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DNA Quality Control by Conformational Readout on the Undamaged Strand of the Double Helix

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Summary

Synthetic DNA probes were incubated in human cell extracts to dissect the early step of bulky lesion recognition in the nucleotide excision repair pathway. Excision was induced upon combination of the target adduct with either a two-sided bulge, involving both the damaged sequence and its undamaged partner strand, or a one-sided bulge, affecting exclusively the undamaged complementary sequence. Surprisingly, the same adduct became refractory to repair when only the modified strand was bulged out of the double helix. Adduct removal was further dependent on an intact opposing strand and, at carcinogen-DNA adducts, the assembly of excision complexes was triggered by a single flipped-out deoxyribonucleotide in the complementary sequence. These findings describe a mechanism of molecular readout in DNA repair that, unexpectedly, is entirely confined to the undamaged side of the double helix.

Introduction

Nucleotide excision repair (NER) removes bulky DNA lesions caused by environmental genotoxic insults, such as UV radiation or chemical carcinogens. In humans, NER deficiencies are associated with several genetic disorders, including xeroderma pigmentosum (XP), Cockayne syndrome, and trichothiodystrophy [1, 2]. The XP syndrome, in particular, is characterized by photosensitivity and a highly elevated incidence of sunlight-induced skin cancer. Seven XP genetic complementation groups (XP-A through XP-G) reflect mutations in NER factors that are involved in two distinct processes. A general pathway designated global genome repair (GGR) operates throughout the chromosomes and reduces the frequency of mutations, which are responsible for the long-term carcinogenic risk. In contrast, a transcription-coupled pathway is directed to DNA lesions in transcribed strands and, thus, is important for the short-term recovery from genotoxic stress [3–5]. XPC is unique among these factors because it is required only for GGR, whereas all the other subunits are involved in both NER pathways [6]. XPC protein constitutes the initial sensor of DNA damage in the general repair mechanism [7–10], although a role in lesion recognition has also been attributed to replication protein A (RPA) and XPA [11, 12]. Upon binding to damaged sites, XPC protein mediates the recruitment of transcription factor IIH (TFIIH), which employs its helicase subunits (XPB and XPD) for DNA unwinding [13–15]. Subsequently, two structure-specific endonucleases, XPF-ERCC1 and XPG, catalyze the dual incision reaction [16, 17].

An astounding feature of the NER machinery is its ability to process a wide diversity of DNA lesions, but the mechanism by which damaged residues are discriminated against the vast background of normal DNA is still a focus of intense debate. No obvious chemical motif of the different DNA adducts exists on which a classic "lock and key" recognition scheme can be based [18, 19]. Instead, the observed substrate versatility implies that the NER complex may recognize conformational distortions imposed on DNA at sites of damage rather than specific base modifications. This hypothesis led to the search for a universal feature of damaged DNA that accounts for the broad substrate range of the NER machinery. There is, for example, a general bias for excision of base adducts that lower the melting temperature of DNA, suggesting that thermodynamic instability caused by DNA damage may facilitate the detection of bulky lesions [20]. However, the degree of duplex destabilization does not correlate with the efficiency of adduct removal [21], and certain types of adducts, recognized by the NER complex, actually stabilize the double helix relative to undamaged sequences [22]. Another attractive hypothesis proposes that bulky adducts are detected by sensing an increased local conformational flexibility [23], but it is not evident which specific deviation from the normal dynamics of the double helix may promote the recruitment of NER factors.

An alternative approach to study the process of damage recognition is to analyze the particular manner in which the NER proteins align themselves with respect to the nucleic acid substrate. By dissecting the molecular anatomy of early, dynamic nucleoprotein intermediates, it will be possible to identify structural elements of damaged DNA that provide the critical initial binding sites for NER factors [24]. To that end, the strand-specific orientation of NER complexes was analyzed by manipulating the conformational and chemical properties of synthetic DNA probes (i.e., by changing the strand-specific location of the accompanying DNA distortion and by introducing different modifications in the opposing complementary sequence). This experimental strategy led to the unexpected finding that the NER complex is loaded onto damaged sites through interactions with a portion of distorted but undamaged DNA

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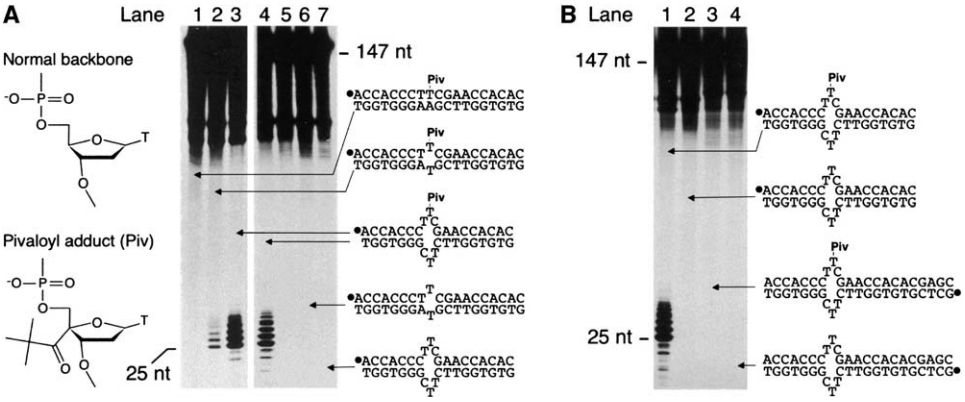

Figure 1. Synthetic Probes for the Analysis of NER Assembly

Excision assays were performed by incubating internally labeled DNA fragments of 147 base pairs (5 fmol; 75,000 dpm) in human cell extract (80 μg) for 40 min at 30°C. The reaction products were separated on denaturing polyacrylamide gels and visualized by autoradiography.

