Long-range oxidative damage to DNA: effects of distance and sequence

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Introduction: Oxidative damage to DNA *in vivo* can lead to mutations and cancer. DNA damage and repair studies have not yet revealed whether permanent oxidative lesions are generated by charges migrating over long distances. Both photoexcited *Rh(III) and ground-state Ru(III) intercalators were previously shown to oxidize guanine bases from a remote site in oligonucleotide duplexes by DNA-mediated electron transfer. Here we examine much longer charge-transport distances and explore the sensitivity of the reaction to intervening sequences.

Results: Oxidative damage was examined in a series of DNA duplexes containing a pendant intercalating photooxidant. These studies revealed a shallow dependence on distance and no dependence on the phasing orientation of the oxidant relative to the site of damage, 5′-GG-3′. The intervening DNA sequence has a significant effect on the yield of guanine oxidation, however. Oxidation through multiple 5′-TA-3′ steps is substantially diminished compared to through other base steps. We observed intraduplex guanine oxidation by tethered *Rh(III) and Ru(III) over a distance of 200 Å. The distribution of oxidized guanine varied as a function of temperature between 5 and 35°C, with an increase in the proportion of long-range damage (> 100 Å) occurring at higher temperatures.

Conclusions: Guanines are oxidized as a result of DNA-mediated charge transport over significant distances (e.g. 200 Å). Although long-range charge transfer is dependent on distance, it appears to be modulated by intervening sequence and sequence-dependent dynamics. These discoveries hold important implications with respect to DNA damage *in vivo*.

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Introduction

Radicals generated by exogenous chemicals, radiation and metabolic waste products such as peroxides can cause severe damage to DNA by generating base and sugar modifications, as well as strand breaks and DNA–protein cross-links. Although much of the damage can be reversed by the repair mechanisms present in both eukaryotic and prokaryotic cells, some of the damage that escapes the repair machinery can persist and lead to mutations, carcinogenesis or cell death [1].

The role that electron transfer through the DNA π stack plays in radical-induced oxidative damage still needs to be established. Radiation biologists have used both γ irradiation [2–9] and high-energy ultraviolet light [10–14] to study the formation of nucleobase radicals on DNA. Both γ irradiation and high-energy light are capable of generating holes in any of the bases, but in fact both result in reactions primarily at guanine residues, suggesting that some charge migration might occur. DNA-bound radical traps have also been proven to be useful for the study of charge mobility [15–19]. In these studies, which have been performed under a variety of conditions (i.e. frozen, dehydrated or aqueous), the estimates of the distances of charge migration have ranged from 2–200 base pairs (bp).

The photochemical oxidation of DNA in solution at ambient temperatures has been particularly valuable in establishing long-range oxidative DNA damage. It was first observed that a variety of potent photooxidants, including substituted anthraquinones [20–22], ethidium bromide with methyl viologen [23], napthalimide derivatives [24] and riboflavin [25], all yielded oxidative guanine products at the 5′-G of 5′-GG-3′ sites even though these species bound DNA without sequence specificity. It is understandable that guanine is damaged preferentially because it has the lowest oxidation potential of the four bases: the oxidation potential of the dG nucleoside as measured by pulse radiolysis is 1.29V, lower than that of dA (1.42V), dC (1.6V) and dT (1.7V) [26]. *Ab initio* molecular orbital calculations indicate that for the stacked guanine doublet, the bulk of the highest occupied molecular orbital (HOMO) is located on the 5′ guanine, and therefore the 5′ guanine may be more easily oxidized than the 3′ guanine [27]. On the basis of empirical as well as theoretical findings, therefore, reaction at the 5′-G of

guanine doublet sites has become a signature for oxidative DNA base chemistry.

Oxidation of guanine generally results in the formation of 7,8-dihydro-8-oxoguanine (8-oxoG) through a reaction of the cation-radical intermediate with water, although imidazolone and oxazolone products might also be formed by alternate pathways [28,29]. 8-oxoG itself appears to be only somewhat piperidine labile, but the products of further oxidation of 8-oxoG by dioxygen or other available oxidants are highly piperidine labile, as are the imidazolone and oxazolone lesions [30,31]. The second oxidation of 8-oxoG to a piperidine-labile guanine lesion is facile because of the low oxidation potential of 8-oxoG [29]. Moreover, according to molecular orbital calculations, this low redox potential might be further diminished by stacking with a 3′ guanine residue, so that 5′-8-oxoGG-3′ sequences can be easily converted to piperidine-sensitive lesions [32]. The first one-electron-oxidized guanine radical intermediate that is formed by photooxidation has been observed directly by transient absorption spectroscopy; the transient spectrum obtained for a Ru(III) intercalator bound to poly d(GC) closely matches the spectrum of the neutral (deprotonated) radical of the guanosine and guanine monophosphate, as measured by pulse radiolysis [33].

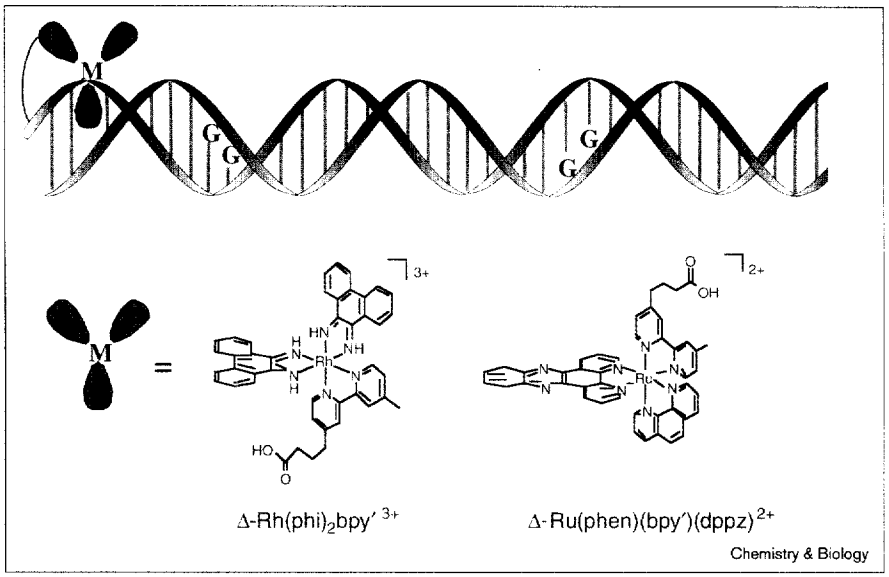
Oxidative damage to DNA from a distance was demonstrated first in an oligonucleotide assembly constructed so as to contain two 5′-GG-3′ sites spatially separated from the photooxidant, a tethered rhodium intercalator [34]. Phenanthrenequinone diimine (phi) complexes of rhodium had been shown to bind DNA avidly by intercalation [35]. Upon photoactivation at high energy (313 nm), the metallointercalator promotes direct strand cleavage of DNA, whereas with lower energy irradiation (365 nm) photooxidation of 5′-GG-3′ sites results in piperidine-sensitive DNA lesions [34,36]. Importantly, in the DNA assemblies prepared, it was shown that the 5′-GG-3′ sites were oxidized even when the rhodium complex was covalently tethered to one end of the DNA assembly at a distance of 10 or more base pairs away, corresponding to a distance for charge migration of 34 Å. The oxidation products produced by this long-range charge migration from guanine to photoexcited rhodium were piperidine-sensitive and included 8-oxoG. Moreover, in a rhodium-modified DNA assembly containing two 5′-GG-3′ sites, slightly greater oxidative damage was observed at the distal (34 Å) than the proximal site (17 Å), suggesting that, over this distance range, the yield of oxidative damage might be relatively insensitive to the distance of charge migration. This long-range reaction was, however, found to be sensitive to the stacking of the intercalator into the helix, as the right-handed (Δ) isomer, which fits more deeply into the major groove of a right-handed DNA helix, generates more oxidized product than does the left-handed (Λ) isomer. Long-range oxidation is also sensitive to disturbances in the stacking generated by intervening bulges in DNA between the oxidant and the 5′-GG-3′ site [37]; large bulges that do not stack well into the helix (for example, the 5′-ATA-3′ bulge) were shown to diminish the distal oxidation significantly.

