Generation of oligonucleotides containing site-specific cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidinone photoproducts by immunoprecipitation

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Synthetic oligonucleotide substrates have recently been used in studies investigating the biological effects of specific UV-induced photoproducts in DNA (1). To date, defined damaged substrates have been produced by UV-irradiating synthetic oligonucleotides (ranging from 6 to 11 bases) and separating the damaged from the undamaged fragments using high performance liquid chromatography. After biophysical characterization, the damaged fragments are ligated into larger oligonucleotides (2). These substrates were used in several studies to determine the specificities and affinities of damaged DNA binding proteins and repair enzymes for different UV photoproducts in DNA [e.g., *uvrA*, *uvrB*, XPE factor and (6-4) photoproduct photolyase] (3–5). Using well-characterized antibodies against UV-irradiated DNA, we developed an immunoprecipitation technique to generate oligonucleotides containing a site-specific UV-photoproduct. Since the integrity of the lesions thus produced is dependent on accurate characterization of the binding specificity of the antibodies used for immunoprecipitation, we further developed a mobility shift immunoassay technique for visualizing the specificities of antibody binding to the major types of dimeric photodamage. Previous data determining antiserum specificity comes from our work on the effects of lesion-specific biochemical and enzymatic treatments on antibody binding (6). Loss of antibody binding after *in vitro* digestion of UV DNA with T4 endonuclease V or photoreactivating enzyme demonstrates specificity for the cyclobutane pyrimidine dimer (CPD) (7). Likewise, reduced binding after treatment with hot piperidine or photoisomerizing light (UVB) denotes binding to the pyrimidine(6-4)pyrimidinone photoproduct [(6-4)PD]. This paper describes an adaptation of the electrophoretic mobility shift assay to visualize binding of anti-UV DNA antibodies to UV-damaged substrates. In addition, we describe a novel, facile approach for the generation of damage-specific oligonucleotides that can be used in studies on the interaction of cellular proteins with damaged DNA.

Production of CPD and (6-4)PD immunogen and antisera. Anti-(6-4)PD was produced in rabbits using DNA irradiated with 50 kJ/m² UVC (240–290 nm) light (Philips Sterilamp G8T5) as immunogen. At these high UVC fluences, the yield of (6-4)PD is comparable with the CPD (8,9). The immunogen for

anti-CPD production consisted of DNA diluted in a triplet sensitizer [2×10^{-4} M acetophenone (Sigma)] and irradiated with 50–60 kJ/m² UVB (290–320 nm) light emitted by four Westinghouse FS20 fluorescent sunlamps filtered through cellulose acetate (Kodak) (UV cutoff at 290 nm). Under these photochemical conditions the T(*c,s*)T is the major photoproduct induced (i.e., >95%) (10,11). Salmon sperm DNA (Sigma) was used for all immunizations and was heat denatured prior to coupling to methylated bovine serum albumin (Calbiochem) according to Plescia, 1968 (12). For each antiserum, four rabbits were inoculated at 2 week intervals at 10 subcutaneous sites with a total of 1 ml immunogen (500 µg DNA) mixed with an equal volume of Freund's adjuvant (The University of Texas M. D. Anderson Cancer Center, Science Park/Veterinary Division, Bastrop, TX). Antisera were harvested after the third injection. After preadsorption to single-stranded DNA (Ultragel; IBF Biotechnics, France) to remove non-specific binding, immunoglobulin (IgG) was purified from rabbit antiserum using a protein A column and the Immunopure Gentle Ag/Ab Buffer System (Pierce). After extensive dialysis IgG concentrations were determined from absorbance measurements at 280 nm.

Mobility shift immunoassay of oligonucleotides containing site-specific UV-photoproducts. Evidence for the specificity of the different immunoglobulins came from the electrophoretic mobility shift immunoassay, a technique that allowed us to visualize the binding of affinity-purified polyclonal antisera to synthetic DNA substrates containing only a single major UV photoproduct. The substrates used were the *cis,syn* CPD [T(*c,s*)T], *trans,syn* CPD [T(*t,s*)T], T–T (6-4) photoproduct [T(6-4)T], and the Dewar photoisomer of the T–T (6-4) photoproduct [T(Dew)T] (2). The sequence of the substrate is shown in Table 1. We end-labeled 5–10 pmol of each of the single-stranded oligonucleotide [(T(*c,s*)T, T(*t,s*)T, T(6-4)T and T(Dew)T] (kindly provided by Dr J.-S. Taylor, Washington University, St Louis) with [γ -³²P]ATP (3000 Ci/mmol; Amersham) using T4 polynucleotide kinase (Boehringer-Mannheim). Each end-labeled oligomer (~0.1 pmol) was incubated with increasing concentrations of anti-CPD or anti-(6-4)PD at 37°C for 3 h followed by 4°C overnight in Tris-EDTA (pH 8.0). The bound complexes were separated from the unbound oligonucleotides by