(A) Stimulation of excision repair by the introduction of a site-specific DNA bulge. The nondistorting pivaloyl (Piv) adduct is shown with the central sequence context of each substrate. The ^{32}P-labeled residue (filled circle) was placed in the upper strand. Lane 5, undamaged homoduplex DNA.

(B) Control incubations demonstrating that excision is directed to the damaged sequence. The ^{32}P-labeled residue (filled circle) was placed either in the upper (lanes 1 and 2) or in the lower strand (lanes 3 and 4).

mapping to the complementary strand located across the adduct.

Results

A Modular System of DNA Repair Probes

We developed an assembly line for the construction of NER substrates that combine a site-directed bulky lesion with helical distortions of variable type, degree, localization, and orientation [25]. The common DNA repair target consists of a central deoxythymidine carrying a pivaloyl adduct linked to the C4′ moiety of deoxyribose (Figure 1A). This C4′-modified residue adopts the same conformation as native nucleotides and, hence, the adduct is incorporated into the minor groove of the DNA double helix without disturbing Watson-Crick base pairing interactions [26]. Duplexes of ~150 base pairs were designed to contain this nondistorting pivaloyl adduct in the center and a ^{32}P-labeled phosphodiester bond on the 5′ side, 9–15 nt away from the position of the covalent modification. These internally labeled DNA probes were incubated in a soluble HeLa cell extract that provides all core GGR factors necessary for the dual DNA incision reaction [27, 28]. The resulting oligonucleotide excision products are expected to include the radioactive tracer because the 5′ incision occurs at a distance of 20–25 nt from the lesion [29]. Thus, repair efficiency was monitored by measuring the fraction of oligomeric excision products, relative to the amount of full-length substrate, after electrophoretic separation and autoradiography.

When embedded into homoduplex DNA, a single C4′ pivaloyl-modified deoxythymidine is not processed in human cell extracts, indicating that such a nondistorting adduct remains undetected by the general NER system. In this case, only full-length substrate, as well as radioactive bands generated by unspecific nuclease activity, could be observed near the gel origin (Figure 1A, lane 1). However, the same C4′ pivaloyl-modified deoxythymidine became an NER substrate when it was combined with an artificial DNA distortion constructed by the insertion of one base mismatch (Figure 1A, lane 2) or three contiguous base mismatches (lanes 3 and 4). In both cases, the incubation in human cell extract yielded typical oligonucleotide excision products in the size range of 25–30 residues. Minor amounts of smaller oligomeric fragments result from partial degradation of the main excision products [18]. This excision activity is dependent on the simultaneous presence of both structural alterations (i.e., an artificial DNA distortion as well as the C4′ pivaloyl adduct), because substrates containing only one or three base mismatches, without any covalent modification, were not processed by the NER complex (Figure 1A, lanes 6 and 7). These observations indicate that a local bulge that interrupts the canonical DNA helix is necessary for the recruitment of NER factors.

The strand selectivity of this repair reaction has been examined by labeling either the covalently modified top strand or the unmodified bottom strand (Figure 1B). The ^{32}P-label was inserted at the 9th phosphodiester bond on the 5′ side to the adduct in the top strand, and at the 15th phosphodiester bond on the other side in the bottom strand. These control experiments revealed an absolute level of strand specificity, as only the sequence containing the adduct was excised (Figure 1B, lane 1), whereas the undamaged strand opposite to the lesion remained protected from endonucleolytic cleavage (lane 3). Additional control assays showed that oligonucleotide excision was absent in extracts prepared from NER-deficient XP-A or XP-C fibroblasts, but the activity was restored in mixture experiments involving equal amounts of cell extract from the two different XP complementation groups (data not shown).

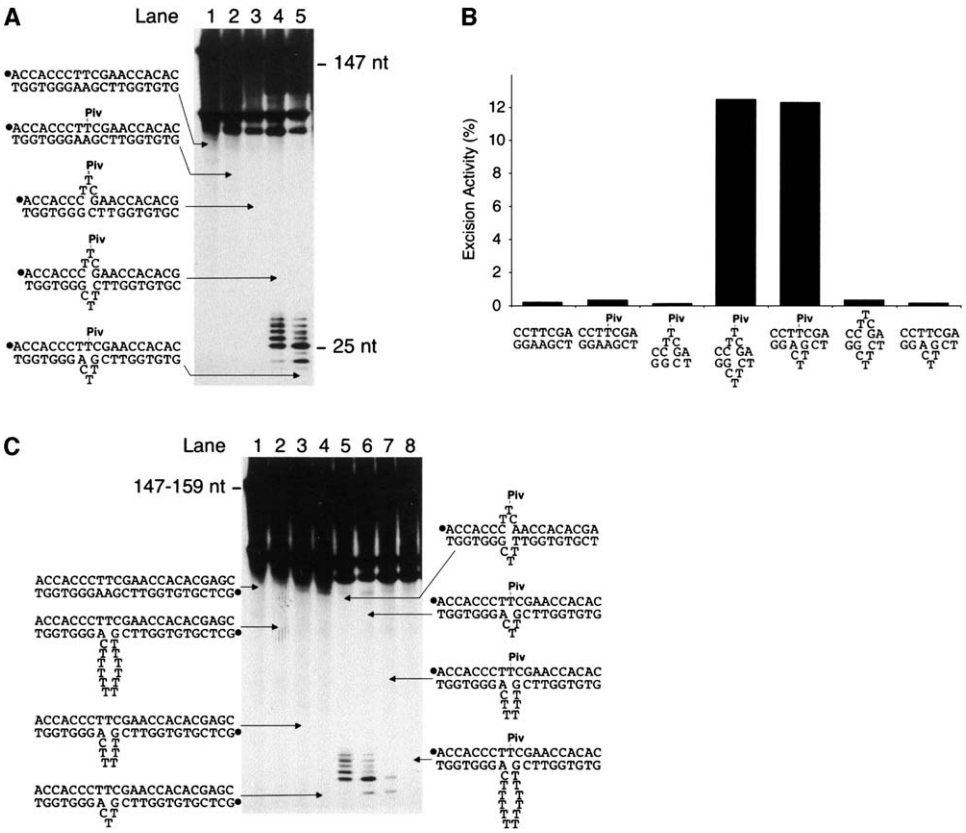

Figure 2. Recruitment of NER Activity to Targets with Distinct DNA Strand Topography

