Recognition of DNA Adducts by Human Nucleotide Excision Repair

EVIDENCE FOR A THERMODYNAMIC PROBING MECHANISM*

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The mechanism by which mammalian nucleotide excision repair (NER) detects a wide range of base lesions is poorly understood. Here, we tested the ability of human NER to recognize bulky modifications that either destabilize the DNA double helix (acetylaminofluorene (AAF) and benzo[a]pyrene diol-epoxide (BPDE) adducts, UV radiation products) or induce opposite effects by stabilizing the double helix (8-methoxypsoralen (8-MOP), anthramycin, CC-1065 adducts). We constructed plasmid DNA carrying a defined number of each of these adducts and determined their potential to sequester NER factors contained in a human cell-free extract. For that purpose, we measured the capacity of damaged plasmids to compete with excision repair of a site-directed NER substrate. This novel approach showed differences of more than 3 orders of magnitude in the efficiency by which helix-destabilizing and helix-stabilizing adducts sequester NER factors. For example, AAF modifications were able to compete with the NER substrate 1740 times more effectively than 8-MOP adducts. The sequestration potency decreased with the following order of adducts, AAF > UV > BPDE > 8-MOP > anthramycin, CC-1065. A strong preference for helix-destabilizing lesions was confirmed by monitoring the formation of NER patches at site-specific adducts with either AAF or CC-1065. This comparison based on factor sequestration and repair synthesis indicates that human NER is primarily targeted to sites at which the secondary structure of DNA is destabilized. Thus, an early step of DNA damage recognition involves thermodynamic probing of the duplex.

Nucleotide excision repair (NER) is an essential pathway for removing bulky base modifications from DNA. This repair mechanism involves endonucleolytic cleavage at two phosphodiester bonds, one 3' and the other 5' of the site of damage, followed by excision of DNA damage as the component of a single-stranded fragment (1–5). The excised oligonucleotide is replaced by DNA repair synthesis, and DNA continuity is re-established by ligation. In mammalian cells, the major sites of incision are at the 5th phosphodiester bond 3' and the 24th phosphodiester bond 5' to the lesion (6).

Human patients deficient in NER suffer from xeroderma pigmentosum (XP), a hereditary disease characterized by photosensitivity, increased incidence of skin cancer, and frequently neurological abnormalities (7, 8). At the biochemical level, XP individuals are impaired in the removal of radiation products induced by the UV component of sunlight (9). Somatic cells obtained from these patients are also defective in excision repair of bulky DNA adducts resulting from genotoxic chemicals (1, 2, 4). Recent in vitro studies using cell-free extracts showed that the range of base lesions processed by mammalian NER extends to nonbulky adducts, and even abasic sites are susceptible to excision repair by this pathway (10).

Biochemical reconstitution experiments demonstrated that mammalian NER is catalyzed by the coordinated action of at least 30 polypeptides (11, 12). Several of these factors have been implicated in the recognition step of NER, primarily a complex made up of XPA and the three subunits of RPA (p70, p34, p11) (13); in addition, the multisubunit protein complex TFIH and XPE protein may participate in damage recognition (1–4). The molecular mechanism by which these proteins discriminate a large number of chemically unrelated DNA lesions as substrates for NER is unknown. However, the versatility of NER led to the assumption that this system recognizes conformational changes imposed on DNA at sites of damage rather than specific base modifications (2, 5). In this report, we compared recognition of DNA adducts where detailed structural information is available and identified a molecular determinant triggering initiation of the mammalian NER pathway.

