM) were performed in 10 or 500 μl of 40 mM Tris-HCl buffer (pH 7.6), containing 50 mM NaCl and 7 mM DTT (buffer B) in the presence or in the absence of 10 mM $CaCl_2$. Reaction mixtures were preincubated for 10 min at 37ºC and 20 min on ice. Photo-cross-linking was induced with a XeCl eximer laser [22] operating at 308 nm, pulse duration 50 ns, repetition rate 0.3 Hz, pulse energy 20 mJ and beam diameter 4 cm. Reactions were conducted in 0.5 ml micro test tubes containing 10 μl aliquots or in quartz cuvette (l 0.5 cm) containing 500 μl aliquots on ice for 1.3-40 min. To vary of the laser intensity probes were settled at different distances from the focal point of the collecting lens (f 2m). Reactions were analyzed by 12% SDS-PAGE. Gels were analyzed by authoradiography or by Coomassie blue staining followed by authoradiography. The cross-linking yields were determined as the ratio of the covalent conjugate radioactivity to the total radioactivity of conjugate and unbound oligonucleotide. Radioactivity of gel slices was determined by Cherenkov counting.

Ultraviolet light-induced degradation of 5-iodo-2'-deoxyuridine

Aqueous solutions of 1.5 M IdU (0.1 ml) were placed into the 1.5 ml micro test tubes and

irradiated at intensity of 5×10^5 W/cm^2 or 1×10^7 W/cm^2. After 10-30 min of irradiation solutions were diluted tenfold with water. Absorption spectra were obtained using a Hitachi 150-20 spectrophotometer (Japan).

RESULTS AND DISCUSSION

Binding of R•*Eco*RII to IdU-substituted DNA duplex

We use a 14-mer DNA duplex I, in which dT within the recognition sequence of *Eco*RII (**CCA/TGG**) was replaced by a photo-label IdU as a reagent for photo-cross-linking to *Eco*RII.

5'GCCAA**CCXGG**CTCT 3'
3'CGGTT**GGACC**GAGA 5' (I), where X=IdU

The substrate properties of modified DNA and its ability to specifically bind to *Eco*RII were examined. It was found that introduction of IdU into the center of *Eco*RII recognition site does not abolish activity of the enzyme (data not shown). In the absence of Mg^{2+} ions, the canonical substrate formed two complexes with *Eco*RII characterized by specific electrophoretic mobilities as revealed by gel retardation assay [20]. Addition of Mg^{2+} ions resulted in disappearance of the "lower" complex and led to accumulation of the "upper" complex. At catalytic Mg^{2+} ions concentration (5-10 mM) only "upper" complex was formed [21] (O.V. Petrauskene and E.S. Gromova, unpublished data). We carry out photocrosslinking experiments with IdU-DNA duplex I in the conditions of the formation of one type of complexes, an "upper" one. However, in the presence of Mg^{2+} ions this duplex was cleaved, therefore the use of Ca^{2+} ions, which are cofactor analog, seems reasonable. Ca^{2+} ions do not behave as a cofactor of restriction enzymes but replace Mg^{2+} ions in the enzyme catalytic center and thereby to inhibit hydrolysis [22]. *Eco*RII does not cleave the canonical 14-mer substrate with one recognition site in the presence of Ca^{2+} [17]. To study formation of IdU-substituted DNA - *Eco*RII complex with Ca^{2+} ions, a gel mobility shift experiment was performed (Fig.1). In the range of DNA/*Eco*RII monomer molar ratio ~3:1-1:1 one type of complexes was formed (Fig.1, lanes 1-5). In the presence of 5 mM Mg^{2+} ions (lanes 6-7) and 10 mM Mg^{2+} (lanes 8-9) one can see IdU-DNA duplex I – enzyme complexes, traces of starting IdU DNA duplexes and products of their cleavage

by *Eco*RII. The electrophoretic mobilities of IdU-substituted DNA-*Eco*RII-Ca^{2+} complexes (lanes 1-5) are the same as those of (IdU-DNA)-*Eco*RII-Mg^{2+} complexes (lanes 6-9). Thus, the complex of IdU-substituted DNA with *Eco*RII in the presence of Ca^{2+} ions is a pre-reactive one and consists of dimeric *Eco*RII and two IdU-DNA duplexes.