Long-range charge-transfer chemistry in DNA appears to be general and has now been demonstrated with a range of tethered oxidants. A tethered, intercalating ground-state ruthenium (III) species generated *in situ* was shown to oxidize guanine doublets over a comparable distance [38]. Moreover, when the single guanine doublet was replaced by a 5′-GC-3′ sequence, damage was observed essentially equally at all the guanine sites. This observation graphically underscored the notion that the radical could equilibrate over sites of equal oxidation potential on a time scale that was fast compared to the irreversible trapping reaction, which occurs on the μs to ms time scale. Analogous long-range piperidine-sensitive damage to guanine and 8-oxoG was subsequently observed by photoexcitation of covalently bound anthraquinones, associated in a capped position on the duplex [20]. Most recently, long-range oxidative damage to DNA has been established by photooxidation of tethered ethidium, the classic organic intercalator [39], as well by a uridine base modified with a cyano-benzophenone derivative [40]. It is now, therefore, clear that oxidative damage to DNA can result, generally, through DNA-mediated electron transport, yielding oxidation from a distance. Moreover, this long-range chemistry is not simply a function of the oxidant employed, but instead represents a unique feature of the DNA itself.

Given that charge migration can occur through DNA, it now becomes critical to establish those factors that control DNA-mediated charge transfer and ultimately lead to permanent base lesions. The distances over which charge transfer between guanine and an intercalator had been observed previously were between 5 and 15 bp (17–51 Å), which are on the order of a single protein-binding site. It becomes more interesting to examine whether charge transfer through DNA occurs over still longer distances, corresponding to promoter regions, or perhaps through whole genes. In this distance regime, it is also interesting to consider various damage-detection and signaling mechanisms occurring through DNA-mediated charge transfer as a means to transmit chemical information over long molecular distances. Furthermore, in long pieces of DNA containing more than just a few intervening base pairs, issues of the sequence dependence upon charge transfer become much more important. Some sequences might better mediate charge transport than others, based upon their redox potentials and base-stacking characteristics, so that we might envision traps, blocks and bumps, as well as regions of smooth sailing, along the electronic DNA 'pi-way'. We have therefore begun to address the dependence on distance and sequence of DNA-mediated electron

Figure 1

A schematic structure of metal-conjugated oligonucleotide duplex assemblies. $Rh(phi)_2bpy'^{3+}$ and $Ru(phen)(bpy')(dppz)^{2+}$ were attached to the 5′ end of one strand of the duplex by a nine-carbon linker from the appended arm on the bpy′ ligand (phi, phenanthrene diimine quinone; bpy′, 4-butyric acid-4′methylbipyridine; phen, phenanthrene; dppz, dipyridophenazine). Each assembly contained a tethered intercalating oxidant, as well as spatially separated 5′-GG-3′ sites on the complementary strand for oxidative damage.



transfer from guanine to an intercalated photoxidant using metallointercalator-conjugated oligonucleotides (Figure 1).

Results and discussion

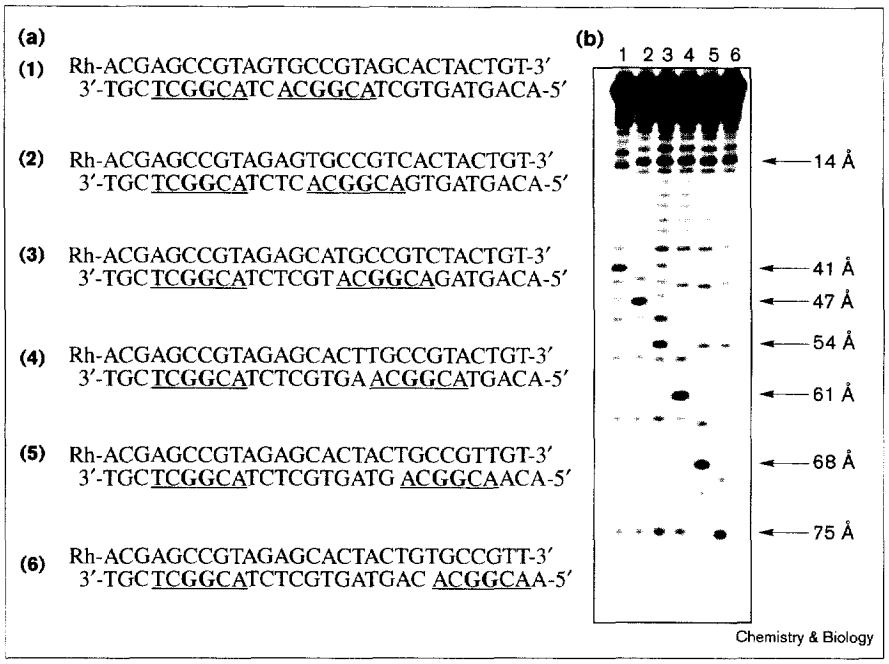
Systematic variation of the intercalator–GG distance

A series of six 28 bp DNA duplexes with $Rh(phi)_2(bpy')^{3+}$ covalently tethered to one end was synthesized (Figure 2). Each contained two 5′-GG-3′ sites of potential oxidation. The location and sequence context of the 5′-GG-3′ site proximal to the tethered intercalator were not changed in order to facilitate quantitative comparison among the six duplexes, whereas the distal site was moved systematically away in 2 bp increments from the rhodium-modified end of the duplex. Because the DNA bases flanking the 5′-GG-3′ sites can influence the oxidation potential and hence the yield of oxidative damage, the two flanking base pairs on either side of both 5′-GG-3′ sites were also kept constant. Additionally, to keep the six duplexes as similar as possible, the sequence composition was identical; the only change was the location of the six base pairs containing the distal 5′-GG-3′.