The acetylaminofluorene (AAF), benzo[a]pyrene diol-epoxide (BPDE), 8-methoxypsoralen (8-MOP), anthramycin, and CC-1065 moieties are illustrated in Fig. 1. Melting temperature studies have shown that these bulky base adducts alter the thermodynamic characteristics of DNA in different ways. AAF adducts (14, 15), BPDE adducts (16, 17) and UV radiation products (18) destabilize the DNA double helix relative to non-modified DNA, whereas 8-MOP (19, 20), anthramycin (21), and CC-1065 adducts (22) stabilize the DNA duplex. In the predominant adduct formed by N-acetoxy-2-acetylaminofluorene (AAF-C8-guanine), the modified base is rotated out of the helix axis, and the duplex is locally denatured (14, 23). Reaction of anti-7,8-diol 9,10-epoxy-benzo[a]pyrene with double-stranded DNA generates mainly (+)-trans-anti-BPDE-N2-guanine adducts with quantitatively minor lesions resulting from (−)-trans, (+)-cis, and (−)-cis additions to the same position N2 of guanine (24, 25). Depending on their stereochemistry, these BPDE-N2-guanine adducts are either accommodated in the minor groove (24) and cause DNA unwinding (17) or assume a base-displacement configuration with localized base pair disruption (25). For comparison, we also generated helix-destabilizing modifications by irradiating DNA with UV light at 254 nm, producing as major lesions cyclobutane pyrimidine dimers.

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The abbreviations used are: NER, nucleotide excision repair; AAF, N-acetyl-2-aminofluorene; BPDE, benzo[a]pyrene diol-epoxide; 8-MOP, 8-methoxypsoralen; RPA, replication protein A; UV, ultraviolet; XP, xeroderma pigmentosum; pyrimidine(6-4) photoproduct, 6-(1,2)-dihydro-2-oxo-4-pyrimidyl)-5-methyl-2,4-(1H,3H) photoproduct.

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and pyrimidine(6-4) photoproducts in a ratio of about 3:1 (26).

Treatment of DNA with 8-MOP and long wavelength UV light (>320 nm) yields psoralen monoadducts and a small proportion of psoralen diadducts (27). This photoaddition reaction occurs between the 5,6 double bond of pyrimidine bases and either the 3,4 (pyrrole) or the 4′,5′ (furan) double bond of the psoralen. Modifications with psoralen induce helical distortion by unwinding the duplex and enhancing backbone flexibility (28) but fail to destabilize the secondary structure of DNA. On the contrary, thermostability measurements showed that site-directed modifications with psoralen induce helical distortion by mediating stacking interactions between the psoralen moiety and the surrounding base pairs (19, 20, 28). Helix-stabilizing adducts were also obtained using anthramycin, a pyrrolo[1,4]benzodiazepine antibiotic, and CC-1065, a composite compound consisting of three pyrroloindole subunits joined by amide linkages (Fig. 1). Anthramycin binds selectively to N\textsuperscript{3} of guanine through aminal bonds and forms covalent adducts with essentially no distortion of the DNA helix (21, 29). CC-1065 displays a cyclopropyl ring that alkylates DNA at position N\textsuperscript{3} of adenine, generating covalent adducts that cause bending and winding of the double helix (22, 30). Both anthramycin and CC-1065 adducts enhance duplex stability through noncovalent interactions derived from hydrogen bonds (anthramycin) or van der Waals and hydrophobic forces (CC-1065) within the minor groove of DNA (21, 22, 30).

Recognition of these bulky adducts was compared by monitoring their capacity to sequester NER factors. To that end, we have developed a competition assay that measures the efficiency by which damaged plasmids compete for NER factors operating on a site-directed substrate (31). As a source of NER activity we exploited a standard soluble extract from human cells (32, 33). Under the conditions used in our study, this cell-free extract does not support chromatin assembly or transcription, thereby eliminating nuclear activities that modulate the intrinsic capacity of NER to recognize DNA damage (34, 35). This system revealed >1000-fold differences in the capacity of the tested adducts to sequester human NER factors. Those lesions that destabilize the DNA helix (AAF or BPDE adducts, UV radiation products) were effective competitors. In contrast, those adducts that stabilize the helix (8-MOP, anthramycin, and CC-1065 adducts) displayed minimal competing effects. In parallel, site-specifically placed CC-1065 adducts were unable to detectably stimulate synthesis of DNA repair patches. These results indicate that an early subset of NER recognition factors is attracted to structural defects associated with unfavorable thermodynamic changes of the DNA double helix. As a consequence, human NER is preferentially targeted to sites of helical instability.