Formation of the photo-cross-linked *Eco*RII-DNA complex

We used a XeCl eximer laser, which is a powerful source of monochromatic light (308 nm) with 50 ns pulses and frequency of 0.3 Hz [19] for photocrosslinking of *Eco*RII to 14-mer IdU-containing DNA. The 308 nm-emitting XeCl eximer laser with other parameters has been used previously in protein-RNA[23] and protein-DNA[24,25] cross-linking.

***UV cross-linking*.** The ^{32}P–labeled DNA duplex containing IdU for dT substitution in the recognition site was irradiated with 308-nm monochromatic light in the presence of *Eco*RII and Ca^{2+} ions, the DNA/enzyme ratio being ~1:1, i.e. under conditions when only one type of enzyme-substrate complex was formed (Fig.1, lane 5). Aliquots of reaction mixtures were analyzed by denaturig polyacrylamide gel electrophoresis (PAGE). Irradiation of IdU DNA-*Eco*RII-Ca^{2+} complex resulted in formation of only one product migrating in gel more slowly as compared to free ^{32}P–labeled oligonucleotide (Fig.2A). This band representing a cross-linked *Eco*RII-DNA complex was observed both on the Coomassie blue stained gel and on autoradiogram. We failed to obtain *Eco*RII–DNA crosslink without Ca^{2+} ions (Fig.2A).

The formation of a crosslink in the presence of Ca^{2+} suggests that cross-linking occurs at the catalytic step. A conformational transition of the enzyme may occur in the presence of Ca^{2+} ions thus bringing an amino acid residue of *Eco*RII closer to IdU of the central base pair of *Eco*RII recognition site in DNA. As a result, the iodouracil residue and the amino acid residue took a suitable orientation that favored crosslinking. In the case of chemical crosslinking of *Eco*RII to DNA duplex containing monosubstituted pyrophosphate internucleotide bond crosslinking yields strongly depended on Mg^{2+} concentration, supporting the conformational transition of *Eco*RII [15].

When instead of IdU 5-bromo-2'-deoxyuridine residue (BdU) was introduced into the center of the *Eco*RII recognition site, the yield of *Eco*RII-DNA cross-link in the presence of Ca^{2+} dramatically reduced (Fig.2B). This experiment was performed at intensity ~1×10^6 W/cm^2. The difference in the cross-linking efficiency with IdU- and BdU-containing 14-mer DNAs may be due to the higher absorption of the IdU chromophore at 308 nm and the excellent leaving group reactivity of iodide [8,9].

***Photocrosslinking as a function of laser light intensity*.** To improve efficiency of covalent attachment of *Eco*RII to IdU-substituted DNA duplex, the time courses of the cross-linking reaction at different laser light intensities were monitored (Fig.3A and B). All experiments were performed in the presence of Ca^{2+} ions. A lot of products were formed upon irradiation of DNA-enzyme complex with high-intensity laser light (~1.5×10^7 W/cm^2) during 10-30 min. Photocrosslinking yields decreased with the time of irradiation. Products moving on the gel faster than the *Eco*RII–DNA covalent complex were observed (Fig. 3A). When the irradiation intensity was reduced to ~1×10^6 W/cm^2, the number of undesirable products of photodamage diminished (Fig. 3A). The yield of EcoRII–DNA crosslink increased with time and leveled off at ~20% in 9 min of irradiation. Only one band representing a crosslinked complex was observed, both on the Coomassie blue stained gel and on autoradiogram, when the laser operated at ~5×10^5 W/cm^2 (Fig. 3A). However, in this case longer incubation times were necessary to achieve ~ 11% crosslinking of total DNA to EcoRII. It is noteworthy that crosslinking experiments were performed at DNA–EcoRII monomer molar ratio ~1 : 1 to have stoichiometry of the active DNA–enzyme complex.[5] However, in these conditions only part of total DNA was bound to the enzyme (Fig. 1, lane 5). Hence, the resulting crosslinking yields calculated relatively to bound DNA should be larger.