All reactions were performed by incubation (30°C, 40 min) of internally labeled DNA substrates (5 fmol; 75,000 dpm) in human cell extract (80 μg). Excision products were detected by gel electrophoresis and autoradiography.

(A) Requirement for a DNA bulge in the undamaged complementary sequence. The ^{32}P-labeled residue (filled circle) was placed in the upper strand of each substrate.

(B) Laser scanning quantification of excision activity from two independent experiments. The strand composition in the center of each substrate is indicated.

(C) Control incubations demonstrating that DNA loops are not excised. The ^{32}P-labeled residue (filled circle) was placed either in the lower (lanes 1–4) or in the upper strand (lanes 5–8).

Strand-Specific Orientation of the Incision Complex

To dissect the molecular process leading to bulky lesion recognition into its minimal constituents, we synthesized DNA probes in which the accompanying distortion was inserted in a strand-specific manner (Figure 2A). In one case, the bottom unlabeled strand was designed to lack three nucleotides across the lesion, such that the deoxythymidine residue containing a bulky adduct remained unpaired and was, therefore, extruded from the duplex structure into a short extrahelical loop. The general NER factors were not able to process this asymmetric deletion substrate, in which only the damaged strand was curved outwards (Figure 2A, lane 3). The analogous substrate with opposite polarity was constructed by introducing three additional nucleotides in the bottom strand, such that a 3 nt insertion loop was formed in the undamaged sequence opposite to the adduct. Interestingly, this one-sided bulge of three undamaged nucleotides led to substantial excision of the target lesion (Figure 2A, lane 5), generating a similar pattern of oligonucleotide products as the control substrate, where the adduct was combined with the standard two-sided bulge composed of three base mismatches (Figure 2A, lane 4; also in Figure 1A, lanes 3 and 4).

Laser scanning quantifications of duplicate experiments showed that the asymmetric insertion loop, involving only the undamaged strand, and the two-sided bulge, comprising both strands, yielded nearly identical levels of excision activity in response to the pivaloyl adduct (Figure 2B). Instead, the one-sided bulge with opposite strand polarity, involving only the damaged sequence, failed to trigger the NER reaction (Figure 2B). These results reveal for the first time that NER activity is completely dependent on a local DNA distortion that maps to the complementary strand across the lesion. This strand bias indicates that the correct orientation of repair intermediates, relative to the double helix, is critical for the formation of a productive excision complex.

We also excluded that a DNA loop on its own may serve as a NER substrate. For that purpose, short, sin-

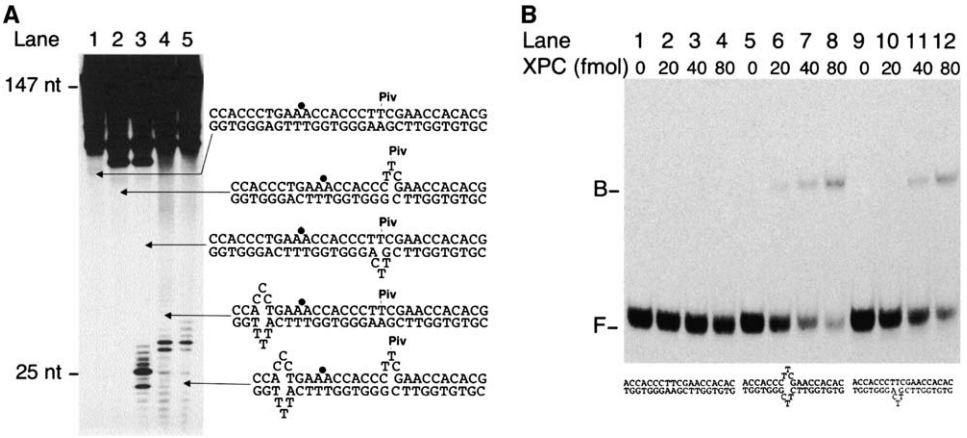

Figure 3. Docking Site for Recruitment of the Repair Complex

(A) Excision assays (30°C, 40 min) were performed with 5 fmol (75,000 dpm) of linear substrate, carrying the radiolabel in the upper strand, and 80 μg of human extract proteins. Lane 3: control incubation with an efficiently excised target. Lanes 4 and 5: the intractable adducts in the DNA fragments of lanes 1 and 2 are converted to excision substrates in the presence of a DNA bulge in the flanking duplex. Excision products were analyzed by gel electrophoresis and autoradiography. The position of the radiolabeled phosphodiester bond is indicated (filled circle).