EXPERIMENTAL PROCEDURES

Materials—Compound CC-1065 was provided by Dr. J. P. McGovren (The Upjohn Co.). Anthramycin was a gift from Dr. F. Sorter (Hoffmann-La Roche). N-Acetoxy-2-acetylaminofluorenone, [\textsuperscript{3}H]N-acetoxy-2-acetylaminofluorenone (457 mCi/mmol), and (±)-anti-benz[a]pyrene-r, t,6-dihydriodiol-t,9,10-epoxide were purchased from the NCI Chemical Carcinogen Repository. 8-MOP was from Sigma and 8-[methoxyl-H]Methoxypsoralen (80 Ci/mmol) from Amersham Corp. Stock solutions of these chemicals were prepared in dimethyl sulfoxide or methanol and stored at −80 °C. Plasmid DNA, competent cells, polynucleotide kinase, and cell culture media were purchased from Life Technologies, Inc. Ribonuclease A, creatine kinase, and calf intestinal phosphatase were from Boehringer Mannheim. Restriction enzymes were obtained from New England BioLabs. Protease inhibitors, phosphocreatine, ATP, and deoxyribonucleotides were obtained from Sigma. Synthetic oligonucleotides were from MWG-Biotech. [\textsuperscript{3}H]Methoxypsoralen (80 Ci/mmol) and [\textsuperscript{32}P]ATP (3000 Ci/mmol) were from DuPont NEN. All other chemicals used were of the highest purity commercially available.

Cell Lines and Extracts—HeLa cells were kindly provided by the Institute of Virology (Zurich). The human lymphoid cell lines GM1250 (XP-A) and GM1263 (XP-C) were obtained from the NIGMS Human Genetic Mutant Cell Repository. HeLa and lymphoid cells were grown in RPMI 1640 supplemented with 7 and 15% fetal bovine serum, respectively. Cell extracts were prepared as described (32, 33).

Site-directed Substrates—M13 double-stranded DNA containing a site-directed AAF adduct (M13-AAF) was constructed using a gapped DNA intermediate as outlined previously (36). To obtain site-specific CC-1065 adducts, a short double-stranded fragment containing a single CC-1065 modification was inserted into pUC19 by a previously described method (37), with the following modifications. The oligonucleotide 5′-GATCTCGGATTAGCCGGCCG at 5′-end (38) and annealed with an equal amount of the oligonucleotide 5′-TCGACCCTGGGCTTAATCGGGA, which still had a 5′-OH terminus. The resulting double-stranded fragment (0.21 mg/ml) was reacted with CC-1065 (160 μM) in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl for 24 h at room temperature. This incubation results in covalent modification of the most 3′-adjacent of the 5′-GATTA sequence in the upper strand, whereas the lower strand does not contain such a high affinity site for CC-1065 binding (39). The fragment was recovered by ethanol precipitation and ligated with BamHI-di- and ligated with BamHI-di- gested pUC19 using a 50-fold molar excess of the fragment. The reaction products were digested by Sall, precipitated with 6% (w/v) polyethylene glycol 6000 and 450 mM NaCl, phosphorylated with ATP and T4 polynucleotide kinase and circularized as indicated (37). The obtained pUC19 derivative (designated pUC19-CC-1065) was purified as covalently closed circular DNA by CsCl centrifugation (40). Nondam-

![Chemical structure of the bulky DNA adducts tested in the present report.](image)
aged pUC19 derivative (pUC19-Control) was prepared with nonmodi-
fied gels for the identical purpose. A small amount of these derivatives was analyzed by cleavage with Smal, followed by treatment with calf intestinal phosphatase and 5' end labeling of the resulting fragments with polynucleotide kinase and [γ-32P]ATP (38). After incubation of these fragments at 95°C for 20 min to induce strand breaks at sites of CC-1065 modification (22), the resulting products were resolved on 20% denaturing polyacrylamide gels.