The yield of piperidine-sensitive lesions in each of the six duplexes following 365 nm irradiation was determined using gel electrophoresis and phosphorimagery (Figure 2). It is clear from the gel that the yield of oxidative damage at the distal site does not change significantly with increasing separation. In order to account for experimental variation that affects the total amount of oxidation from sample to sample, for example gel loading, the piperidine-sensitive cleavage at the distal 5′-GG-3′ site was normalized to that at the proximal site. Over the series of duplexes, the ratio of distal to proximal oxidative damage is only slightly diminished over a variation in distance separating oxidant and 5′-GG-3′ doublet from 41 to 75 Å along the helical axis (Figure 3).

One goal in studying the distance dependence of oxidation in 2 bp increments was to determine if DNA helical phasing influenced the yield of oxidative damage. It was possible that the relative orientation of the dipoles on the base and the intercalating ligand or the through-space distance between the edge of the base and the intercalator might affect long-range charge transfer. By moving the 5′-GG-3′ site out from the intercalation site by 2 bp increments along approximately one turn of the helix, we could determine whether oxidation was dependent upon the 5′-GG-3′ site being stacked on a particular side of the helix in relation to the intercalator. (One turn of the helix in B-form DNA contains 10.4 bp and is approximately 35 Å long.) As is evident from Figures 2 and 3, phasing did not appear to be a significant factor. For example, in duplex 2 the distal and proximal sites were 10 bp apart and were therefore on the same face of the DNA helix, whereas in duplex 5 they were 16 bp apart and on opposite sides. Despite these differences in phasing, the oxidation ratios observed in duplexes 2 and 5 were the same, within experimental error. The same argument holds for duplexes 1 and 4 and duplexes 3 and 6. In fact, the levels of oxidation appeared the most dissimilar for the set of guanine doublets that are close, namely duplexes 3 and 4. Therefore the position of the intercalator relative to the guanine doublet did not measurably affect the yield of oxidized guanine. This result suggests that rather than a single, concerted long-range transfer event occurs that might be dependent upon phasing of donor–acceptor, the hole is first introduced

Figure 2



The distance dependence of long-range oxidative DNA damage tested in a series of Rh-modified DNA duplexes. **(a)** Each duplex contained two 5′-GG-3′ sites of preferential oxidation. The distance to the proximal site was identical for each of the six duplexes, whereas the distance to the distal site was increased in 2 bp increments. The two base pairs on either side of the double guanine sites were kept constant as indicated by the underlined sequences. **(b)** Rhodium-induced DNA oxidative damage as revealed by polyacrylamide gel electrophoresis is shown, where the numbers above the lanes correspond to the sequence numbers in (a). Samples were irradiated with 365 nm light for 1 h and then treated with piperidine to reveal sites of guanine modification after denaturing polyacrylamide gel electrophoresis. The 5′-guanines of the 5′-GG-3′ sites are indicated with arrows. Because the strands containing the 5′-GG-3′sites were ^{32}P-labeled at the 5′ end, the invariant proximal site is nearest to the top of the gel (14 Å) and the distal site (varied between 41 and 75 Å) is near the middle and bottom. The following concentrations were used: 20 µl samples contained 8 µM Rh-DNA, 15 mM sodium phosphate buffer, pH = 7, and 50 mM NaCl.

rapidly into the DNA bridge, and it is the characteristics of the bridge that determines the yield of reaction.

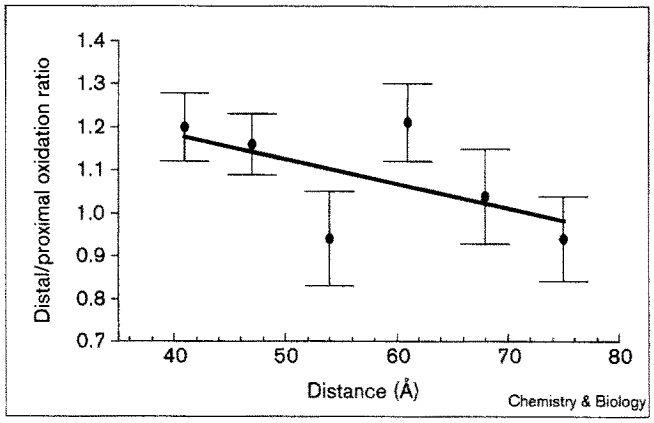
The effect of intervening DNA sequence on hole migration

We considered that the small but reproducible changes in yield for the set of duplexes described above might arise from differences in the intervening sequence between each set of 5′-GG-3′ doublets. In order to examine this sequence effect more carefully, more dramatic changes in the intervening sequence were introduced. Work in our laboratory had suggested that TA-rich sequences, and 5′-TATA-3′ specifically, might be particularly disruptive to long-range hole migration (M.E.N., D.B.H. and S. Rajski, unpublished observations). As shown in Table 1, a single G–C to T–A base-pair mutation in duplex 4 resulted in the formation of a 5′-TATA-3′ site between the proximal and distal 5′-GG-3′ sites. Duplexes with an intervening 5′-TATATA-3′ or 5′-TTTA-3′ site were also made by only slight alterations in sequence. The investigation of the oxidation in these sequences revealed that slight changes in sequence had a surprisingly large effect on charge transfer through the helix (Table 1). The single base-pair change to create a 5′-TATA-3′ site reduced the ratio of distal to proximal oxidation by ~40%, from 1.21 to 0.75 (Table 1). The TAAA-containing duplex, like the TATA-containing duplex, was constructed by a single base-pair change, but oxidation at the distal site was essentially unchanged by this substitution. Surprisingly, changing the intervening sequence to a 5′-TATATA-3′ did not have a major inhibitory effect upon long-range charge transfer through the helix, as charge transfer through this sequence was only slightly diminished compared with the parent duplex.

To explore the observation that sequences containing multiple 5′-TA-3′ steps were poor media for hole transfer through DNA, we also constructed duplexes containing three intervening 5′-AT-3′, 5′-TA-3′, or alternating 5′-TT-3′ and 5′-AA-3′ sequences that are not immediately adjacent to one another (Table 2). A significant decrease in long-range oxidation through the sequence that contained three 5′-TA-3′ steps was reproducibly demonstrated compared with distal oxidation through the other base sequences. These results confirmed that the presence of multiple 5′-TA-3′ steps reduced electron transfer through the DNA helix, dependent on the phasing and context of these steps. Because all of the sequences have intervening A–T base pairs, these results also confirm that the diminution in oxidation is not due to the absence of intervening guanine 'stepping stones' in an charge-transfer relay, but instead is due to the stacking of base pairs on their neighbors [41].