(B) DNA binding reactions (30°C, 30 min) were conducted with 1 fmol of radiolabeled 147-mer substrate and the indicated amounts of XPC fusion protein. The fraction of protein-bound DNA fragments was visualized by separation on a native 5% polyacrylamide gel followed by autoradiography.

gle-stranded loops of 3–12 nt were inserted in one strand of duplex substrates and the radioactive tracer was incorporated at a distance of 11 nt from each loop, on the 5′ side of the same strand (Figure 2C). None of the substrates carrying a DNA loop alone was processed by the NER machinery (Figure 2C, lanes 2–4). Only in combination with a C4′ pivaloyl adduct were the 3 nt loop (Figure 2C, lane 6) and, to a minor extent, also the 6 nt loop (lane 7) able to promote excision activity, resulting in oligonucleotide release. Surprisingly, no excision was detected when the loop size was further increased to 12 nt (Figure 2C, lane 8), suggesting that the recruitment of NER complexes is based on an affinity for small bulges while ignoring large single-stranded loops.

Restoration of NER Activity on Composite Substrates

We identified two types of constructs from which the target lesion could not be excised. The first intractable substrate consisted of a single, nondistorting C4′ pivaloyl adduct incorporated into fully homoduplex DNA (Figure 1A; Figure 3A, lane 1). Another type of intractable substrate was generated by constructing a deletion duplex, such that the C4′ pivaloyl adduct was extruded into a 3 nt extrahelical loop (Figure 2A; Figure 3A, lane 2). The next experiment was prompted by the notion that a common feature of these two different "stealth" lesions is the lack of a DNA bulge in the complementary strand across the adduct. Therefore, we tested whether excision repair could be restored by the addition of a site of distorted DNA flanking the covalent modification. For that purpose, three contiguous base mismatches were incorporated to generate a DNA bulge at different positions in the 5′ direction from the C4′ pivaloyl adduct. The three mispaired bases were progressively moved away from the adducted backbone residue and, surprisingly, NER activity was maintained even when the mismatched bases were placed at a distance of ~15 nt from the otherwise intractable adducts (Figure 3A, lanes 4 and 5). These composite substrates were processed with a slightly different excision pattern (compare, for example, with the reaction of Figure 3A, lane 3), but the dominant repair products (composed of 28 and 29 nt oligomers) remained within the characteristic 24 to 32 nt range elicited by the human NER complex. Thus, an adjacent DNA bulge generated by base mismatches at a distance of ~15 nt is sufficient to restore excision of bulky lesions that, on their own, are refractory to the NER process. These results are consistent with a DNA bulge being indispensable for the initial recruitment of NER subunits rather than for subsequent conformational rearrangements along the formation of an active excision complex. In fact, the successful repair of composite substrates demonstrates that, once the recognition factors are loaded onto DNA by interaction with a structural bulge, the resulting nucleoprotein intermediates can easily progress toward the excision reaction.

Our findings also suggested that the primary recognition subunit (XPC protein) binds to DNA bulges even if no adducts are located in immediate proximity to the initial site of recruitment. To test this hypothesis, a gel mobility shift assay was performed by incubating recombinant human XPC protein with radiolabeled 147-mer duplexes containing variable nucleotide compositions in the center. The affinity of purified XPC protein for these distorted substrates was compared to its interaction with a homoduplex fragment of the same length. Electrophoretic analysis of the resulting nucleoprotein complexes indicated that the XPC subunit binds preferentially to the substrates carrying a two-

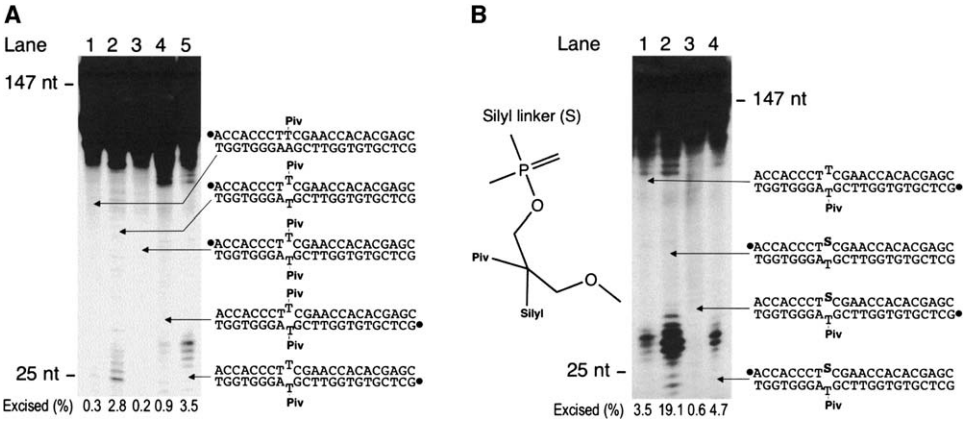

Figure 4. Requirement for an Intact Complementary Backbone

Doubly damaged DNA substrates were constructed by combining a pivaloyl-deoxythymidine (Piv) in the top strand with an identical adduct in the bottom strand (A), or by placing a silyl linker (S) opposite to the pivaloyl adduct (B). The linear DNA fragments of 147 base pairs display alternating locations of the radiolabeled residue (filled circle), as indicated. These substrates (5 fmol; 75,000 dpm per reaction) were incubated (30°C, 40 min) in human cell extract (80 μg), and excision products were detected by gel electrophoresis and autoradiography. The percentage of excised fragments was determined by laser densitometry (mean values of three experiments).

sided bulge (Figure 3B, lanes 6–8) or a one-sided bulge (lanes 10–12), relative to the homoduplex control (lanes 2–5). This selectivity coincides with the hypothesis that the human NER complex is loaded onto damaged substrates through interactions of the XPC subunit with structural bulges containing an undamaged DNA sequence.