**DNA Repair Synthesis Assay—**Recognition of DNA Adducts

Preparation of Competitor DNA and Adduct Quantification—Plasmid pUC19 (2868 base pairs) was purified by CsCl and sucrose gradient centrifugation. To obtain AAF-DNA adducts, pUC19 (50 µg/ml) was reacted with 0.1 mM -9,10-epoxide in 2 mM sodium citrate, pH 7.0, at 25°C for 3 h. The unreacted carcinogen was extracted five times with ether as described (41), and DNA was precipitated with ethanol and purified through a 5–20% sucrose gradient (40). Using [3H]-N-acetoxy-2-acetylaminofluorene in 2 mM sodium citrate, 0.12 or 0.21 mM (Fig. 2), light bulbs type F20T12BLB. After sucrose gradient purification, we identified by Cerenkov counting. The obtained values were used to calculate the percentage of covalently bound, 32P-bound, 32P-free, 32P-g of bovine serum albumin. After heating the plasmids to 95°C and analysis of the resulting strand breakage products (22) on alkaline agarose gels (data not shown).

8-MOP adducts were obtained by incubating pUC19 (333 µg/ml) with 8-(N-methyl-N′-nitro-5-nitroso-2-imidazolines (0.83 mM; 7 × 10−9 dm3/mmol) in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA. Aliquots (100 µl) were placed on ice in a closed plastic Petri dish and irradiated for 6 min with near UV light (peak output at 365 nm) 17 cm below two Sylvania black light bulbs type F20T12BLB. After sucrose gradient purification, we found that this procedure yielded 14.5 8-MOP adducts/pUC19 molecule (Fig. 2C).

To prepare anthracycin-DNA adducts, pUC19 (333 µg/ml) was incubated with 3.3 µM anthracycin in 1.5 mM sodium citrate, pH 7.0, and 15 mM NaCl for 90 min at 37°C. The modified DNA was purified by ethanol precipitation and 5–20% sucrose gradient centrifugation. The reaction of anthracycin with DNA is accompanied by a gradual change in the anthracycin absorption peak (43), which shifts from 333 to 343 nm and decreases in amplitude (Fig. 2D). Thus, the fractions of free and DNA-bound anthracycin were measured from the absorbance (A) at 321 nm (the maximum of the difference spectrum) and at 349 nm (the isobestic point). With the equations A321,A349

**Results**

**NER Substrate for Repair Competition Assays—**In this report, we compared the capacity of various bulky DNA adducts to sequester NER recognition factors contained in a human cell-free extract. For that purpose, we developed a competition assay that measures the efficiency by which damaged plasmid pUC19 inhibits excision repair of a specific NER substrate. This substrate consists of M13 double-stranded DNA containing a site-directed AAF-guanine adduct (Fig. 3A) and was constructed by ligating into a gapped M13 intermediate a 19-mer oligonucleotide with a single AAF-guanine modification in the center (36). The site-directed AAF adduct is flanked by the recognition sequences for Smal and PstI that were introduced to generate a fragment of 37 base pairs (Fig. 3A). Nondamaged control M13 DNA was prepared by ligating into the gapped intermediate a nonmodified 19-mer of the same sequence. Repair incubations were performed in a NER-proficient HeLa cell extract (33) supplemented with ATP, an ATP-regenerating system, and all four deoxynucleoside triphosphates, of which dCTP was radiolabeled. After incubations of 3 h at 30°C, DNA was restricted with Smal, PstI, and AvaII, and NER patches were detected by monitoring the incorporation of radiolabeled dCMP in the region of the substrate where the AAF adduct is located, i.e. in the 37-base pair Smal-PstI fragment (Fig. 3B, lanes 1 and 2). A biochemical complementation experiment proved that damage-specific nucleotide incorporation into this 37-base pair Smal-PstI region was catalyzed by the NER system. Incorporation of dCMP was abolished in extracts prepared from NER-deficient XP-A (GM2250) or XP-C (GM2634) cell lines (Fig. 3B, lanes 3 and 4). However, DNA repair synthesis was reconstituted by coincubating the two different XP cell extracts in the same repair reaction (Fig. 3C, lanes 5 and 6, in duplicate).