It should be noted that the mechanism of photodecomposition of 5-iodo-2'-deoxyuridine in aqueous solution depends on laser light intensity (Fig. 4). Irradiation at different intensities resulted in progressive loss of the characteristic absorption peak near 280 nm. After 30 min of irradiation at ~1.5×10^7 W cm^{-2} (Fig. 4B, curve 3) the photoproduct shows a peak near 260 nm which is

characteristic of the absorption spectrum of 2'-deoxyuridine. At ~1.5×10^5 W cm^{-2} the loss of absorbance of IdU at ~280 nm was not accompanied by the appearance of a peak at ~260 nm (Fig. 4A, curve 3). This result probably fits the photodimerization of dU monomers in solution.[26]

The formation of photodamage products at laser intensities higher than ~5×10^5 W/cm^2may be mainly due to protein photodegradation as it follows from mobilities of degradation products in the gel (Fig.3), in agreement with the available data on enzyme-DNA complexes irradiated at 308 nm [9,10]. The mechanism of protein photodamage at intensities higher than ~5×10^5 W/cm^2 is apparently associated with absorption of additional quantum by preexcited Trp residues owing to high laser intensities. The sum of energies of two 308 nm photons is about 8 eV, which is enough for a Trp excited electron to be expelled from the protein. Thus, the protein converts into a very reactive ion radical and may thus give rise to photodamage conversions. Accordingly, the decrease of intensity to ~1.5×10^5 W/cm^2 allowed us to avoid the photodamage of the enzyme (Fig. 3A, right window). The correlation between the intensity of Nd-YAG laser (266 nm) irradiation and the degradation of nucleic acid in the DNA–protein complex has been described elsewhere and attributed to biphotonic excitation by high intensity pulsed lasers.[8]

At intensities higher than ~1.5×10^5 W cm^{-2} (for example, at ~1.0×10^6 W cm^{-2}), degradation occurred at 2 min of irradiation (Fig. 3A). Similar results have been reported for the methylase EcoRI–(IdU-DNA) complex.[10] Here, at ~1.5×10^5 W cm^{-2}, even at 30 min of exposure, we did not detect degradation of the crosslinked complex.

Photocrosslinking reaction yields as a function of laser light intensity at the fixed dose of energy were examined. We increased irradiation time as the intensity decreased. The data were fitted to an exponential decay function, revealing that photocrosslinking yields dramatically depend on laser light intensity (Fig. 5).

Thus, we established the conditions for the photoaffinity modification of *Eco*RII by IdU-substituted DNA with XeCl laser (308 nm) avoiding protein and DNA degradation.

CONCLUSIONS

The 308 nm-emitting XeCl excimer laser induced crosslinking of *Eco*RII to DNA-duplex containing IdU in the center of *Eco*RII recognition site in catalytic conditions, i.e. in the presence of Ca^{2+} as substitute for the natural cofactor, Mg^{2+}, indicating a close proximity between the protein and the central thymine base in the recognition site of the catalytic *Eco*RII-DNA complex. Cross-linking yields strongly depend on the laser light intensity. Yield (~11%) was maximal at ~5×10^5 W/cm^2 during 30 min. In these conditions biopolymers did not show photodamage.

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FIGURES

Fig. 1. Binding of *Eco*RII to IdU-containing DNA duplex I in the presence of Ca^{2+} or Mg^{2+} ions. Autoradiogram of 7.5% non-denaturing PAG. The binding reactions contained 0.5 μM ^{32}P-labeled DNA, *Eco*RII with indicated concentrations (0.15-0.48 μM), buffer A and indicated concentrations of $CaCl_2$ or $MgCl_2$.