Requirement for Intact DNA in the Complementary Strand

The results of Figure 2 indicated that a one-sided DNA bulge in the undamaged strand is necessary and sufficient to promote the damage-specific assembly of active NER complexes. To characterize this conformational requirement in more detail, DNA substrates were designed to contain a C4′ pivaloyl-deoxythymidine lesion on both sides of the double helix. We first tested constructs containing a single pivaloyl-modified deoxythymidine in either the top strand (Figure 4A, lane 2) or the bottom strand of duplex DNA molecules (Figure 4A, lane 5). These substrates were radiolabeled at the 9th phosphodiester bond, when the top strand was modified, or at the 15th phosphodiester bond when the lesion was placed in the bottom strand. Interestingly, the two singly modified substrates yielded excision products of different lengths. In the presence of a C4′ pivaloyl-modified deoxythymidine in the top strand, the major excision product consisted of 25-mer oligonucleotides (Figure 4A, lane 2; see also Figure 1A, lane 2) but, with a bottom-strand modification, the dominant band of excision products consisted of 30-mer oligonucleotides (Figure 4A, lane 5). Nevertheless, the rates of excision from the top or bottom strand (2.8% and 3.5% of excised substrates in 40 min reactions, respectively) were very similar (mean values of three experiments).

To monitor excision of two simultaneous lesions separately, the radioactive tracer was incorporated either in the upper or in the lower strand of doubly damaged duplexes. Surprisingly, we found that, in comparison to singly modified duplexes, NER activity toward doubly modified substrates was strongly inhibited. In fact, oligonucleotide release from the top strand was reduced to 0.2% in 40 min (Figure 4A, lane 3), a value that is indistinguishable from background activity. A substantial inhibition of excision activity was also observed in the bottom strand (Figure 4A, lane 4), where the excision rate reached only 1.1% (mean value of three experiments). Thus, the overall excision activity from the two strands of doubly damaged substrates was significantly lower than the ~3% level of excision observed with singly modified substrates.

The same experiment was repeated with another backbone lesion (a silyl linker) that is more susceptible to NER activity than the pivaloyl-modified deoxythymidine. As shown in Figure 4B, the replacement of a single nucleotide with a silyl linker generated a duplex substrate that was excised at a rate of 19.1% (mean value of three experiments) in 40 min reactions (lane 2). When this silyl linker was combined with a pivaloyl-deoxythymidine modification in the bottom strand, however, NER activity was markedly diminished. In fact, the excision rate from the top strand of such doubly modified substrates, containing the silyl linker, was reduced to 4.7% (Figure 4B, lane 4), whereas removal of the pivaloyl-modified residue from the bottom strand of these doubly modified substrates was completely suppressed (Figure 4B, lane 3). The poor repair of both types of doubly damaged duplexes indicates that the presence of a second backbone modification in the opposite complementary strand interferes with the effective recruitment of NER factors.

To gain further insight into the structural requirements for damage recognition, we constructed doubly modified probes containing a pivaloyl-modified deoxythymidine residue on the one side and nonhybridizing base analogs on the other side of the duplex. As illustrated in Figure 5A, nonhybridizing pyrimidine or purine analogs retain an aromatic ring structure similar to

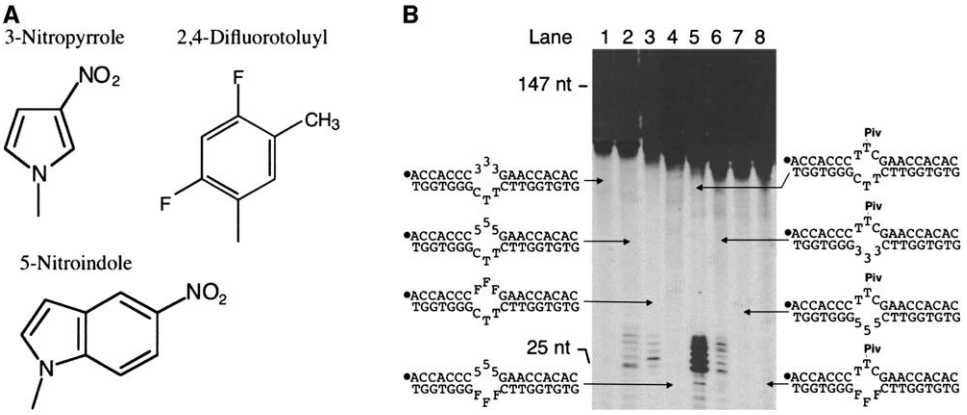

Figure 5. Requirement for Native Bases in the Complementary Strand

(A) Structure of nonhybridizing base analogs.

(B) Inhibition of NER activity following the introduction of three consecutive 3-nitropyrrole (lane 6), 5-nitroindole (lane 7), or 2,4-difluorotoluyl residues (lane 8) in the complementary strand across the pivaloyl adduct. Lane 5: control incubation with unmodified bases. Lanes 2 and 3: 5-nitroindole and 2,4-difluorotoluyl residues on their own are excised in human cell extract. Lane 1: 3-nitropyrrole analogs are not excised. Each reaction (30°C, 40 min) contained 5 fmol (75,000 dpm) of linear substrate, radiolabeled in the upper strand, and 80 μg of cell extract proteins.

that of natural bases, but lack hydrogen acceptor and donor groups for canonic Watson-Crick pairing interactions. DNA duplexes containing only a single base analog in one of the two strands were not excised in the human cell extract (data not shown). However, two of these analogs (5-nitroindole and 2,4-difluorotoluyl) were converted to excision substrates when three consecutive residues were clustered in the same strand (Figure 5B, lanes 1–3). Therefore, a local bulge of nonhybridizing residues around the target adduct was generated by replacing three bases opposite to the lesion with either 3-nitropyrrole (Figure 5B, lane 6), 5-nitroindole (lane 7), or 2,4-difluorotoluyl analogs (lane 8). Relative to the control substrate, with a standard mismatch-induced bulge (Figure 5B, lane 5), excision was in all cases severely reduced, or completely abrogated, following the insertion of base analogs in the complementary strand (lanes 6–8). These results show that the effective recruitment of NER complexes depends on the presence of native bases in the complementary strand opposite to the target lesion.