**Repair Competition Assay with AAF-DNA Adducts—**We first tested the capacity of pUC19 DNA containing 10.2 AAF adducts/plasmid to compete with the single AAF adduct located on M13 DNA. The formation of NER patches in the 37-base pair Smal-PstI region of the site-directed substrate was progressively inhibited when increasing amounts of multiply AAF-damaged competitor were added to the repair reactions (Fig. 4). Partial inhibition was observed at molar ratios of AAF-damaged pUC19 to M13 DNA ranging from 0.06 to 0.54, and essentially complete inhibition was detected at molar ratios between the two DNA molecules of 1.35 or higher. In contrast, little or no inhibition was observed when nondamaged pUC19 DNA was added to the repair reactions even in a large molar
excess over M13 DNA substrate. The typical gel shown in the inset of Fig. 4 illustrates that NER in the 37-base pair SmaI-PstI region of the M13 substrate was reduced to background levels when AAF-damaged pUC19 was added in a 1:1 mass ratio, corresponding to a pUC19 to M13 DNA molar ratio 2.7:1 (lane 3). We consistently noted that only the specific NER response in the 37-mer SmaI-PstI fragment was suppressed, whereas the nonspecific nucleotide incorporation unrelated to NER in the adjacent 330-base pair AvaII-SmaI region remained unaffected. These different responses of the two M13 DNA fragments confirmed that the competition between M13 substrate and damaged pUC19 was selective for NER factors.

Fig. 2. Competitor pUC19 DNA. Plasmid pUC19 (2686 base pairs) was exposed to DNA damaging agents and purified as covalently closed DNA by 5–20% sucrose gradients. A, quantification of AAF adducts. Treatment with 0.1 mM [3H]-acetoxy-2-acetylaminofluorene yielded an average of 10.2 ± 0.9 AAF adducts per plasmid (mean value of three independent determinations). B, quantification of BPDE adducts. Plasmid pUC19 was incubated with 0.12 or 0.21 mM BPDE, purified by sucrose gradients, and analyzed by UV spectroscopy. The spectrum shown was obtained with BPDE-modified pUC19 (530 μg/ml, upper line) and nonmodified pUC19 at the same concentration (lower line). Using the absorption peak at 346 nm (42), we determined the formation of 5.0 and 16.7 BPDE adducts per plasmid molecule. C, the frequency of 8-MOP-DNA adducts as a function of irradiation time was determined using 8-[methoxyl-3H]methoxypsoralen. Based on the shown time course, we irradiated for 6 min and obtained an average of 14.5 8-MOP adducts/pUC19 after sucrose gradient purification. D, absorption spectrum of anthramycin before (upper line) and after (lower line) reaction with pUC19 DNA. The covalent binding of anthramycin (3.3 μM) to DNA (330 μg/ml) is accompanied by a change in the anthramycin absorption peak, which shifted from 333 to approximately 340 nm and decreased in amplitude (43). The frequency of anthramycin modification of DNA (11.9 adducts/pUC19) was calculated from the absorbance at 321 nm (the maximum of the difference spectrum) and at 349 nm (the isosbestic point). E, UV absorption spectrum of CC-1065-modified DNA (100 μg/ml, upper line). The amount of DNA-bound CC-1065 (9.6 adducts/pUC19 molecule) was determined from its absorbance at 365 nm (44). The lower line shows the corresponding absorption spectrum of nondamaged pUC19.
By interpolation of the data shown in Fig. 4, we estimated that approximately 50% inhibition is expected at a molar ratio of AAF-damaged pUC19 to M13 substrate of 0.09. This number translates to a 10.8-fold molar excess of M13 substrate over pUC19 competitor. Because each M13 DNA molecule contains an average of 10.2 adducts/molecule (Fig. 2A), incubation with site-specifically modified substrate (M13-AAF) and damaged pUC19 (with an average of 10.2 AAF adducts per plasmid), in which NER of the substrate was suppressed. The molar ratio of damaged pUC19 competitor to M13 substrate was 2.7:1.

