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Factors influencing resistance of UV-irradiated DNA to the restriction endonuclease cleavage

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Abstract

DNA molecules of pUC19, pBR322 and ΦX174 were irradiated by various doses of UV light and the irradiated molecules were cleaved by about two dozen type II restrictases. The irradiation generally blocked the cleavage in a dose-dependent way. In accordance with previous studies, the (A + T)-richness and the (PyPy) dimer content of the restriction site belongs among the factors that on average, cause an increase in the resistance of UV damaged DNA to the restrictase cleavage. However, we observed strong effects of UV irradiation even with (G + C)-rich and (PyPy)-poor sites. In addition, sequences flanking the restriction site influenced the protection in some cases (e.g. *Hin*dIII), but not in others (e.g. *Sal*I), whereas neoschizomer couples *Sma*I and *Ava*I, or *Sac*I and *Ecl*136II, cleaved the UV-irradiated DNA similarly. Hence the intrastrand thymine dimers located in the recognition site are not the only photoproduct blocking the restrictases. UV irradiation of the A-form generally made the irradiated DNA less resistant to restrictase cleavage than irradiation in the B-form and in some cases, the A-form completely protected the UV-irradiated DNA against the damage recognized by the restrictases. The present results also demonstrate that the UV irradiation approach used to generate partial digests in genomic DNA studies, can be extended to the (G + C)-rich and (PyPy)-poor restriction sites. The present extensive and quantitative data can be used in genomic applications of UV damage probing by restrictases.

Keywords: DNA; Structure; Damage; UV light; Restrictases

1. Introduction

Type II restriction endonucleases (restrictases) belong among the most specific proteins recognizing nucleotide sequences in DNA. The recognized sequences are mostly six, four or eight nucleotides in length and they are palindromatic, which reflects homodimeric restrictase structure. Restrictases mostly cut both DNA strands in the target sequence, which together with the high nucleotide sequence specificity makes them a key tool in many molecular biology labs. More than 3000 restrictases have already been described (for recent information see database REBASE [1], http://rebase.neb.com) and some of them have also been characterized regarding the molecular structures of their complexes with DNA [2–5]. Restrictases bind to DNA either in the major groove, e.g. *Eco*RI [2] and *Bam*HI [5], or in the minor groove, e.g. *Eco*RV [3] and *Pvu*II [4]. Restrictase contacts to DNA extend beyond the target sequence, e.g. *Eco*RI binds to 12 bp of the DNA of which only 6 bp are specifically recognized [6]. Restrictase binding frequently bends and unwinds DNA [7–9]. Restrictase *Nae*I simultaneously binds to multiple sites on DNA to form DNA loops [10]. It is interesting that restrictases are evolutionary relatives of recombinases [11] and topoisomerases [12].

The highly specific cleavage of DNA by restrictases is influenced by a number of factors. First of all, restrictases require divalent (mostly magnesium) cations to cleave the target sequence in DNA. Other divalent cations, alkaline pH, glycerol and other organic solvents reduce the restrictase specificity (e.g. *Eco*RI [13] and *Eco*RV [14]). This reduced specificity brings about restrictases that cleave DNA at sites slightly differing from the target sequence (so-called star activity). The primary and secondary structure of DNA are another important factors. Regions flanking the target site influence the rate of cleavage. A flanking oligopurine tract causes nicks in a pseudo-*Eco*RI target sequence (GAATTA) [15]. Restrictases cleave neither the left-handed Z-form [16] nor triplex DNA [17]. Restrictase cleavage is also hindered by the target site methylation [18,1]. Another factor

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hindering restrictase cleavage is the damage induced in DNA by UV irradiation.

The first reports showing that UV irradiation of DNA inhibits its cleavage by some restriction enzymes appeared in the early eighties [19–21]. In these studies, supercoiled plasmid pBR322 and viral SV40 DNAs as well as linear phage SPP1, SPP2 or lambda DNAs were irradiated with UV light (254 nm) doses up to 4 kJ m^{-2} and cleaved with restriction enzymes *Hin*dIII (AAGCTT), *Taq*I (TCGA), *Eco*RI (GAATTC), *Bam*HI (GGATCC), *Sal*I (GTCGAC), *Hha*I (GCGC) and *Hae*III (GGCC) [19,21], *Eco*RI (GAATTC), *Hin*dIII (AAGCTT) and *Hae*III (GGCC) [20]. *Eco*RI (GAATTC) and *Hin*dIII (AAGCTT) were the most UV-sensitive restrictases. They had two thymine nucleotide dimers in their recognition sites. *Bam*HI (GGATCC) and *Sal*I (GTCGAC) were less sensitive and they had two TC in the recognition sites. Cleavage of *Hae*III (GGCC) and *Hha*I (GCGC) sites was not influenced by the UV irradiation. This led to a conclusion that the relative efficiency with which restrictase cleavage was blocked, corresponds approximately to the relative frequency of pyrimidine photodimer formation in the recognition sequence [19]. Later, Cleaver [21] concluded that the effectiveness of irradiation was directly proportional to the number of potential pyrimidine nucleotide photodimer sites in the irradiated DNA. The fraction of restrictase-resistant DNA molecules was a linear function of the pyrimidine dimer yield [21]. The restrictase cleavage blocking was the same (as with the 254 nm light irradiation) when DNA was irradiated in the presence of acetophenone, a photosensitiser specifically introducing thymidine dimers to DNA at 365 nm light irradiation, suggesting that thymine dimers were responsible for the restriction cleavage inhibition [20]. The influence of flanking sequences as well as formation of other photoproducts than the cyclobutane pyrimidine dimers was considered to explain the higher than expected (based on nucleotide sequence of the restriction site) UV light-sensitivity of *Taq*I or *Hin*dIII [19,21]. The range of pyrimidine nucleotide photodimer influence was determined to be 1–3 bp depending on whether the cleavage position was on the same, or on the opposite strand with respect to the photodimer [21]. In some cases UV damage prevented complete cleavage but did permit cleavage of one DNA strand.