A Universal Determinant for the Detection of Carcinogen-DNA Adducts

Previous structural studies demonstrated that a local two-sided bulge is formed naturally at guanine adducts of benzo[*a*]pyrene (B[*a*]P) or acetylaminofluorene (AAF) through disruption of base pairing interactions, accompanied by displacement of both the modified guanine and its cytosine partner from their normal position inside the double helix [30, 31]. The results of the present study, based on synthetic probes, indicate that it is the particular distortion localized on the undamaged side of the double helix that leads to recruitment of the general NER complex. To test this hypothesis, modified DNA molecules were constructed in which the deoxyribonucleotide residue opposite to B[*a*]P-dG adducts was removed, generating deletion duplexes in which only the modified dG is looped out of the double helix [32]. The isomeric (–)-*cis*- and (+)-*cis*-B[*a*]P-dG adducts, placed into normal homoduplex substrates, were processed in human cell extract with comparable efficiency but a slightly different distribution of excision products (Figure 6A, lanes 2 and 4). On the other hand, there was only poor repair of the same (–)-*cis*- and (+)-*cis*-B[*a*]P-dG isomers when these adducts were incorporated in deletion duplexes where the opposing dC residue had been omitted (Figure 6A, lanes 3 and 5).

The role of an unstacked deoxyribonucleotide situated in the undamaged complementary strand was confirmed using site-specific AAF-dG lesions. We removed the critical dC residue opposite to each AAF-dG adduct, either by deletion, as in the previous experiments with B[*a*]P adducts, or by replacement with a C_3 molecular spacer that maintains the normal distance between neighboring backbone repeats (Figure 6B). In both cases, the absence of an unstacked deoxyribonucleotide across the modified guanine, on the undamaged side of the double helix, resulted in nearly complete loss of excision activity (Figure 6B, lanes 2 and 4). By labeling the complementary strand, it was shown that the C_3 spacer remains intact throughout the incubation period (Figure 6B, lane 5).

Time-course experiments confirmed that both the deletion (Figure 6C, lanes 1–3) and C_3 spacer substitution of the opposing dC residue (lanes 4–6) interfered with the assembly of active NER complexes at carcinogen-DNA adducts. By increasing the reaction time to 60 min in an experiment, the results of which are presented in Figure 6C, there was a higher background level of unspecific substrate degradation, but still no detectable oligonucleotide excision products when the opposing dC residue was missing. In contrast, single base replacements, which generate dG/dG, dG/dA, and dG/dT mismatches and increase the degree of local destabilization, exert opposite effects by stimulating the excision reaction (Figure 6D). In summary, these results

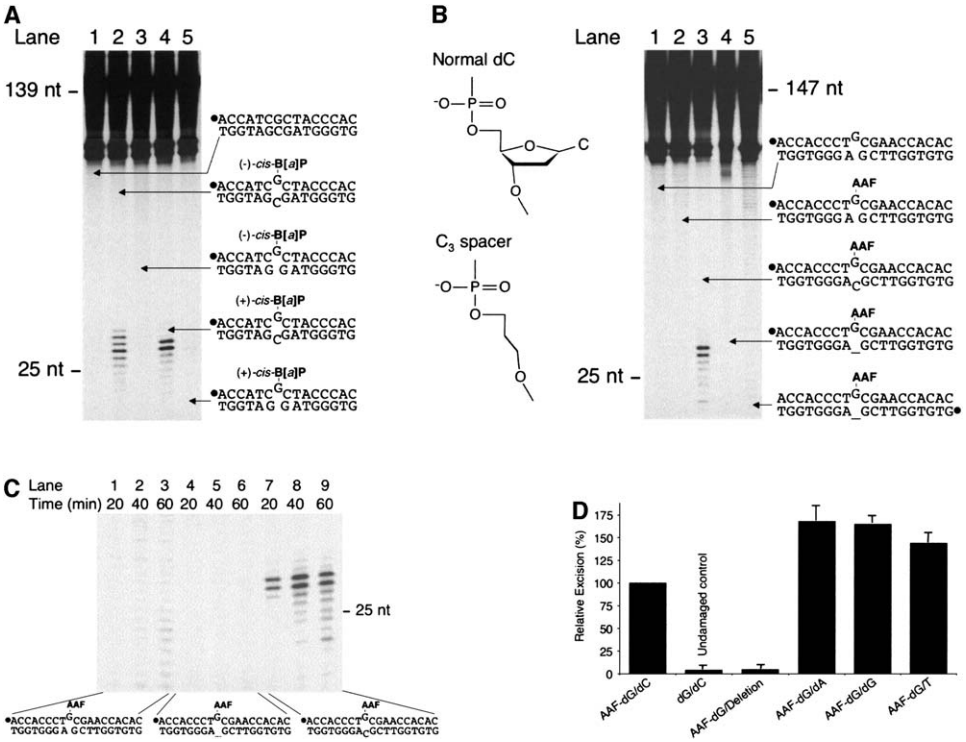

Figure 6. Recruitment of NER Activity to Carcinogen-DNA Adducts

Excision assays were carried out at 30°C with 5 fmol (75,000 dpm) linear substrate, radiolabeled in the upper strand, and 80 μg of human extract proteins. Reaction products were detected by gel electrophoresis and autoradiography.

(A) Inhibition of excision of (–)-*cis*- and (+)-*cis*-B[a]P-dG by a deoxyribonucleotide deletion in the complementary strand (lanes 3 and 5). The reactions were stopped after 40 min.