The intervening DNA sequence therefore influenced the migration of holes through the DNA helix. Intervening sequences containing multiple 5′-TA-3′ steps appeared to be especially poor media for electron transfer. It has long been known that there is very little overlap between bases

Figure 3



A plot of the ratio of distal to proximal guanine damage as a function of distance separating the tethered rhodium oxidant from the 5′-G of the guanine doublet. The mean yield of distal damage relative to the proximal damage is shown with error bars that represent the standard deviation based upon at least four trials using the assemblies shown in Figure 2. A very shallow distance dependence was observed over a range of separations of 34 Å. The long-range oxidative DNA damage also showed no apparent dependence on phasing along the DNA of the oxidized base with respect to the metal complex. The differences between the damage yields at individual guanine doublet separations and the best fit line probably reflect sequence-dependent effects on charge transfer through the helix (for more details, please see text).

Table 1

Dependence of long-range oxidation on intervening sequence.

	Sequence	Distal/proximal oxidation*
(4)	Rh-ACGAGCCGTAGAGCACTTGCCGTACTGT-3′ 3′-TGCTCGGCATCTCGTGAACGGCATGACA-5′	1.21 ± 0.09
	Rh-ACGAGCCGTATAGCACTTGCCGTACTGT-3′ 3′-TGCTCGGCATATCGTGAACGGCATGACA-5′	0.75 ± 0.06
	Rh-ACGAGCCGTAAAGCACTTGCCGTACTGT-3′ 3′-TGCTCGGCATTTCGTGAACGGCATGACA-5′	1.17 ± 0.14
	Rh-ACGAGCCGTATATAGCTTGCCGTACTGT-3′ 3′-TGCTCGGCATATATCGAACGGCATGACA-5′	1.06 ± 0.09

Samples representing variants of sequence 4 were prepared, irradiated and analyzed using PAGE as described in the Materials and methods section. *The ratio of the percent cleavage at the distal guanine doublet to that at the proximal doublet was determined using phosphorimagery. Shown are the mean and standard deviation of the distal/proximal ratio based on at least four trials.

at 5′-pyrimidine–purine-3′ steps in B-form DNA [42]. As a result, these steps are especially susceptible to bending and can act as a hinge or kink, as seen in the TATA-binding protein site, for example [43]. We might, therefore, expect 5′-TA-3′ (but not 5′-AT-3′) steps to act as poor conduits for charge transfer through the helix, either due simply to poor π–π orbital overlap between the thymine and adenine, or due to an induced kink in the DNA that interrupts hole transfer through the π stack. If the 5′-TA-3′ step induces a kink in the DNA, multiple 5′-TA-3′ steps that are in phase or out of phase with each other might interact constructively, generating a larger overall bend in the DNA, or destructively, canceling out the effect of the others and generating straight DNA. Although it is difficult to rationalize why 5′-TATATA-3′ caused less of a reduction in distal oxidation than 5′-TATA-3′, the spacing, relative orientation and sequence context of the 5′-TA-3′ steps apparently influenced the overall stacking of the base pairs and long-range DNA damage.

Clearly, therefore, long-range charge transfer through DNA depends on sequence. These sequence-dependent effects could be a result of the differences in π-orbital overlap at various base steps, or due to static conformational differences (i.e. rolling, twisting and kinking) at particular base steps. Alternatively, the variations we observed could have been caused by sequence-dependent flexibilities (i.e. dynamic bending, twisting and base flipping). Although we have just begun to explore these parameters, charge transfer through DNA could provide a completely new means to gauge sequence-dependent effects on both static and dynamic changes in base stacking (see below).

Distance dependence of guanine oxidation on long DNA duplexes

We constructed a metallated 63 bp assembly containing several guanine doublets. These duplexes contained one of two tethered octahedral metallointercalators, $Rh(phi)_2bpy'^{3+}$ or $Ru(phen)(bpy')(dppz)^{2+}$ (dppz = dipyridophenazine; Figure 1). Excited-state rhodium(III) complexes

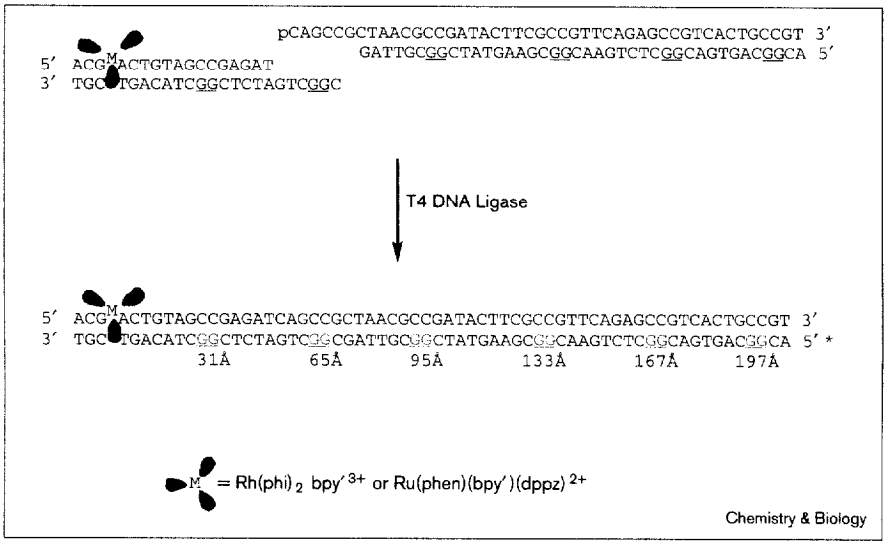
Table 2

Dependence of long-range oxidation on intervening TA base pairs.

Sequence	Distal/proximal oxidation*
Rh-ACGAGCCGTCATGATCATCTGCCGTTGT-3′ 3′-TGCTCGGCAGTACTAGTAGACGGCAACA-5′	0.97 ± 0.07
Rh-ACGAGCCGTCTTGAACTTGTGCCGTTGT-3′ 3′-TGCTCGGCAGAACTTGAACACGGCAACA-5′	1.05 ± 0.05
Rh-ACGAGCCGTCTAGTACTAGTGCCGTTGT-3′ 3′-TGCTCGGCAGATCATGATCACGGCAACA-5′	0.75 ± 0.01

Samples were prepared, irradiated and analyzed using PAGE as described in the Materials and methods section. *The ratio of the percent cleavage at the distal guanine doublet to that at the proximal doublet was determined using phosphorimagery. Shown are the mean and standard deviation of the proximal/distal ratio based on at least four trials.