The sensitivity of some restriction enzymes to UV-irradiated DNA led to a development of a method of restriction mapping based on UV-mediated partial digestion of DNA [22]. The method was improved by Nobile [23–25]. During these studies UV-sensitivity of other restriction enzymes was demonstrated – *Xmn*I (GAANNNNTTC), *Hpa*I (GTTAAC), *Pvu*I (CGATCG), *Nru*I (TCGCGA) as well as rare cutting restrictases *Pac*I (TTAATTAA), *Asu*II (TTCGAA), *Swa*I (ATTTAAAT), *Cla*I (ATCGAT) and *Xho*I (CTCGAG). The inhibition of restrictase cleavage of UV-irradiated DNA was also observed in vivo. Irradiation of cells led to a lower frequency of chromosomal aberrations detected by subsequent in vivo cleavage of DNA with UV-sensitive *Dra*I (TTTAAA) but it did not influence the frequency of aberrations when UV-resistant *Hae*III (GGCC) was used [26]. The same results were obtained when DNA was irradiated in vivo and digested after isolation from cells. With this paper we substantially extend the previous experiments to show that the effect of UV irradiation on restrictase cleavage of the irradiated DNA is a complex process in which the pyrimidine dimer content is only one of many factors deciding the final effect. The influence of various factors is demonstrated on particular examples. Using the present study, we are developing a method based on resistance to cleavage by restrictases of UV-damaged DNA. The method provides a map of B-to-A transition along plasmid DNA (Nejedlý et al., in preparation) and it can in principle be used to monitor conformational transitions along kilobase regions of genomic DNA both in vitro and in vivo.

2. Materials and methods

2.1. Materials

Plasmid pUC19, pBR322 and phage ΦX174 DNAs were purchased from MBI Fermentas. Restriction endonucleases were purchased from the following producers: MBI Fermentas (*Bam*HI, *Bgl*I, *Dra*I, *Ecl*136II, *Eco*RI, *Eco*88I, *Eco*255I, *Ehe*I, *Nde*I, *Pae*I, *Pvu*II, *Sal*I, *Sca*I, *Ssp*I, *Xba*I), New England Biolabs (*Bam*HI, *Bst*NI, *Kpn*I, *Pst*I, *Rsa*I, *Sac*I, *Sma*I, *Xmn*I), Promega (*Ava*I, *Hin*dIII, *Nar*I), Stratagene (*Eco*RV, *Mlu*I) and Angewandte Gentechnologie Systeme (*Mva*I).

2.2. DNA linearization and UV light irradiation

The DNAs were linearized with an excess of appropriate restriction enzyme, precipitated with ethanol and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) to get a 30–80 μg/ml concentration of DNA. DNA samples (20 μl volume) were placed on a thermostatically maintained plastic plate (3–8 °C) and irradiated with a set of four 15 W germicidal bulbs (Philips; predominantly emitting light at 254 nm) at an incident fluence rate 20.8 J m^{-2} s^{-1}, as determined by the IL254 germicidal photometer (International Light Inc., Newburyport, MA, USA). The samples were irradiated for different periods of time to produce the doses shown in the figures.

UV irradiation of the A-form of plasmid pUC19 DNA was performed in the following way. The DNA was first linearized with an excess of restrictase and purified by phenol, phenol/chloroform extraction and ethanol precipitation to remove all proteins, including the restrictase. DNA samples (0.2–0.25 μg in final 20 μl) were dissolved in 0.1 mM EDTA and the appropriate amount of EtOH to get the required concentration (usually 80% [v/v]) necessary for A-form stabilization. These samples were irradiated by UV light with an incident fluence of 15 kJ m^{-2}. The samples irradiated in the B-form DNA were dissolved at an EtOH concentration

Table 1
Restriction enzyme target sites, their flanking sequences, positions of cleavage, and the percentage of DNA molecules resistant to restrictase cleavage owing to the dose of 40 kJ m^{-2} of UV irradiation