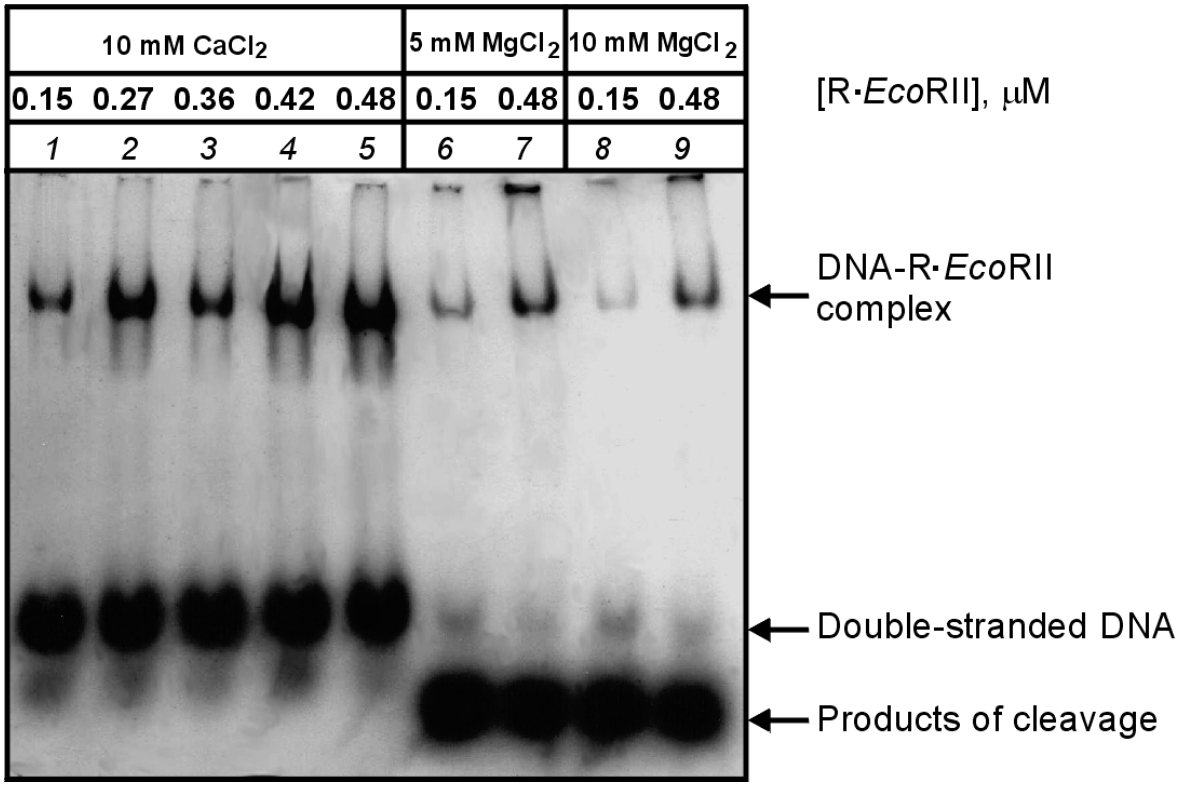

Fig. 2. Photo-cross-linking of *Eco*RII to DNA-duplexes containing IdU (I) or BdU (II) in the center of *Eco*RII recognition site at 308 nm. Products were separated by 12% SDS-PAGE. ***A***, 0.5 μM ^{32}P–labeled DNA duplex I and 0.7 μM of *Eco*RII in the presence (*lane 3*) or in the absence (*lane 2*) of 10 mM $CaCl_2$ after irradiation. *Lane 1*: ^{32}P–labeled DNA-duplex I. ***B***, 0.7 μM ^{32}P–labeled DNA-duplexes I (*lane 1*) or II (*lane 2*) and 0.7 μM *Eco*RII in the presence of 10 mM $CaCl_2$ after irradiation. DNA-duplex II:

5'GCCAA**CCXGG**CTCT 3'
3'CGGTT**GGACC**GAGA 5' , where X=BdU

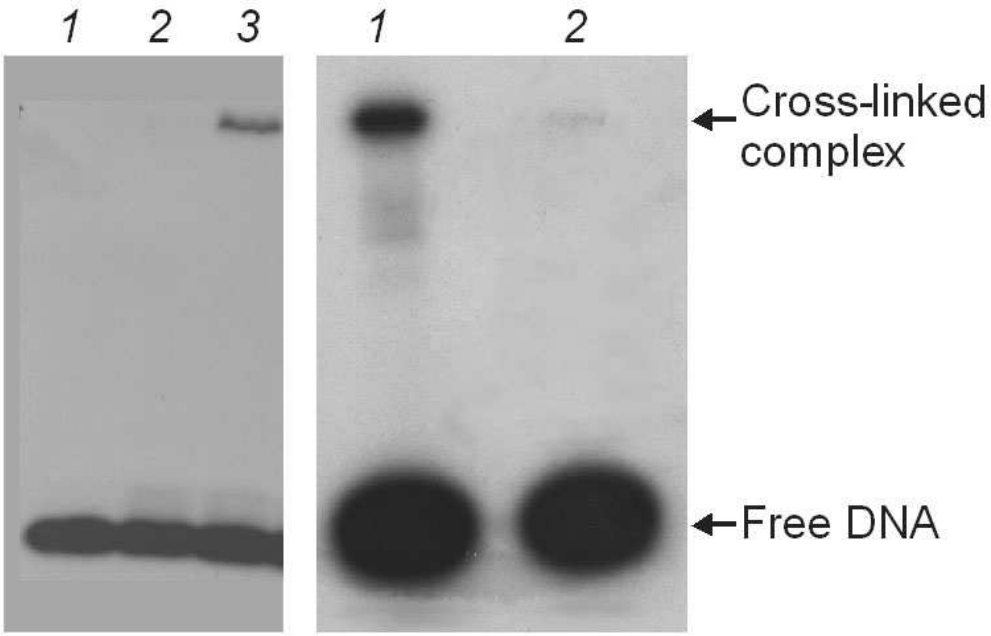

Fig. 3. Photocrosslinking of *Eco*RII to IdU-DNA duplex I as a function of irradiation time at different laser light intensities. ***A***, autoradiograms of 12% SDS-PAGE displaying products after irradiation of reaction mixture containing 0.5 μM ^{32}P–labeled DNA-duplex I and 0.5 μM *Eco*RII in buffer B in the presence of 10 mM $CaCl_2$ at 308 nm. Irradiation times, light intensities (I) and photo-crosslinking yields are indicated. ***B***, photocrosslinking yields as a function of reaction times at corresponding intensities.