Figure 4



Sequence and synthetic strategy for a metallointercalator-tethered 63-bp assembly. The duplex assembly contains a tethered rhodium or ruthenium octahedral intercalator (red) and six guanine doublets spaced approximately 10 bp apart (magenta). The tether for the metal complexes is as described in Figure 1. The duplex was assembled from smaller oligonucleotides prepared by solid-phase chemical synthesis, which were then enzymatically ligated using T4 DNA ligase. The 63-base strand containing the guanine doublets was 5′-end-labeled with ^{32}P at the farthest end of the duplex from the metal (denoted by *), so the oxidation products on the bottom of each gel were produced by oxidation at the furthest end of the duplex from the rhodium or ruthenium.

might serve as a model for oxidative damage *in vivo* by exogenous photosensitizers, whereas ground-state ruthenium(III) complexes could be considered analogous to solution-borne physiological oxidants such as the hydroxyl radical. Of the six 5′-GG-3′ sites contained in the assembly, the first 5′-GG-3′ site was located 9 bp from the proposed intercalation site of the tethered metal and each of the others were located roughly 10 bp apart at 31, 65, 95, 133, 167 and 197 Å separation from the intercalation site (Figure 4). Each guanine doublet was flanked by cytosine on the 3′ and 5′ sides.

The metallointercalator-bound 63-mer duplexes were synthesized in pieces and then ligated together. Metallointercalator-tethered oligonucleotides longer than ~35 bp cannot be synthesized directly in good yield and in pure form. Therefore a metallointercalator-tethered 17-bp oligonucleotide was ligated first to a 46-bp oligonucleotide using T4 DNA ligase in the presence of complementary oligonucleotides with sticky ends. The guanine doublets were situated on the strand complementary to the strand containing the tethered metallointercalator. Using a similar strategy, a metal-conjugated 46-bp assembly was also constructed with a sequence identical to the first 46 bases of the 63-mer from the metallated end.

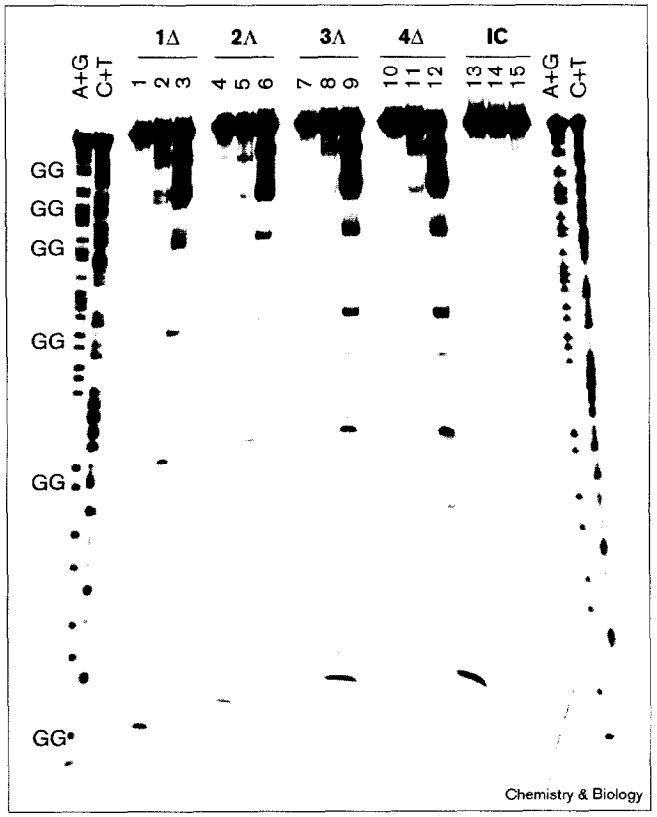
Long-distance oxidation by ruthenium(III)

A 63-mer containing the tethered ruthenium(II) intercalator was constructed to examine the distance over which guanine oxidation could be observed with the ground state oxidant. Here the ground state Ru(III) oxidant is generated by oxidative quenching of the photoexcited *Ru(II) by nonintercalating methyl viologen [33,38]. As can be seen in Figure 5, oxidation at the 5′ guanines of all six guanine doublets was observed after irradiation at 436 nm in the presence of methyl viologen. Hence, Ru(III) generated *in situ* can oxidize guanine doublets through almost 200 Å of the DNA π stack. It is noteworthy that long-range oxidation was only somewhat diminished with increasing distance over the range of separation of 100 to 200 Å from the intercalation site (see below).

To demonstrate that oxidation 200 Å from the intercalation site is generated by electron transfer through the helix, it was important to establish that the reaction was intraduplex. First we demonstrated that the ruthenium complex is intercalated at the end of the duplex to which it is tethered. Irradiation of the ruthenium complex without methyl viologen leads to the sensitization of singlet oxygen, which is reactive only with guanine residues in the immediate vicinity of the intercalation site, given the limited lifetime and rate of diffusion of $^1O_2$ [38]. When the ruthenium-tethered 63-bp assembly was irradiated without quencher, so as to generate damage with singlet oxygen, the damage was confined to one end of the duplex (Figure 5, lanes 2, 5, 8 and 11). This result was fully consistent with our assignment of the intercalation site to 2–3 bp from the end of the duplex based on molecular modeling. Low DNA concentrations were used in these experiments to disfavor interduplex intercalation. We expected metal intercalation to occur exclusively at the end of the duplex nearest the tether because of the rod-like biophysical behavior of short DNA oligonucleotides [44], and the singlet oxygen results supported the contention that the metallointercalator was bound only intraduplex.

The efficiency of guanine oxidation varied between all four diastereomeric metal-conjugated strands, with the 4Δ

Figure 5



Oxidation of the 5′ G of guanine doublets by ground-state Ru(III) observed over 197 Å. Illustrated here are the phosphorimagery results for metallated duplexes after photooxidation, piperidine treatment and elution through a 14% denaturing polyacrylamide gel. The duplexes were 5′-^{32}P-end labeled on the strand complementary to that containing the tethered metal. Each set of three lanes contains the following, respectively: a dark control (without irradiation); a ruthenium-tethered sample irradiated for 1 h without quencher, which marks the site of ruthenium intercalation by singlet oxygen chemistry; and a ruthenium-tethered duplex irradiated for 10 min in the presence of methyl viologen to generate Ru(II) by flash quench. All six guanine doublets were oxidized by the Ru(III) intercalator, with the largest amount of damage near the metallated end of the assembly (lanes 3, 6, 9 and 12). The majority of the singlet oxygen damage occurred at one end of the duplex (lanes 2, 5, 8 and 11), indicating that the metal was intercalated at the end of the duplex to which it was tethered. Note that singlet oxygen was also formed in the presence of quencher, but in very low yield on this time scale. The efficiency of oxidation depended on the diastereomer of the metal-conjugated strand. This order of reactivity, $4\Delta > 1\Delta \approx 3\Lambda > 2\Lambda$, agreed with previous studies indicating that the right-handed Δ isomers are better able to fit into the major groove and intercalate into the π stack. A mixture of assemblies containing those exclusively metallated but not radiolabeled, and those radiolabeled but lacking the tethered metal was irradiated to demonstrate that the reaction we observe occurs intraduplex. Lanes 13–15 containing the intermolecular control (IC) samples show that the long-range oxidation was not due to intermolecular intercalation of the metal complex nor to a diffusible reactive species. The 2Λ diastereomer was used in the intermolecular control samples. Irradiation conditions for all samples were 2 μM metal-tethered assembly, 75 mM Tris–HCl pH 8.3.

isomer oxidizing guanine most efficiently and the 2Λ isomer oxidizing guanine the least efficiently. This order of reactivity, $4\Delta > 1\Delta \approx 3\Lambda > 2\Lambda$, agreed with previous studies indicating that the right-handed Δ isomers are better able to fit into the major groove, intercalate into the π stack and oxidize nucleotide bases. In donor–acceptor systems that use ruthenium intercalators, the efficiency of quenching followed the same order as well [45].