Restriction enzyme	DNA	Restriction site, its flanking sequences and the position of cleavage (*)	Non-cleaved restriction sites (%)[a]
*Ava*I	pUC19	tcggtaC*CCGGGgatcct	74 ± 2
*Bam*HI	pUC19	acccggG*GATCCtctaga	54 ± 1
	pBR322	tcctgtG*GATCCtctagc	54 ± 1
*Bg1*I	pUC19	ccattcGCCATTC*AGGCtgcgca	85 ± 5
	pUC19	cagccaGCCGGAA*GGGCcgagcg	93 ± 3
*Dra*I	pUC19	cagaacTTT*AAAagtgct	96 ± 1
*Eco*RI	pUC19	gccagtG*AATTCgagctc	85 ± 3
	pBR322	cttcaaG*AATTCtcatgt	72 ± 1
*Eco*RV	pBR322	ttgcggGAT*ATCgtccat	70 ± 2
*Eco*255I	pUC19	ctggtgAGT*ACTcaacca	91 ± 4
	pBR322	ctggtgAGT*ACTcaacca	87 ± 3
*Ecl*136II	pUC19	gaattcGAG*CTCggtacc	91 ± 2
*Hin*dIII	pUC19	gcatgcA*AGCTTggcgta	58 ± 1
	pBR322	atcgatA*AGCTTtaatgc	92 ± 5
*Nar*I	pUC19	gcatcaGG*CGCCattcgc	76 ± 2
	ΦX174	caaactGG*CGCCgagcgt	45 ± 1
	ΦX174	gctggtGG*CGCCatgtct	70 ± 4
*Pst*I	pUC19	gtcgacCTGCA*Ggcatgc	21 ± 5
	pBR322	ccattgCTGCA*Ggcatcg	14 ± 4
	ΦX174	tccaacCTGCA*Gagtttt	32 ± 1
*Pvu*II	pBR322	ttaccgCAG*CTGcctcgc	16 ± 2
*Sac*I	pUC19	gaattcGAGCT*Cggtacc	89 ± 2
*Sal*I	pUC19	tctagaG*TCGACctgcag	44 ± 2
	pBR322	gagagcG*TCGACcgatgc	43 ± 1
*Sma*I	pUC19	tcggtaCCC*GGGgatcct	68 ± 3
*Ssp*I	pUC19	tttttcAAT*ATTattgaa	93 ± 3
	pBR322	tttttcAAT*ATTattgaa	84 ± 1
	ΦX174	atgtctAAT*ATTcaaact	81 ± 1
*Xba*I	pUC19	ggatccT*CTAGAgtcgac	71 ± 4

[a] The average ± S.D. of at least three independent experiments.

preceding the onset of B-to-A transition (usually 60–65% [v/v]). To prevent both volume and EtOH concentration changes during the irradiation, the samples were irradiated in Eppendorf tubes that were immersed in a precooled ethanol bath (−20 °C), which ensured that the sample temperature did not exceed −12 °C at the end of irradiation.

2.3. Restriction endonuclease cleavage

Immediately after the irradiation, the DNA samples were complemented by the respective 10× concentrated buffer and the appropriate restrictase (12–30 units per μg of DNA; final volume 25 μl), and the samples were digested for 1.5 h at the optimum temperature recommended by the producer. The nucleotide sequences of restrictases recognition sites in DNA, the flanking sequences of these sites and the positions of DNA cleavage by each enzyme are summarized in Table 1. The digestion was stopped by the addition of a 6× concentrated loading buffer complemented with 50 mM EDTA. Samples irradiated in EtOH were dried out in Speed-Vac (Savant), dissolved in water and complemented with the respective 10× concentrated buffer and the restrictase solution (20–30 units per μg of DNA; final volume 10 μl). Cleavage proceeded for 2 h at the optimum temperature. The digestion was terminated as above.

2.4. Electrophoresis and densitometry

DNA samples were electrophoresed in 1.0–1.5% (w/v) agarose gels in TBE buffer (89 mM Tris–H_3BO_3, 2 mM EDTA, pH 8.1). The gels contained 0.5 μg/ml of ethidium bromide. After electrophoresis, the gels were briefly rinsed in distilled water, visualized using a UV transilluminator (TM36 Model, UVP Inc., San Gabriel, CA, USA) and

photographed using an orange filter. The negatives were quantified using a Personal Densitometer SI, model 375A, and the ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

The DNA of pUC19 was linearized by an appropriate (mostly *Dra*I) restrictase, irradiated by various doses of UVC light up to 40 kJ m^{-2}, and the irradiated samples were cleaved by various other restrictases. We carried out the irradiations under standard conditions, i.e. in the TE buffer. The presence of Tris [tris(hydroxymethyl)aminomethane] was essential, because otherwise, DNA was nicked at the high UV doses [27]. The amount of restrictase was always large enough for a complete digestion of unirradiated control DNA. The products of cleavage were then separated on non-denaturing agarose gels. Representative results obtained with the *Eco*RI, *Bam*HI and *Pst*I restrictases are shown in Fig. 1. The gels demonstrate in all three cases that the 1975 bp fragment, originating from the pUC19 DNA linearization by *Dra*I, was totally cleaved, giving two shorter fragments in the absence of irradiation. However, even low irradiation (1 kJ m^{-2}) caused incomplete cleavage. The amount of DNA resistant to the cleavage generally increased with the UV dose, but the sensitivity was different with the three restrictases (Fig. 1). The experiments described above were carried out with 17 various type II restrictases and various linearized plasmid (pUC19 and pBR322) or phage (ΦX174) molecules of DNA. Each experiment was repeated at least three times to get quantitatively reliable results. The results obtained with the highest dose of UV irradiation (40 kJ m^{-2}), giving the maximum effects, are summarized in Table 1. First of all, we were interested if there was a relation between the (A + T) content of the restrictase recognition sites and the sensitivity of their cleavage to UV irradiation. The strongest inhibition of cleavage caused by UV irradiation was observed for the *Dra*I restrictase whose recognition site is composed of (A + T) bases only. This extreme sensitivity was probably due to the presence of three consecutive thymines in the *Dra*I recognition site. The *Ssp*I site (AATATT), which has 100% content of (A + T) too, was also very sensitive, but the same sensitivity was exhibited by a *Bgl*I site (GCCGGAAGGGC) whose (A + T) content was only 18%. The *Ecl*136II recognition sequence (GAGCTC) is another very sensitive site whose (A + T) content is very low (33%). There are still other examples in Table 1 demonstrating that the (A + T) content of the recognition site is not the only factor deciding the inhibition of restrictase cleavage due to UV irradiation, though the two properties certainly correlate in the sense that the inhibition frequently increases with the increased (A + T) content of the cleavage site. This rule, however, has many exceptions. For example, the *Nar*I site (GGCGCC) contains no AT pairs, but UV irradiation causes