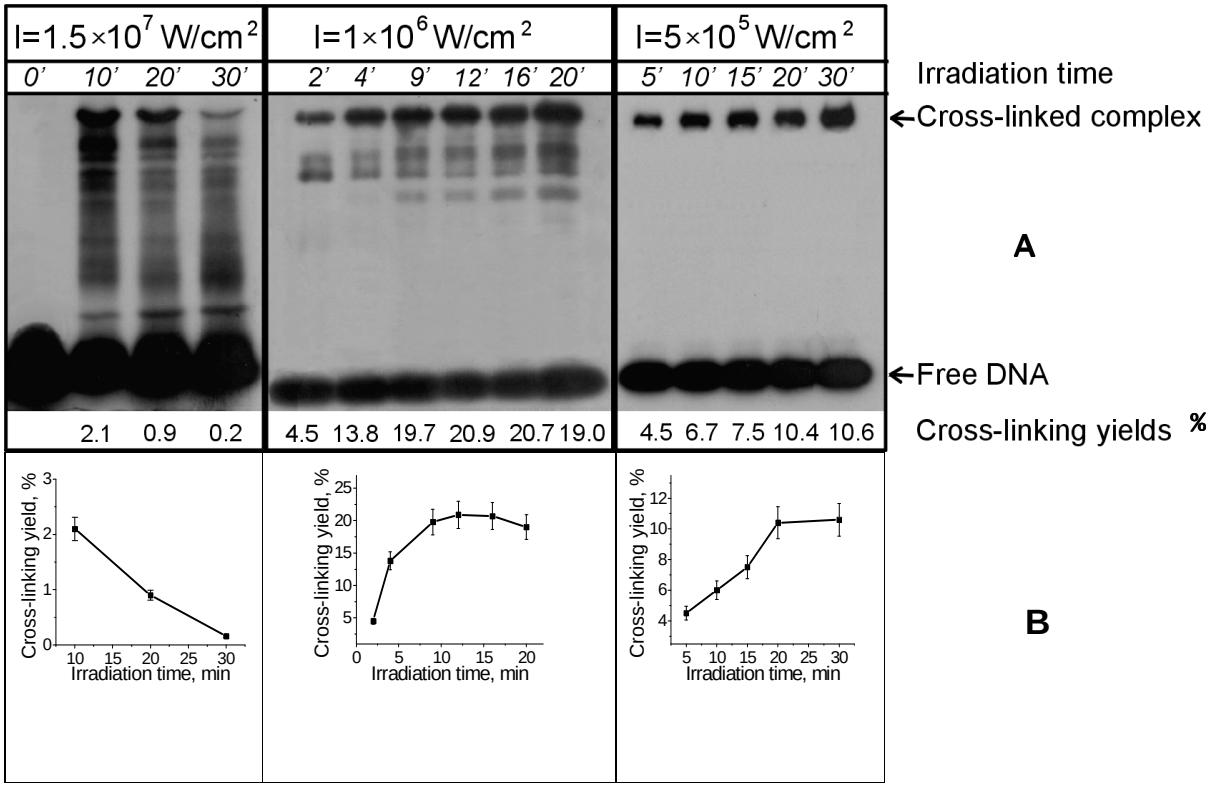

Fig. 4 UV-absorption spectra of 0.15 M 5-iodo-2'-deoxyuridine in aqueous solution without irradiation or after irradiation with UV laser light. Spectra 1(—)-without irradiation; spectra 2 (···) and spectra 3 (---)-irradiation for 15 and 30 min, respectively. Laser light intensities were 5×10^5 W/cm^2 (***A***) or 1×10^7 W/cm^2 (***B***).

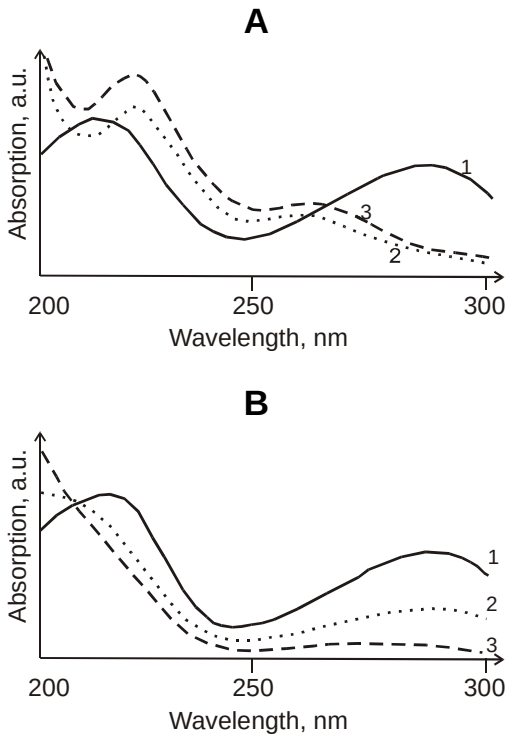

Fig. 5. Dependence of photo-cross-linking of *Eco*RII to IdU-DNA duplex I on laser light intensity at dose absorbed kept constant. 0.5 μM ^{32}P–labeled DNA-duplex I and 0.5 μM *Eco*RII were irradiated at 308 nm for 1.3, 2.5, 5.0, 10 and 40 min at intensities 3.6×10^6, 1.8×10^6, $1\times10^6$and 0.12×10^6 W/cm^2 respectively in buffer B in the presence of 10 mM $CaCl_2$.