In order to confirm more rigorously that the electron-transfer reaction occurred intraduplex, we also examined guanine oxidation of a radiolabeled 63-bp assembly bearing no conjugated metal complex in the presence of an assembly with the same DNA sequence and a conjugated ruthenium complex but no radioactive label. These samples (Figure 5, lanes 13–15) showed little oxidation above background, indicating that the ruthenium complexes did not intercalate into assemblies other than the ones to which they were tethered and that the reactive species that oxidizes guanine was not diffusible. Importantly, this result also indicated that the oxidation that we observe is not due to a direct reaction of the DNA with the light used to photoexcite the metal complex. Therefore, oxidation of 5′-GG-3′ sites in DNA by Ru(III) intercalators is mediated by the DNA duplex over a distance of 197 Å.

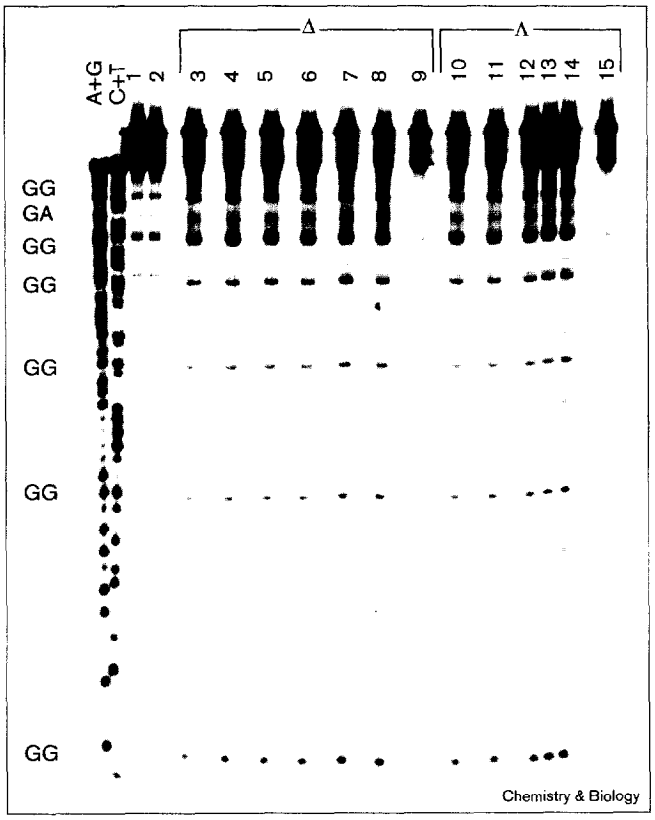
Long-distance oxidation by photoexcited rhodium(III)

A rhodium-tethered 63-bp assembly was also constructed in order to examine the distance over which oxidation by photoexcited rhodium can occur. With Rh(III), no diffusible quencher is required, as photoexcited *Rh(III) is a potent oxidant for DNA. This DNA assembly was identical to the ruthenium-tethered assembly except for the identity of the metallointercalator, which has not only a different metal center but different ligands and a higher redox potential (+1.6 V for Ru(III) versus +2.0 V for *Rh(III) [38,46,47]. Unlike Ru(III), which can oxidize only purines because of its potential, *Rh(III) can oxidize all of the DNA bases.

After irradiation of the rhodium-conjugated 63-bp assembly at 365 nm and treatment with aqueous piperidine, approximately equal oxidation by both the Λ and Δ isomers of rhodium was observed at all six guanine doublets, 31, 65, 95, 133, 167 and 197 Å away from the putative intercalation site of the metal complex (Figure 6). In addition to this guanine damage, some oxidative damage was observed also between the two proximal guanine doublets at a 5′-GA-3′ site, which *ab initio* molecular orbital calculations have indicated is the next most easily oxidized site after 5′-GG-3′ [27].

Irradiation of phi complexes of rhodium at 313 nm induces direct strand scission at the site of intercalation without piperidine treatment, providing an excellent marker for rhodium intercalation sites on DNA. Rhodium-tethered 63-bp assemblies were damaged extensively at the extreme 3′ end of the strand but only minimally at the guanine doublets when irradiated at 313 nm (Figure 6, lanes 1,2),

Figure 6



Oxidation of the 5′ G of guanine doublets by photoexcited $Rh(phi)_2bpy'^{3+}$ observed over 197 Å. Illustrated here are the phosphorimagery results for metallated duplexes after irradiation, piperidine treatment and separation in a 14% denaturing polyacrylamide gel. The duplexes were radioactively labeled on the nonmetallated strand. The metal was tethered to the far end of the assembly from the radioactive label (top of the gel). Lanes 1 and 2 show the direct photocleavage by the metal complex (Δ and Λ isomers, respectively) resulting from irradiation at 313 nm for 10 minutes. In these samples, which were not piperidine treated, almost all of the cleavage is observed at the end of the assembly closest to the metal. The samples in lanes 3–15 were irradiated at 365 nm and piperidine cleavage to reveal the sites of guanine damage. Lanes 3–9 show the long-range oxidation of guanine doublets by the Δ-rhodium diastereomer over a range of time points (60, 70, 80, 90, 100, 110 and 0 min, left to right), and lanes 10–15 show the same for Λ-rhodium diasteriomer (60, 70, 80, 90, 100 and 0 min, left to right). The efficiencies of oxidation by the Λ and Δ isomers were approximately the same, so in later experiments mixtures of both isomers were used. The oxidation at all of the guanine doublets increased as a function of irradiation time. Irradiation conditions for all of the samples were 2 μM metal-tethered assembly, 75 mM Tris–HCl pH 8.3.

demonstrating that the rhodium complexes were intercalated near one end of the duplex and not within neighboring duplexes nor into the far end of the duplexes to which they were tethered.

We considered that multiple cleavage events on the same DNA duplex would complicate analysis of the distribution of oxidation, and therefore we monitored the guanine oxidation at all of the doublets as a function of time. The amount of oxidation increased linearly as a function of irradiation time for all six of the guanine doublets, indicating that multiple cleavage events on the same strand did not occur over the range of irradiation times used in this experiment. The possibility of multiple oxidation events at a single site caused by some preferential oxidation of 8-oxo-G formed in a separate oxidation event cannot be completely ruled out [28–32]; this would require catalytic oxidation by the rhodium complex, however.