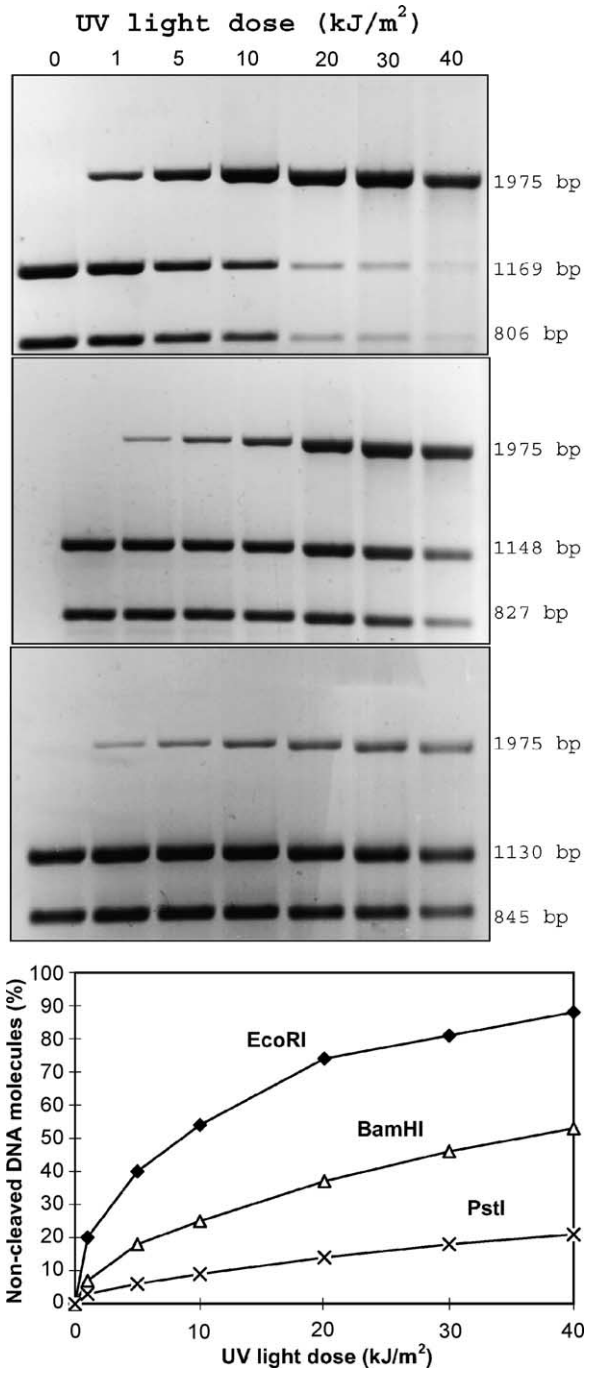

Fig. 1. Dose-dependences of the restriction enzyme cleavage of UV light-irradiated DNA. *Dra*I-digested pUC19 DNA was irradiated by an increasing dose of UV light and then cleaved with the restriction enzymes *Eco*RI (top gel), *Bam*HI (middle gel) or *Pst*I (bottom gel), all having a unique recognition site in the 1975 bp *Dra*I fragment.

an almost five times higher inhibition of cleavage than with the *Pvu*II site (CAGCTG), whose recognition site contains two AT pairs (Table 1). Another restrictase without AT pairs in its recognition site, *Eco*88I, exhibits UV-induced inhibition comparable to *Hin*dIII (67% AT pairs) and a non-negligibly higher inhibition than *Bam*HI or *Kpn*I (33% AT pairs; not shown).