(B) Inhibition of excision activity (40 min) toward AAF-dG adducts following a deoxyribonucleotide deletion (lane 2) or its replacement by a C_3 spacer (lane 4). Lane 5: control reaction demonstrating the stability of the strand containing a C_3 spacer.

(C) Time course of excision of AAF-dG adducts after deletion of the opposing dC (lanes 1–3) or its replacement with a C_3 spacer (lanes 4–6). Lanes 7–9: control reaction with the AAF-dG adduct in the normal homoduplex sequence.

(D) Summary of three independent assays with DNA substrates containing a nucleotide deletion or a mismatched base across the AAF-dG residue. The repair activity was determined by laser scanning densitometry after 40 min incubations and expressed as the percentage of oligonucleotide excision observed in response to the adduct in fully complementary sequences (±SD).

reveal a strict requirement for a single, flipped-out deoxyribonucleotide in the complementary sequence for the correct loading of NER complexes onto damaged substrates.

Discussion

Previous Studies on Bulky Lesion Recognition

The early step of damage recognition in the general NER pathway appears mechanistically very intriguing, primarily because this enzymatic system is able to process a wide array of chemically unrelated base adducts. It has been shown in previous studies that the protein complex formed by XPC and hHR23B (a human homolog of Rad23) is the initial DNA damage binding protein essential for the recruitment of all subsequent factors involved in the GGR process, including TFIIH, XPA, RPA, XPG, and ERCC1-XPF [7–9]. Consistent with XPC-hHR23B being the first damage-specific sensor in the general NER pathway, this heterodimer has been shown to bind selectively to various types of modified DNA substrates containing, for example, 6-4 photoproducts induced by UV irradiation or AAF adducts [33–35]. Recombinant XPC protein itself possesses this substrate binding activity, whereas hHR23B alone does not have any overt affinity for DNA.

Subsequent analysis of the DNA binding properties of XPC protein suggested that this factor also displays a preference for small helical distortions, regardless of the presence or absence of damaged bases [34]. For example, XPC protein binds preferentially to a two-sided DNA bulge generated by the introduction of three base mismatches, or to a one-sided bulge formed by the insertion of three deoxyribonucleotides in only one strand of duplex DNA (Figure 3B). Scanning force microscopy showed that XPC protein induces a bend in the linear double-helical structure [36], and DNase I footprints demonstrated that XPC-hHR23B protects both strands of DNA around bulky lesions [35]. Inspection of the DNaseI cleavage patterns suggested that XPC-hHR23B can bind to the DNA double helix with at least two different orientations, but, in the presence of a one-sided bulge consisting of three inserted deoxyribonucleotides, this interaction with DNA is restricted

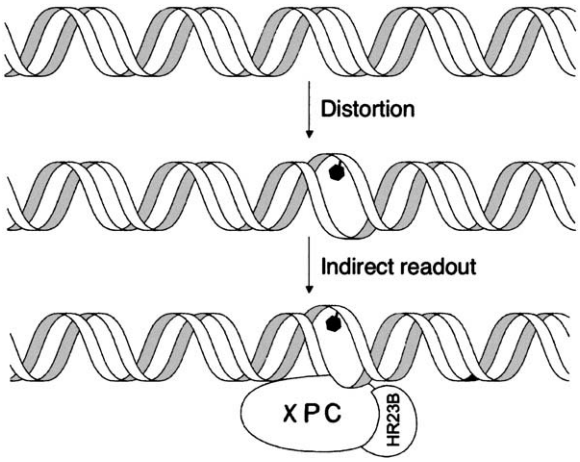

Figure 7. Schematic Model of DNA Quality Control through Conformational Readout on the Undamaged Side of the Double Helix

The NER pathway is initiated when XPC-hHR23B complex recognizes a local DNA bulge in the undamaged complementary strand. The subsequent anchoring of XPC on substrate DNA generates a nucleoprotein complex in which both strands are partially protected from DNaseI digestion. This model of indirect readout by XPC protein accounts for both the substrate versatility and the strand selectivity of the NER machinery. Also, this model implies that an additional recognition function, downstream of XPC protein, is required to confirm the presence of a bulky lesion.

to only one specific orientation [35]. However, in these preceding studies, it was not possible to clarify the molecular mechanism of action of XPC protein. It was not known what structural constituent of distorted DNA might be responsible for its recruitment to damaged sites or what particular orientation or damage-specific location of XPC protein may lead to the assembly of active NER intermediates.

Conformational Readout on the Undamaged Strand

To overcome the limitations of previous DNA binding and footprinting studies, an enzymatic strategy was exploited to screen for critical DNA elements that induce the assembly of functional NER complexes. This approach provides the advantage of discriminating against silent nucleoprotein intermediates that may be formed in simple reconstituted systems without being able to trigger the dual DNA incision reaction. A comparison of NER activities in response to a set of synthetic probes, which differ only in the type, localization, or orientation of the accompanying DNA distortion, led to the unexpected model of bulky lesion recognition based on an initial interaction with unstacked or flipped-out deoxyribonucleotides on the undamaged side of the double helix (Figure 7). The experimental evidence in favor of this novel model of damage recognition in DNA repair is as follows. First, a one-sided DNA bulge in the undamaged complementary strand is necessary and sufficient to convey a nondistorting DNA adduct into the excision reaction (Figure 2). In contrast, a bulge that allows for interactions with only the damaged strand is not compatible with NER activity (Figure 2). Second, this helical deformation is absolutely required for the early recruitment of NER factors to the DNA substrate molecules, rather than for a later step during the assembly of active incision complexes at the lesion site. In fact, a DNA bulge in a flanking region of the duplex substrate is again sufficient to redirect an otherwise intractable adduct to the excision pathway (Figure 3A). Third, the effective recruitment of NER factors to adducted sites is absolutely dependent on the integrity of the complementary strand. Single backbone modifications (Figure 4), as well as changes of the hydrogen bonding properties of the opposing strand (Figure 5), interfere with loading of NER complexes, indicating that close contacts with both the backbone and bases of the complementary undamaged sequence are required to mediate substrate recognition. Fourth, the assembly of NER complexes was completely abolished following the removal of a single flipped-out deoxyribonucleotide situated across carcinogen-DNA adducts on the undamaged side of the double helix. This loss of NER activity was observed regardless of whether the single, unstacked deoxyribonucleotide in the undamaged strand was simply deleted or, alternatively, replaced by a molecular spacer of appropriate length (Figure 6). This model of damage recognition by readout on the undamaged side of the double helix is consistent with the known biochemical properties of XPC protein; in particular, with its ability to form stable complexes in conjunction with DNA duplexes containing a one-sided bulge. Indeed, our functional study reveals, for the first time, the significance of this unique binding modality of XPC protein. Crosslinking studies might provide a future approach to determining if separate NER subunits are recognizing the lesion and the nearby perturbation of the unmodified strand.