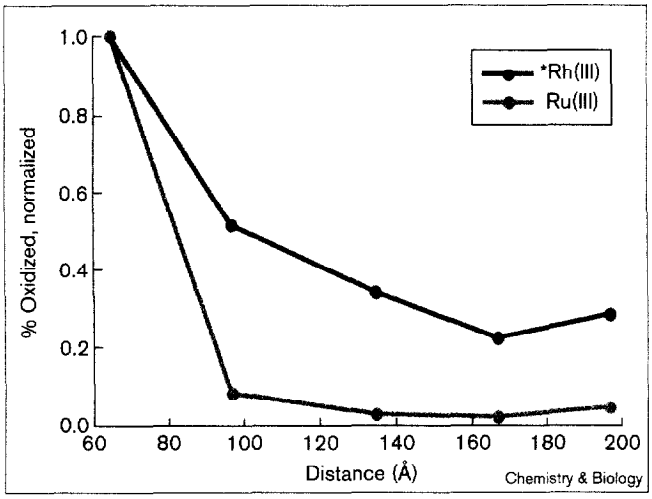
As in the case with the ruthenium-tethered assembly, the long-range guanine oxidation occurred intraduplex. When an assembly with a conjugated rhodium complex but no radioactive label was irradiated in the presence of an assembly with the same DNA sequence labeled at the 5′ end but bearing no metal, no oxidation was observed, which indicated that the reaction was intramolecular and therefore was mediated by the DNA duplex (data not shown).

Thus both excited-state rhodium and ground-state ruthenium intercalators can oxidize guanine bases through at least 200 Å of DNA. This overall distance for charge transport is not dissimilar to that found in the photosynthetic reaction center and across the mitochondrial electron-transport chain [48]. A distance of 200 Å through DNA furthermore corresponds to the distance between various conserved sites in a prokaryotic promoter, or approximately 20 amino acids worth of genetic code. Thus DNA may offer an important macromolecular medium for the transmission of chemical information over long molecular distances within biological systems. Whether charges migrate in DNA over longer distances corresponding to eukaryotic genes, regulatory regions or introns is a question that we are actively pursuing.

Comparison of oxidation by *Rh(III) and Ru(III)

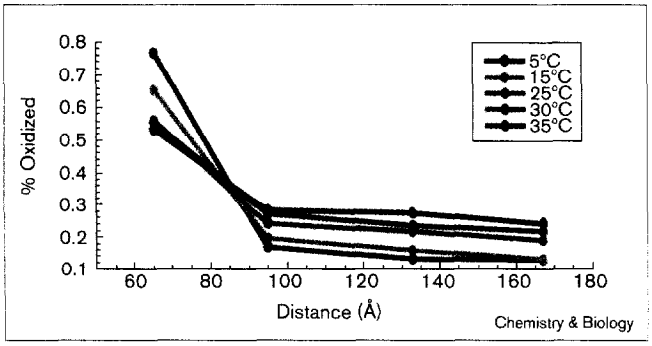
Both *Rh(III) and Ru(III) were both shown to oxidize DNA over at least 200 Å, and the patterns of oxidative damage by the two oxidants showed other similarities as well. Damage with both oxidants at the most distal guanine doublet was slightly higher than the oxidation at the two doublets that are located closer to the metal (Figure 7). Damage at the guanine doublet nearest the end of an assembly generally is higher than at more proximal doublets ([34], Figure 3), which might be due to better trapping of the guanine radical intermediate at the frayed ends of duplexes. Oxidation by both oxidants was strong in the 20–30 bp nearest the metal and dropped off gradually past that distance, a phenomenon that was not observed previously, as all of the duplexes studied were shorter than 30 bp in length. Interestingly, the distribution of oxidized guanines generated by *Rh(III) and Ru(III) as a function of distance displayed one interesting difference. Although oxidation decreased gradually with distance from the metal in

Figure 7



Plot of the distance dependence of long-range guanine oxidation by Ru(III) and *Rh(III). Ratios for the distal to proximal oxidation in the assemblies described in Figure 6 and Figure 7 were compared for the ruthenium- and rhodium-tethered assemblies. The ratios at 60 Å were used for normalization because the cleavage bands at shorter distances were intense and difficult to isolate for the purposes of quantitation. Oxidation of guanine by both tethered metallointercalators decreased as a function of distance. Interestingly, this distance dependence was more pronounced for ruthenium than for rhodium.

Figure 8



Temperature dependence of long-range guanine oxidation. The distance dependence of guanine oxidation by *Rh(III) decreased as a function of increasing temperature over the range of 5–35°C. Charge transfer was not affected by temperature at a point at which the lines cross, approximately 90 Å from the intercalation site of the metal.

assemblies containing either intercalator, the rhodium complex was better able to oxidize guanine at longer distances from the intercalation site than was ruthenium. The difference in distance dependence could be caused by any of several factors, including the difference in intercalating ligand, the ease of back-electron transfer from the metal, or the difference in the redox potential of the photooxidant. The latter possibility would be consistent with a 'hopping' model of charge transfer through DNA (see below) [49].

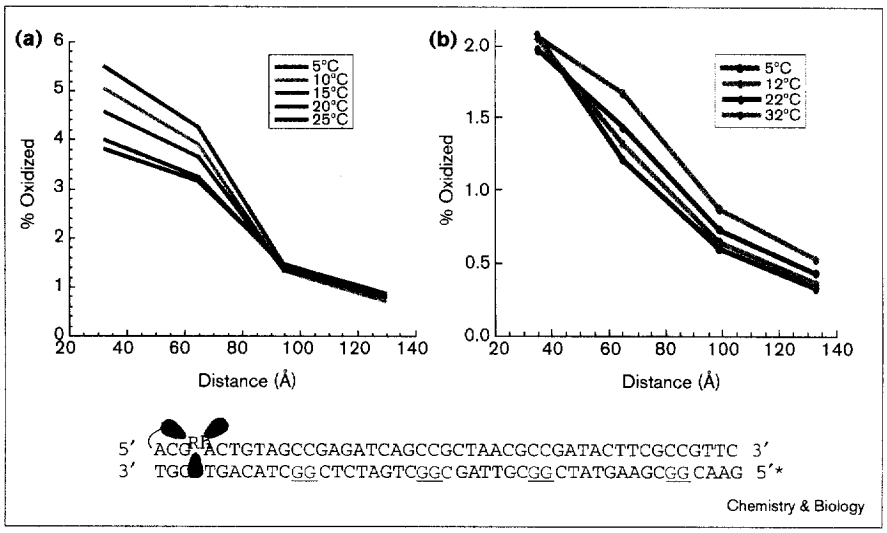
Temperature dependence of long-distance oxidation

The temperature dependence of guanine oxidation by rhodium over a range of physiologically relevant temperatures was also examined. Over this range of temperatures (5–35°C), a 63-bp duplex should remain fully stacked. In general, the total amount of damaged guanine generated by photo-excitation of rhodium increased slightly with increasing temperature, probably reflecting an increased intercalation over this range of temperatures. More interestingly, the distribution of oxidized guanine products changed with increasing temperatures. The amount of damaged guanine formed at the distal end of the duplex increased with increasing temperature, whereas the amount of damaged guanine formed at the proximal end of the duplex decreased (Figure 8). These two regions intersected at a distance of approximately 90 Å from the rhodium complex, where there was no change evident in oxidation over this temperature range. Although this crossover point corresponded to the center of the assembly, we determined that it was not location-specific but instead sequence-specific. The temperature dependence of oxidation in a 46-bp assembly (which was identical to the 63-bp assembly except for the last missing bases) contained a crossover point at the same sequence position, despite the fact that this point was no longer in the middle of the duplex (Figure 9a). Thus this crossover point did not represent a central node of an oscillating string model of DNA [50]. Interestingly, the sequence at this position contained one of only three 5′-TA-3′ sequences in the assembly.