Secondly, we were interested in how the data given in Table 1 correlate with the (PyPy) dinucleotide content of the restriction site and its neighbourhood. The most often used restrictase sites in our study had the (PyPy) dinucleotide content of 20% (Table 1). The amount of DNA resistant to restrictase cleavage in these sites due to UV irradiation, however, ranged from 14% (the *Pst*I site in pBR322) to 93% (the *Ssp*I site in pUC19) (Table 1). Another group contained 40% of (PyPy) in the restriction site and the percentage of resistant molecules ranged from 54% (*Bam*HI site in pUC19) to 96% (*Dra*I site in pUC19) (Table 1). The 40% (PyPy)-content group consists of 12 members and the average amount of non-cleaved DNA is 75% within the group, which is significantly higher than the average 58% over 15 members of the 20% (PyPy)-content group. The same is true if the flanking 5 bp segments are taken into account. Hence, the conclusion is similar to the above with the (A + T)-content, i. e. the extent of cleavage protection on average increases with the amount of the (PyPy) dimers in the restriction site and its immediate neighbourhood, but many exceptions exist to indicate that other factors are important as well. Neither the TT dimer occurrence in the restriction site nor in its immediate neighbourhood is the decisive factor. After finishing the extensive experiments, whose part is summarized in Table 1, we noticed that all tested restrictases contained at least one pyrimidine dinucleotide in the target site. That is why we also examined *Mlu*I (A*CGCGT) and *Rsa*I (G*TAC) restrictases containing no pyrimidine dinucleotide in the target sequence, but found no significant difference in their incapability to cleave UV-irradiated DNA in comparison with the restrictases in Table 1.

Having described the general information following from the above analysis, we will present examples illustrating the not negligible role of the various particular factors in more detail. The first example concerns flanking regions of the restriction site. We took the *Hin*dIII restrictase, cleaving DNA within the (AAGCTT) hexamer, to cut UV-irradiated DNAs of pUC19 and pBR322 where the target hexamer was flanked by different sequences. Fig. 2 demonstrates that the protective effect of UV damage strongly depended on the surrounding sequence, being much more pronounced in pBR322 DNA. Strong effects of the flanking sequences were also observed with *Eco*RI and *Nar*I (Table 1). On the other hand, the effect of the sequence context was negligible with *Bam*HI and *Sal*I (Table 1). The 11 bp recognition site of the *Bgl*I restrictase is degenerate in the five central bases, but variability of this central sequence and the flanking bases also exerted no significant influence on the cleavage of UV-damaged DNA by this restrictase (Fig. 3). Isoschizomers *Mva*I and *Bst*NI (not shown), or neoschizomer couples of *Sma*I and *Ava*I, or *Sac*I and *Ecl*136II, were equally sensitive to the UV damage of DNA (Fig. 4), indicating that neither the restrictase identity nor the position of cleavage within the same recognition site was decisive for the extent of resistance of UV-damaged DNA to the restrictase cleavage. On the contrary, the restrictase *Ehe*I was about three times

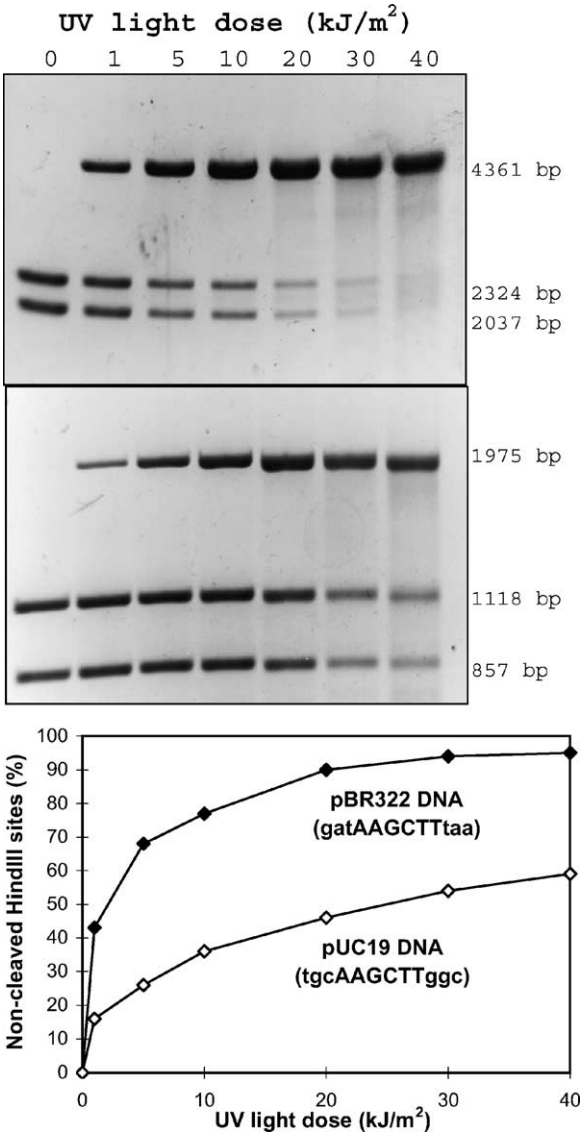

Fig. 2. Effect of the flanking sequences on the inhibition of the *Hin*dIII site cleavage caused by UV irradiation. *Pvu*II-cleaved pBR322 (top gel) and *Dra*I-cleaved pUC19 (bottom gel) DNAs were irradiated with an increasing dose of UV light and cleaved with *Hin*dIII.

less sensitive to UV damage in the DNA in comparison with its neoschizomer *Nar*I (not shown), which may reflect different lengths of surrounding sequences recognized by these neoschizomers.