Significance

The early process of DNA damage recognition in the nucleotide excision repair (NER) pathway is highly controversial. This study reveals, for the first time, a universal mechanism that explains how a single protein subunit can detect a wide range of bulky DNA lesions. In fact, we found that the general NER pathway is recruited to sites containing bulky DNA adducts through interactions with a local bulge in the undamaged complementary strand. This mechanism may be defined as indirect conformational readout, because a DNA repair subunit, in this case XPC protein, binds to the double helix through recognition of conformational features in the undamaged sequence rather than by direct interactions with the functional groups at damaged sites. This indirect mode of DNA quality control presents the obvious advantage that the initial damage sensor does not have to rely on molecular interactions with the adducts themselves, and actually avoids such intimate contacts with abnormal residues, thereby broadening the range of modifications that can be channeled into the same enzymatic pathway. Also, the two sides of damaged duplexes are not equivalent and, to avoid inappropriate cleavage of undamaged strands, it is conceivable that the assembly of NER factors in an incorrect orientation will be prematurely aborted. A readout mech-

anism based on the recognition of a DNA distortion on the undamaged side of the double helix may support the strand discrimination capacity of the NER system. According to this model, XPC protein contributes to strand specificity through its initial anchorage to the undamaged partner sequence, such that the subsequent excision enzymes can be loaded selectively onto the damaged target strand. This new conceptual framework for the mechanism of bulky lesion recognition will facilitate further molecular analyses to understand how the individual repair subunits assemble on substrate molecules to form the active excision machinery.

Experimental Procedures

Materials

Building blocks for oligonucleotide synthesis, containing a C4′ pivaloyl substitution or a pivaloyl-silyl linker, were synthesized as previously described [26]. Oligonucleotides containing base analogs or a C_3 spacer were purchased from MedProbe (Lund, Sweden). [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq) was from ICN (Costa Mesa, CA). T4 polynucleotide kinase and DNA ligase were from Invitrogen (Paisley, UK). A site-directed AAF-dG adduct was produced by reacting the oligonucleotide 5′-ACCACCCTTCGAACCA CAC-3′ with *N*-acetoxy-2-acetylaminofluorene (National Cancer Institute Chemical Carcinogen Reference Standard Repository) as outlined previously [37]. The resulting AAF-modified oligonucleotides were isolated by electroelution from a 20% polyacrylamide gel. The oligonucleotides 5′-CCATCGCTACC-3′, containing B[*a*]P adducts, were a generous gift from Dr. N.E. Geacintov (New York University). Human recombinant XPC protein was expressed in sf9 cells and purified as a fusion construct with maltose binding protein, as previously described [38].

Substrates

The following oligomers were synthesized by the cyanoethyl phosphoramidite method: 5′-ACCACCCTT^{9}CGAACCACAC-3′, containing a C4′ pivaloyl adduct at the position T^{9}; 5′-GCTCGTGTG GTTCGT^{15}AGGGTGGTTCAG-3′, containing a C4′ pivaloyl adduct at the position T^{15}; and 5′-GCTCGTGTGGTTCGS^{15}AGGGTGGTT CAG-3′, containing a single silyl linker instead of the regular nucleotide at position 15. Internally labeled duplex DNA fragments of 141–147 base pairs were constructed by ligating 6 partially overlapping oligonucleotides as outlined previously [17, 18]. Prior to ligation, the central oligonucleotide in one of the two strands was 5′ end-labeled by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase. Sequence heterologies were generated by the addition, replacement, or deletion of bases in the unmodified strand.

Cell-Free Extracts and Repair Assay

Soluble extracts [27] were prepared from HeLa cells and the human lymphoid cell lines GM2250 and GM2634 were derived from XP-A and XP-C patients, respectively [20]. Excision assays were performed by incubating cell extract proteins (50 μg) with internally labeled DNA fragments (5 fmol, 75,000 dpm) at 30°C [17, 28]. The resulting excision products were resolved on 10% polyacrylamide denaturing gels, visualized by autoradiography, and subsequently analyzed by laser scanning densitometry (Imagequant software, Molecular Dynamics, Sunnyvale, CA). NER activity is expressed as the percentage of excised products in the size range of 24–32 nt relative to the total amount of substrate molecules.

Gel Mobility Shift Assay

Radiolabeled DNA fragments of 147 base pairs (1 fmol) were incubated with XPC fusion protein (20–80 fmol) in reactions of 10 μl containing 40 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, and 100 ng duplex poly[dI·dC]. After incubations of 30 min at 30°C, the bound fractions of DNA were separated from free substrates by electrophoresis on native 5% polyacrylamide gels, as previously described [33].

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