**Repair Competition Assay with BPDE-DNA Adducts and UV Radiation Products**—We next exploited the competition assay to test the capacity of human NER to recognize BPDE adducts. M13 DNA substrate with the site-directed AAF-guanine modification was coincubated with pUC19 containing either 5.0 or 16.7 BPDE adducts per plasmid molecule. Control reactions were performed with equivalent amounts of nondamaged pUC19 DNA. The molar ratios of pUC19 competitor to M13 substrate were 2.7 and 8.1. The results from four to six independent determinations were quantified by scanning densitometry and plotted as the percentages of DNA repair synthesis obtained in control reactions incubated with nondamaged pUC19 (Fig. 5A). At a modification frequency of 5.0 BPDE adducts/pUC19 and a competitor to substrate molar ratio of 8.1:1, NER in the 37-base pair Smal-PstI region of the substrate was reduced by 53%. At a competitor to M13 DNA molar ratio of 2.7:1, the same plasmid carrying 5.0 BPDE adducts was unable to significantly inhibit NER operating on the substrate (Fig. 5A). We also tested pUC19 containing 16.7 BPDE adducts per plasmid. As one would expect from the 3-fold higher frequency of modification, this latter plasmid yielded approximately 50% inhibition at a molar ratio of pUC19 to M13 substrate of 2.7:1 and exerted a more pronounced inhibition of NER activity on the substrate at a molar ratio of pUC19 competitor to M13 DNA of 8.1:1 (Fig. 5A). The representative gel of Fig. 5B illustrates the partial inhibition of NER activity in the 37-base pair Smal-PstI region of the M13 substrate when pUC19 (16.7 BPDE adducts/plasmid) was added to the reactions in a 2.7-fold (lane 3) or in an 8.1-fold molar excess over the substrate (lanes 4 and 5). In contrast, no reduction of NER in the 37-base pair region was found when the reactions were incubated with nondamaged pUC19 (lanes 6 and 7).

We then calculated the stoichiometric excess of BPDE adducts over AAF modifications required to inhibit NER of the substrate. Assuming ~50% inhibition at an 8.1-fold molar ex-
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**Fig. 5. Repair competition assay, comparison between BPDE-, UV-, and AAF-damaged DNA.**

A, standard repair competition reactions were incubated in the presence of pUC19 DNA containing BPDE adducts (5.0 or 16.7/molecule), UV radiation products (11.5/molecule), or AAF adducts (10.2/molecule). The molar ratio of pUC19 competitor to M13 sub-strate was 2.7:1 or 8.1:1, as indicated. After 3 h at 30 °C, dCMP incorporation in the 37-base pair SmaI-PstI region of the substrate was analyzed by gel electrophoresis, quantified by scanning densitometry of the corresponding autoradiographs, and expressed as the percentage of DNA repair synthesis obtained in control reactions containing identical amounts of nondamaged pUC19 DNA (mean values of four to six determinations ± S.D.). B, representative gel obtained using, as a competitor, pUC19 modified with 16.7 BPDE adducts per plasmid. DNA repair synthesis in the 37-base pair SmaI-PstI region of the M13 substrate was visualized by autoradiography. Standard competition mixtures contained M13 DNA substrate with the site-directed adduct (M13-AAF) or nonmodified M13 DNA (M13-Control), as indicated. Competitor pUC19 was either BPDE-damaged or nondamaged (N), and the molar ratio of pUC19 to M13 was 2.7:1 (lanes 3 and 6) or 8.1:1 (lanes 4, 5, and 7).

cess of pUC19 containing 5.0 BPDE adducts per molecule (Fig. 5A), these calculations yielded a stoichiometry of BPDE to AAF adducts of ~40:1. Nearly the same value is obtained by considering the ~50% inhibition observed when plasmid pUC19 containing 16.7 BPDE adducts/molecule was added to the reactions at a 2.7:1 molar ratio of competitor to substrate (Fig. 5A). In summary, an approximately 40-fold excess of BPDE adducts was required to inhibit NER of the site-directed AAF substrate.