So far, we have described experiments showing that the resistance of UV-irradiated DNA to restriction endonuclease cleavage depends on a combination of properties characterizing the primary DNA structure of the recognition site and its neighbourhood. However, the protein recognizes, in fact, the DNA secondary structure that is influenced by a number of other factors, including pH and dehydrating agents (e.g. ethanol), inducing the B-to-A conformational transition in DNA. In the second part of this study, we were therefore interested in how the conformational changes influence the resistance of UV-irradiated DNA to the restriction

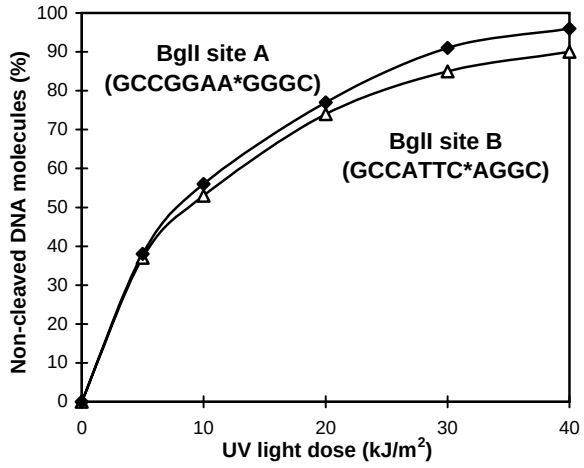

Fig. 3. Dose-dependence of the UV light-induced inhibition of the cleavage of two *Bgl*I sites containing different internal nucleotide sequences. The pUC19 DNA was digested with *Dra*I. The resulting fragments carried *Bgl*I sites A and B containing the indicated internal sequences. DNA was then irradiated with various doses of UV light and cleaved with *Bgl*I.

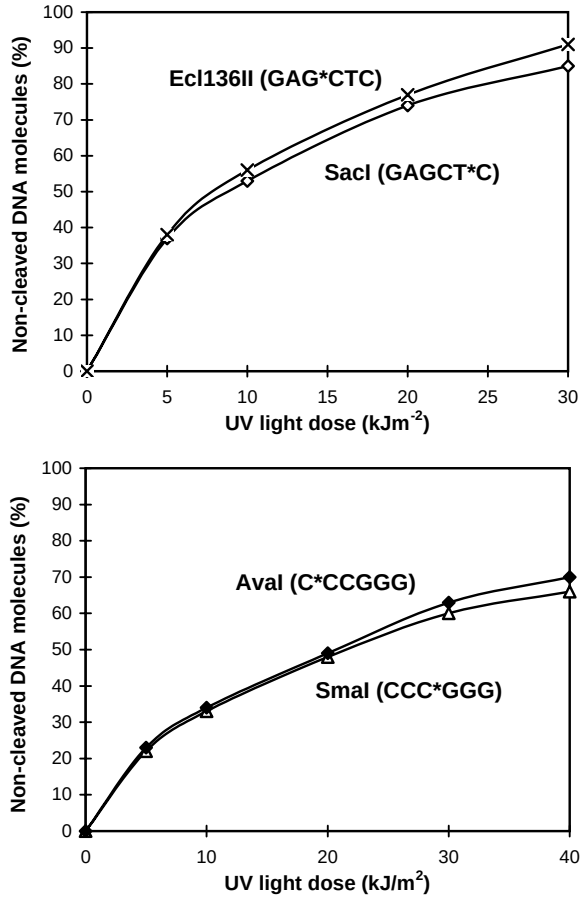

Fig. 4. Dose-dependence of cleavage of UV light-damaged DNA by neoschizomer pairs (top) *Ecl*136II and *Sac*I, and (bottom) *Ava*I and *Sma*I. *Dra*I-linearized pUC19 was irradiated with various doses of UV light and cleaved in parallel with the respective restrictases.

endonuclease cleavage. Firstly, we examined the effect of pH to find no detectable effect on the resistance to *Hin*dIII cleavage of the DNA irradiated by UV light in buffers having the pH values of 6.1, 6.6, 7.1, 7.5, 7.9 and 8.6. This behaviour is in line with our observation that the CD spectrum and hence conformation of natural DNA molecules is invariant if the pH value is changed within the 6.1–8.6 range (not shown). Then, we examined the resistance of the UV-irradiated A-form DNA to restriction endonuclease cleavage.

The A-form is induced in DNA by ethanol [28]. We induced the A-form in linearized pUC19 DNA by ethanol and followed the B-to-A transition using CD spectroscopy, taking advantage of our previous experience (e.g. ref. [29]). The control use of CD spectroscopy is necessary because A-form induction is not trivial in plasmid DNA. As the transition is hindered by proteins, it was necessary to remove the restrictase used for the DNA linearization. The removal of proteins also increased the differences in cleavage by the detection restrictases between the UV-irradiated DNA in the B-form and the A-form. Secondly, salt causes DNA aggregation at ethanol concentrations, inducing the A-form. This aggregation results in DNA–DNA crosslinks following UV irradiation [30], the reason why the experiments were performed at the minimum ionic strength (0.1 mM sodium EDTA). The EDTA is needed to bind traces of divalent cations almost always present in water and sufficient to aggregate DNA at the ethanol concentrations inducing the A-form. We also minimized the aggregation by working with DNA concentrations as low as possible (10–13 μg/ml). These conditions led to the characteristic changes in the CD spectra accompanying the B-to-A conformational transition [29]. We took the DNA samples before the beginning (the B-form) and at the end of the transition, where the DNA was completely transformed into the A-form, and continued as described in Section 2. Results of this work are summarized in Table 2.