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Figure 1. Mobility shift immunoassay to show the binding specificities of the IgGs to 49mers containing site-specific, UV-induced lesions. (A) Anti-CPD IgG at 0.6, 2.0 and 6.0 pmol was allowed to bind 0.1 pmol of each of the 49mers containing no damage (TT) or site-specific photoproducts (T[t,s]T; T[c,s]T; T[6-4]T; T[Dewar]T). The retarded band (represented by an arrow) indicates the immune complex formed and the lower band represents unbound 49mer. (B) Anti-(6-4)PD IgG at 0.6, 2.0 and 6.0 pmol was incubated with 0.1 pmol of each of the 49mers containing site-specific UV-induced lesions as mentioned above.

electrophoresis on 2% agarose gels. The gels were dried and autoradiographed or subject to phosphorimaging analysis.

Table 1. Sequences of the oligonucleotides showing the specific dipyrimidine sites

A. 5' AGCTACCATGCCTGCACG[AA TT AA]GCAATTCGTAATCATGGTCATAGCT 3'		
B. 5' ACATGTGTACGAATAT TT AAAGGTAGACATG 3' AAT TT	<i>SspI</i>	
5' ACATGTGTACGGATAT TC AAAGGTAGACATG 3' GATAT TC	<i>EcoRV</i>	

(A) Hexamer (in parentheses) containing the dipyrimidine TT (in bold) was ligated to flanking sequences to form the 49mer (from J.-S. Taylor). (B) 30mers used in the immunoprecipitation procedure showing the location of dimeric damage within the restriction enzyme recognition sites.

In Figure 1A the binding specificity of the anti-CPD IgG is shown. This antiserum, raised against DNA irradiated with UVB light in the presence of a triplet-sensitizer, binds the CPD exclusively, even at the highest IgG concentration used (i.e., 60 molar excess). In Figure 1B the binding specificity of the

Figure 2. Relative affinity of anti-CPD IgG and anti-(6-4)PD IgG for CPD and (6-4)PD, respectively obtained from MSIA. Linear regression analysis (microsoft excel) showed the correlation coefficient to be 0.87 for the (6-4) IgG ($n = 13$) and 0.71 for the CPD IgG ($n = 16$). The slope (calculated from the nmol/logIgG) was 0.24 and 0.18 for the (6-4)PD and CPD IgG, respectively. The lower detection limit determined from the x-intercept was calculated to be 266 nmol for the (6-4)PD IgG and 1154 nmol for the CPD IgG.

anti-(6-4)PD is shown. This antiserum, raised against DNA irradiated with a high fluence of UVC light, readily bound the (6-4)PD but showed no activity toward any of the other photoproducts even at the highest concentration. The gel shift data in Figure 1B shows a minor, slower-migrating band in addition to the major band. This band is probably associated with multimers resulting from the bivalent binding of the IgG to more than one oligomer.

Binding data from several MSIAs are plotted in Figure 2. The number of photoproducts bound (nmol) was calculated by densitometry of MSIA for the anti-(6-4)PD and the anti-CPD sera. Because we used total IgG purified from polyclonal antisera in the MSIA and have no estimate of the concentration of damage-specific IgG, we can not calculate binding constants using standard techniques. However, by plotting the number of photoproducts bound (in nmol) against the total number of IgG molecules available for binding (in nmol), the relative concentrations and affinities of the antisera for (6-4)PDs and CPDs could be estimated. The x-intercept (no photoproducts bound) represents the lower limit of detection of specific binding to photodamage. This value is 266.4 nmol for the anti-(6-4)PD sera and 1154.4 nmol for the anti-CPD serum or that ~5-fold more anti-CPD IgG is required to bind the same number of photoproducts as the (6-4)PD IgG. A similar trend is seen by comparing the slopes of the binding curves. The (6-4)PD IgG shows significantly greater binding affinity for the (6-4)PD as compared with that of the CPD IgG for the CPD [i.e., 0.248 nmol (6-4)PD bound versus 0.187 nmol CPD bound/log nmol increase of IgG]. These data suggest that the binding constants for the (6-4)PD antibodies are greater than for CPD and that the (6-4)PD antibody population is significantly greater than the CPD population in their respective polyclonal antisera. These results are consistent with previous immunoprecipitation data (13). At relatively low UVC fluences (e.g. < 1 kJ/m²) (6-4)PDs are induced at ~20–40% the frequency of CPDs (8), whereas at higher fluences (e.g. 10–100 kJ/m²) CPDs and (6-4) photoproducts are induced at comparable (or greater) levels (9).