EXCISION REPAIR ASSAY WITH A SITE-DIRECTED CC-1065 ADDUCT—The observation that CC-1065 modifications have an extremely low capacity to sequester NER factors led to the prediction that these adducts are poorly recognized and processed by the human NER system. To test this hypothesis, we exploited the selectivity of compound CC-1065 for the sequence 5'-GATTA*- (22, 39) and constructed circular double-stranded DNA containing a uniquely located CC-1065 adduct (the asterisk denotes the covalently added adenine). Briefly, a duplex oligonucleotide with the CC-1065 modification in one strand was ligated between the BamHI and SstI sites in the polylinker of plasmid pUC19 (see “Experimental Procedures” for details). The desired ligation product (designated pUC19-CC-1065) was purified as covalently closed circular DNA. A control substrate (pUC19-Control) was obtained by ligating nonmodified duplexes of the same sequence into the identical region of the pUC19 polylinker.

The single CC-1065-adenine adduct was situated between two adjacent SmaI sites (Fig. 7A). To demonstrate effective
modification, the 21-base pair Smal fragment was obtained from the substrate by restriction digestion, radiolabeled at its 5' ends, and subjected to heating at 95°C for 20 min. This treatment induces breakage of the adducted strand at the site of CC-1065 modification (22) and is expected to generate a radiolabeled 15-mer oligonucleotide that was resolved by denaturing polyacrylamide gel electrophoresis (Fig. 7B). Quantitative analysis of the resulting autoradiograph showed that a considerable fraction (~35%) of radioactivity shifted from the position of the 21-mer oligonucleotide to a smaller oligomer of 15 residues (Fig. 7B, lane 1). This strand breakage product was not detected when the nonmodified control substrate was heated to 95°C (Fig. 7B, lane 2). Although both 5' ends of the 21-mer duplex were radiolabeled, only the upper strand containing the 5'-GATTAG sequence was modified by CC-1065 and, as a consequence, only this upper strand was subject to nicking during thermal treatment. Thus, the observed fragmentation of 21-mer oligonucleotides translates to a frequency of modification of at least 70%, indicating that a major fraction of pUC19-CC-1065 substrate contained the site-specific CC-1065-adenine adduct at the expected position. This analysis also showed that cleavage by Smal was not inhibited by the CC-1065 modification.

Site-directed substrates containing either the CC-1065 adduct (pUC19-CC-1065) or the AAF adduct (M13-AAF) were incubated in the NER-proficient HeLa cell extract supplemented with 8-MOP-, anthramycin-, or CC-1065-damaged DNA. A, standard repair competition reactions were incubated in the presence of damaged pUC19 DNA containing 14.5 8-MOP adducts, 11.9 anthramycin (Atm) adducts, or 9.6 CC-1065 adducts/molecule. The molar ratio of pUC19 competitor to M13 substrate was 8.1:1, 25:1, or 120:1. After 3 h at 30°C, dCMP incorporation in the 37-base pair Smal-PstI region of the substrate was analyzed as indicated in the legend of Fig. 5 and expressed as the percentage of DNA repair synthesis obtained in control reactions containing same amounts of nondamaged pUC19 DNA (mean values of four independent determinations ± S.D.). For comparison, competition reactions were also performed with AAF-damaged pUC19 (ratio 2.7:1) and BPDE-damaged pUC19 (ratio 120:1). B, typical gel obtained with a 25-fold molar excess of 8-MOP-damaged pUC19 competitor (14.5 adducts/molecule) over M13 DNA substrate. Standard competition reaction mixtures contained modified (M13-AAF) or nonmodified substrate (M13-Control), as indicated. DNA repair synthesis in the 37-base pair fragment of the substrate was visualized by autoradiography. I, pUC19 irradiated for 6 min with near UV light in the absence of 8-MOP; N, nondamaged pUC19. C, representative gel obtained with a 25-fold molar excess of anthramycin (lanes 6 and 7) or CC-1065-damaged pUC19 (lanes 4 and 5). The modification frequency was 11.9 (anthramycin) and 9.6 (CC-1065). Standard competition reactions contained M13 DNA substrate with the site-directed adduct (M13-AAF) or nonmodified M13 DNA (M13-Control), as indicated.
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Fig. 9. Basic design of the repair competition assay. This assay was used to assess the capacity of damaged pUC19 to sequester NER factors contained in the HeLa cell extract. Sequestration was measured by monitoring the competitive inhibition of NER operating on a site-directed AAF-guanine adduct in M13 DNA.