In this part of work, we used 16 restrictases to detect the damage caused by UV irradiation, including two neoschizomer couples (*Sac*I–*Ecl*136II and *Nar*I–*Ehe*I). The restrictases differed by the amount of the DNA resistant to restrictase cleavage owing to UV irradiation (Table 2). Recognition sites of the restriction enzymes exerting the largest amounts of uncleaved DNA when irradiated in B-form were (A + T)-rich, but not every (A + T)-rich recognition sequence provided large amounts of resistant DNA (*Hin*dIII, *Nde*I). The *Eco*RI, *Hin*dIII and *Xmn*I contained TT dinucleotides, and the amount of resistant DNA only decreased by 20–40% during the B-to-A transition. The decrease was much larger (60–100%) with restrictases not containing the TT dinucleotide. The decrease of the fraction of resistant DNA was a general feature of the B-to-A transition. This was observed with 15 of the 16 detection restrictases used in this work. *Ssp*I was the only restriction enzyme whose recognition sequence was cleaved more after the UV irradiation in the A-form compared to the B-form. We tentatively attribute this observation to a possibility that ethanol induced no A-form but another conformation in

Table 2
Resistance to restriction cleavage upon UV light irradiation of pUC19 plasmid DNA in the B- or the A-form

Restriction endonuclease	Restriction site and its position of cleavage (*)	Fraction of non-cleaved DNA (%)[a]	
		In B-form[b]	In A-form
*Bam*HI	G*GATCC	13.8 ± 1.1	1.9 ± 1.2
*Ecl*136II	GAG*CTC	19.1 ± 0.4	1.8 ± 3.5
*Eco*RI	G*AATTC	33.4 ± 2.9	25.0 ± 3.0
*Eco*88I	C*CCGGG	19.2 ± 3.0	0.4 ± 0.4
*Ehe*I	GGC*GCC	6.6 ± 0.1	0.6 ± 0.2
*Hin*dIII	A*AGCTT	22.0 ± 0.1	12.9 ± 2.1
*Nar*I	GG*CGCC	21.8 ± 0.6	8.2 ± 1.2
*Nde*I	CA*TATG	13.8 ± 1.9	1.5 ± 0.7
*Pae*I	GCATG*C	4.3 ± 0.5	0.5 ± 1.1
*Pst*I	CTGCA*G	4.5 ± 1.7	0.5 ± 1.8
*Sac*I	GAGCT*C	22.3 ± 4.8	3.8 ± 0.1
*Sal*I	G*TCGAC	6.2 ± 1.2	1.0 ± 0.7
*Sca*I	AGT*ACT	31.1 ± 0.4	2.7 ± 1.1
*Ssp*I	AAT*ATT	36.0 ± 1.9	41.7 ± 1.9
*Xba*I	T*CTAGA	25.9 ± 0.8	4.7 ± 0.6
*Xmn*I	GAAAA*CGTTC	32.1 ± 2.3	25.7 ± 3.0

Samples of linearized DNA were irradiated with 15 kJ m^{-2} dose of UV light and consequently cleaved with an excess of the indicated restrictase.
[a] Average ± S.D. of three to five independent experiments. 100% stands for cleavage of control, non-irradiated samples.
[b] B-form in 50% aqueous ethanol.

this site. This tentative interpretation was inspired by the 100% (A + T) content of its recognition sequence and its (A + T)-rich neighbourhood (the whole block of 18 bases comprises 16 (A + T)) that is similar to poly(dA).poly(dT)). We also observed that the *Eco*88I and *Ehe*I sites (both are 100% G + C) were completely cleaved by the corresponding restrictases if they were irradiated by 15 kJ m^{-2} in the A-form though the same dose generated a non-negligible amount of resistant DNA if it was irradiated in the B-form.

4. Discussion

UV light is a constituent of our environment accompanying the evolution of the genetic material on the earth for billions of years [31,32]. UV light damages DNA [reviewed in ref. [33]) and the damage is sensitively recognized by various proteins. The response of proteins is many-sided because, for example, UV-damaged DNA lures the basal transcription factor TFIID/TBP [34], while TFIIIA [35] and other transcription factor (E2F, NF-Y, AP-1, NFkB and p53) binding to DNA containing UV-induced pyrimidine dimers is inhibited [36]. In addition, binding of transcription factors creates hot spots for UV photoproducts in DNA [37]. Many other proteins recognize UV photoproducts in DNA and/or influence the DNA damage caused by UV light. The proteins include restrictases that serve as a paradigm of specific protein–DNA recognition [38]. Here, we have analyzed cleavage of UV-damaged DNA by various type II restrictases to show that this process is more complex than it is described in the literature. It will be shown elsewhere (Nejedlý et al., manuscript in preparation) that UV light-induced damage is sensitive to DNA conformation, which fact can be used with advantage to map the B-to-A conformational transition along plasmid DNA. A scanning method was developed that uses restrictases to scan mammalian genomes at about 1 megabase intervals [39]. Incomplete digestion of UV-damaged DNA by restrictases was used to monitor UV damage and follow repair of genes in living lymphocytes and granulocytes [40]. Further development of the genomic applications of the UV damage probing by restrictases requires that as many as possible restrictases are characterized in a reliable quantitative way regarding their sensitivity to the UV damage. Such extensive quantitative data are reported in this paper to complement the analogous previous studies.