Figure 3. Binding of the (6-4)PD IgG to double- and single-stranded 49mers containing a single site-specific (6-4)PD. Data from mobility shift experiments (from the ratio of bound to free oligo) was used to determine the nmoles of photoproducts bound. The higher affinity for the single-stranded substrates was seen in three different sets of experiments performed. Open circles denote single-stranded substrate and closed circles represent double-stranded substrate.

A duplex T(6-4)T substrate was produced by annealing a 10-fold molar excess of the complementary strand at the T_m of the oligonucleotide followed by purification by polyacrylamide gel electrophoresis. MSIA was used to compare the relative binding of (6-4)PD IgG to photoproducts in single- and double-stranded DNA. Comparison of the binding curves indicate that the affinity of the (6-4)PD IgG towards the (6-4)PD in single-stranded DNA is 10-fold greater than in duplex DNA (Fig. 3). These data are in agreement with the competitive binding assays using UV-irradiated single- and double-stranded substrates (8).

Generation of oligonucleotides containing site-specific cyclobutane pyrimidine dimers and (6-4) photoproducts by immunoprecipitation. Based on the specificities of the antibodies, excess immunoglobulin (IgG) purified from rabbit polyclonal antisera was used to immunoprecipitate UV-irradiated oligonucleotides containing the thymine–thymine CPD and thymine–cytosine (6-4)PD. Single-stranded oligonucleotides (30mers) were designed such that they contained either a thymine–thymine (T–T) or a thymine–cytosine (T–C) dipyrimidine within a restriction site (Genosys Biotechnology) (Table 1). Because dimeric damage within a restriction site inhibits recognition and digestion by the appropriate restriction enzyme (14), inhibition of restriction digestion could be used to measure photoproduct yield in immunoprecipitated oligonucleotides. In order to determine the yield of oligonucleotides in the precipitate, we supplemented 50 μ mol of oligonucleotide with 5–10 pmol of the same oligomer end-labeled with [γ - ^{32} P]ATP. The

Figure 4. Inhibition of digestion of immunoprecipitated oligos by restriction endonucleases. Lanes 1 and 2 are *SspI* digests of a T(CPD)T and T–T containing 30mer. The arrows indicate the migration of a 14mer and 16mer produced by *SspI* digestion of the T–T oligonucleotide. Lanes 3 and 4 show an *EcoRV* digest of a T(6-4)C and T–C 30mer respectively. The 13mer and 17mer produced by *EcoRV* digestion are indicated with arrows.

oligomer was then irradiated with 30kJ/m² UVC light and incubated with excess anti-CPD and anti-(6-4) IgG at 37°C for 4–6 h followed by 4°C overnight. IgG was precipitated overnight at 4°C with a 1:20 dilution of goat anti-rabbit IgG (Calbiochem) and carrier IgG (normal rabbit serum). The immune pellet was digested with 0.3 mg/ml proteinase K (Boehringer Mannheim) at 55°C for 4 h, extracted twice with buffer saturated phenol followed by phenol:chloroform (chloroform:isoamyl alcohol 24:1) and then precipitated with ethanol. Double-stranded substrates were produced by annealing a 10-fold molar excess of the complementary strand at the T_m of each oligonucleotide followed by gel purification on 20% polyacrylamide gels. Each of the double-stranded substrates (~50 pmol) was end-labeled with T4 polynucleotide kinase and tested for inhibition of restriction digestion by the respective enzymes.

Inhibition of digestion by the restriction enzymes *SspI* and *EcoRV* at sites containing T–T CPDs and T–C (6-4)PDs respectively is shown in Figure 4. We digested 25 pmol of each of the T(CPD)T oligo and its undamaged counterpart with 0.5 U of *SspI* (Boehringer Mannheim). The T(6-4)C PD and its undamaged control oligomer were digested with 0.5 U *EcoRV* (Boehringer Mannheim). All of the digestions were carried out at 37°C for 2 h. The reactions were heat inactivated and the products were electrophoresed on 20% native polyacrylamide gels. Lanes 1 and 3 show the inhibition of restriction digestion of UV-irradiated oligomers that had been immunoprecipitated with anti-CPD and anti-(6-4)PD IgG, respectively, as compared with their unirradiated controls (lanes 2 and 4). Densitometric analyses showed that the restriction digestion by *SspI* and *EcoRV* was inhibited >95% in the UV-irradiated, compared with

the unirradiated substrates immunoprecipitated with anti-CPD IgG and the anti-(6-4)PD IgG respectively. These data demonstrate the feasibility of using antibodies to generate oligonucleotide substrates containing site-specific lesions not only for the UV-induced class of photoproducts but for any kind of DNA damage to which well-characterized antibodies are available.

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