Fig. 8. DNA repair synthesis at the site of a single bulky modification. A, a representative gel showing that the site-directed CC-1065 adduct is unable to detectably stimulate DNA repair synthesis. Substrate (200 ng) consisting of nonmodified (lanes 1 and 2, in duplicate) or CC-1065-modified pUC19 derivative (lanes 3 and 4) was incubated in HeLa cell extract in the presence of [32P]dCTP. After reactions of 3 h at 30 °C, DNA was recovered, digested with Smal to obtain fragments of 21 base pairs comprising the site of modification, and analyzed by polyacrylamide gel electrophoresis. For comparison, lane 5 illustrates the extent of DNA repair synthesis typically found in the 37-mer Smal-PstI region of M13 DNA (200 ng) carrying the site-directed AAF adduct. The modified strand of the 21-mer fragment contains 6 dCMP residues, whereas the corresponding strand of the 37-mer fragment contains 15 dCMP residues. B, dCMP incorporation into the 21-base pair Smal region of the pUC19 derivative and the 37-base pair Smal-PstI region of M13 DNA. Nucleotide incorporation was stimulated by the site-specific AAF adduct but not by the site-specific CC-1065 adduct (mean values of three independent experiments ± S.D.).
marginal inhibition of NER operating on the substrate. Conversely, efficient competitors such as AAF-damaged pUC19 suppressed NER activity in the covalently modified region of the substrate, without significantly reducing nonspecific nucleotide incorporation in an adjacent DNA segment (Fig. 4). Thus, the competition effect is selective for NER activity. Fourth, the principal finding obtained in the competition assay (differential recognition of thermodynamically diverse lesions) was confirmed by comparing DNA repair synthesis induced by site-specific adducts with either a helix-destabilizing (AAF) or helix-stabilizing (CC-1065) compound (Fig. 8). Finally, previous studies have shown that damage recognition/DNA incision constitute the rate-limiting step of NER in the cell-free extract (47), indicating that competition for repair factors is likely to occur at an early, preincisional level of the pathway.

The repair competition data demonstrated that DNA conformation at the site of damage is of critical importance for recognition. The capacity of the tested adducts to sequester human NER factors decreased with the following order: AAF > UV radiation products > BPDE > 8-MOP > anthramycin, CC-1065. The competition exerted by AAF, UV, and BPDE adducts was 2–3 orders of magnitude stronger than the competition exerted by 8-MOP, anthramycin, or CC-1065 adducts. For example, AAF modifications were able to sequester NER factors 1740 times more efficiently than 8-MOP adducts. This striking hierarchy of sequestration efficiency, combined with the known thermodynamic characteristics of the tested lesions, indicates that mammalian NER is primarily targeted to structural defects that destabilize the double-helical conformation of DNA. In fact, AAF modifications have been shown to completely abolish base pairing between the adducted guanine and its complementary cytosine (14, 15). Analysis with chloroacetate completely abolishes basepairing between the adducted guanine and DNA. In fact, AAF modifications have been shown to completely abolish basepairing between the adducted guanine and DNA. In fact, AAF modifications have been shown to completely abolish basepairing between the adducted guanine and DNA. In fact, AAF modifications have been shown to completely abolish basepairing between the adducted guanine and DNA. In fact, AAF modifications have been shown to completely abolish basepairing between the adducted guanine and DNA. In fact, AAF modifications have been shown to completely abolish basepairing between the adducted guanine and DNA. In fact, AAF modifications have been shown to completely abolish basepairing between the adducted guanine and DNA. In fact, AAF modifications have been shown to completely abolish basepairing between the adducted guanine and DNA. 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