The first present observation, surprising in light of the widely accepted opinions, was the effect exerted on the cleavage of UV-irradiated DNA by restrictases recognizing only GC pairs. This effect was not negligible because, for example, the (AAGCTT) site in pUC19 DNA was less damaged by the UV irradiation and hence less protected from the *Hin*dIII restrictase cleavage than the *Nar*I (GGCGCC) or *Sma*I (CCCGGG) sites. The photoreactivity difference was especially strong between the *Nar*I or *Sma*I sites on one hand, and the *Pst*I (CTGCAG) or *Pvu*II (CAGCTG) sites on the other, because the latter were about three times more photoresistant (Table 1). This fact certainly contributes to the unexpectedly weak and ambiguous correlation of the photoreactivity with the (A + T) content of the recognition sites.

Another interesting point following from the present study is the effect of the regions flanking the restrictase target site. For example, the *Hin*dIII site in pBR322 DNA shows a more than two-fold photoreactivity than the *Hin*dIII site in pUC19 DNA, which should be caused by the flanking regions because the target site and the restrictase are the same in both cases. This is in line with observations of the influence of the flanking regions in restrictase interactions with unirradiated DNA [41]. Though there is not a general consent about the length of flanking sequences necessary for proper binding and cleavage of restrictases, spanning from no extra base pair to more than four base pairs flanking both sides of recognition site [38,42,43], it can be inferred that it depends on the nature of the respective enzyme. In this respect, the photoproducts generated in the very vicinity of the restriction sites can be of the same importance as the ones formed within recognition sequences. Another factor involved with the high specificity of the DNA interactions with restrictases is the strongly bound water molecules that help the restrictases to recognize the binding sites [44]. It is conceivable that the photoproducts alter the water molecule network and disturb the recognition this way. The recognition is also influenced by the deformation caused in DNA by the photoproduct formation. There is quite a lot of different kinds of photoproducts (for review see [45]), but most of them are very rare, so it is reasonable to take into account

only two of them: cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts. Studies performed on these types of lesions suggested that they unwind DNA and induce bending of DNA [46,47]. Recent crystal structure study has shown that a DNA decamer containing a CPD in the middle of its sequence bends DNA by 30° and significantly widens both minor and major grooves both 3′-end and 5′-end of the CPD [48]. These data are consistent with the proposed models for CPD recognition involving base-flipping mechanisms, e.g. for T4 endonuclease V [49] and *E. coli* photolyase [50].

Unfortunately, there are no structural data available concerning the interaction of restriction endonuclease(s) with fragments of DNA bearing UV light-induced photoproducts. Nevertheless, the mechanism by which restriction enzyme recognizes its target sequence is quite different from the above mentioned UV light-induced lesion-repairing enzymes. It involves interaction with at least the whole recognition sequence and does not imply any base-flipping [38]. Both the photoproducts [46,47] and the restrictases [51] bend DNA, but the bends need not be the same [52]. Even restrictases, recognizing the same sequences, can deform DNA in various ways. For example, the *Sma*I and *Xma*I isoschizomers bend DNA in opposite orientations [9]. Besides, it can be expected that UV light-induced bent DNA is not a proper substrate for the restrictase, in addition to the steric hindrances and changes in flexibility imposed by covalent bonds between pyrimidines *plus* the above mentioned injured water molecule network.

In principle there are two extreme explanations of the effects reported here. The first is that restrictases are variable in their sensitivity to the UV damage in DNA. The second explanation considers the richness of photoproducts induced in DNA by various doses of UV light and the sequence dependence of the photoproducts formation as the major factor. Our results with the isoschizomers and the neoschizomers favour the latter possibility. The experiments showing different protective effects in various sequence contexts can simply be explained by inhibitory photoproducts generated in the flanking regions of the recognition site in one type of DNA molecule but not in the other.

The A-form is almost constitutively adopted by double-stranded regions in RNA [53]. DNA adopts the A-form in dehydrated fibers [54,55], aqueous ethanol [28], aqueous methanol [56], *Bacillus subtilis* spores [57], owing to SASP protein binding [58] and in complexes with polymerases [59–66]. The present results demonstrated that DNA photoreactivity was decreased owing to the B-to-A transition. Absence of the (TT).(AA) dinucleotide dimer in the restrictase target site was necessary to observe a substantial increase in restrictase cleavage of the DNA irradiated in the A-form, in comparison to its irradiation in the B-form (Table 2).

Finally, UV irradiation of DNA, resulting in its partial digestion by restrictases, is an approach for rapid restriction site mapping [22], and an identification of restriction site polymorphisms [23]. Owing to the oversimplified interpretation mentioned at the end of Section 1, use of these methods have so far been restricted to the restriction sites containing the TT and TC dinucleotides [24]. The present results extend their applicability to other restriction sites which need not even contain a single thymine.

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