Because 5′-TA-3′ is extremely flexible and frequently kinked due to the poor π overlap between bases [42], we speculated that the 5′-TA-3′ step might act as a temperature-sensitive 'hinge'. At low temperatures, the hinge would be bent, blocking hole transfer to the end of the helix, whereas at increased temperatures the hinge would become more flexible, acquiring other conformations that are more amenable to charge transfer. Fluorescence anisotropy studies have shown that DNA with adjacent mismatched bases is much more flexible than normally base-paired DNA [51], so an assembly was constructed with mismatched bases overlapping the 5′-TA-3′ step in order to change the shape and possibly the flexibility of this region of the assembly. In the presence of this double mismatch, the oxidation of all four guanine doublets increased with increasing temperature and the crossover point disappeared (Figure 9b). This result lends support to the idea that the 5′-TA-3′ step was responsible for the difference in charge distribution across the assembly as a function of temperature by 'gating' the movement of charge through the helix.

Our earlier studies had indicated that charge transfer through the DNA helix can be used to probe unusual

Figure 9



Temperature dependence of guanine oxidation by *Rh(III) in 46-bp assemblies **(a)** without and **(b)** with a pair of base mismatches. The sequence is shown below. (a) The distance dependence of guanine oxidation by *Rh(III) in shorter assemblies decreased as a function of increasing temperature between 5 and 35°C. Considering the possibility that the DNA might bend like an oscillating string with a node at the center, it was interesting that the 'crossover point' was located in the same place as in the longer assembly (Figure 8). (b) When a pair of mismatches were introduced in the center of the assembly, the crossover point disappeared or moved and the guanine oxidation increased as a function of temperature at all three distal guanine doublets.

DNA conformations in B-form Watson–Crick DNA, such as bulges and mismatches, in which the helical stacking is disrupted [37,38]. The dependence of long-range guanine oxidation upon dynamic changes in the bending and flexing of the DNA, now presents a new avenue for exploration of DNA-mediated electron transfer.

Mechanism of electron transfer through DNA

Despite the enormous body of experimental work relating directly or indirectly to electron transfer through DNA [52–63], we still lack an theoretical framework that describes all of the observed data. In fact, several mechanisms might operate in these different systems and over different distance regimes. The studies described here may be represented in the context of a 'hopping' model of charge transfer through DNA [49,64]. According to a hopping model, the electronic 'hole' diffuses along the helix by hopping from base to base until it is trapped by vibrational relaxation at a site with a low oxidation potential; in this case, for example, a 5′-GG-3′ site. These pairs of nucleobase stepping stones have to be electronically coupled, but they do not necessarily need to be adjacent. Indeed, we consider that hopping might even occur between base domains over which charge is delocalized, rather than between discrete base positions.

This general hopping mechanism could certainly be used to describe the data presented here. We might, for example, consider the dependence of long-range hole transfer on the identity of the intercalating metal. If we assume that the hole generated on DNA by *Rh(III) is more energetic than the hole generated by Ru(III), due to the difference in their reduction potentials (+1.6 V for Ru(III) versus +2.0 V for *Rh(III) [44–47]), we would expect that relaxation and trapping of the hole might occur more rapidly (and therefore closer to the intercalation site) for Ru(III) than for *Rh(III). According to this model, the *Rh(III)-generated hole would also be more mobile because it could hop between any two bases (or base domains), whereas the Ru(III)-generated hole could hop only from purine to purine due to its lower potential. The hopping mechanism might also explain the shallow decrease in guanine oxidation that is observed with increasing distance, as some decrease in oxidation with distance would be expected as long as diffusion competes with vibrational relaxation and trapping. The increase in very long-range (i.e. > 100 Å) charge transfer at higher temperatures also favors a hopping model because the electronic hole might use the extra thermal energy to offset vibrational relaxation and therefore be able to diffuse further along the helix before it is trapped. Although far from decisive proof, these data provide some indication that the movement of charge through the helix can be described, at least in part, in the context of a hole-hopping mechanism.

Significance

Charges generated on DNA by UV light, radicals, exogenous chemicals and γ irradiation frequently lead to irreversible damage. It is unclear whether these charges can migrate along the helix over long distances *in vivo*, and, if so, how far they can migrate before they are trapped as a permanent lesion. In this study we examined the distance and sequence dependence of long-range oxidative charge transfer through DNA. These data establish that charge migration can occur and promote damage over very long molecular distances. We showed that oxidative charge transfer has a very shallow dependence on distance. Guanine damage as a result of charge transfer is also not dependent on orientation or phasing along the helix relative to the metallointercalator. The

efficiency of long-range oxidation is sensitive to sequence, however, with multiple 5′-TA-3′ steps being especially poor media for charge transfer. Moreover, we have shown that significant electron transfer occurs through the base stack of DNA oligonucleotides over distances of at least 200 Å. Furthermore, investigations of the temperature dependence of guanine oxidation and of oxidation through disturbances in the π stack illustrate that charge transfer is also sensitive to dynamic disturbances in the helical stacking of B-form DNA. These discoveries have important implications for the study of DNA damage and repair in biological systems. Indeed they show that changes in DNA generated in one location by radicals and oxidants can potentially cause damage at other locations far away in the genome. The longest distance over which guanine oxidation by photo-induced charge transfer through the helix can occur still remains to be shown, as does the effect of DNA-binding by histones and other proteins upon charge transfer. It is possible that nature might use sequence-encoded DNA-mediated charge transfer as a means of signaling or, conversely, use sequence-dependent bending to generate 'traps' for migrating charges around sequences that would be otherwise sensitive to damage. Whether long-range electron-transfer reactions mediated by DNA occur in the cell and whether organisms have evolved a way to use electron transfer through the double helix to their advantage remains to be seen. Given the significant extent of DNA damage that can be generated through charge migration, as well as the ability to modulate such long-range damage by the intervening sequence, as seen here, it becomes important now to consider these potential reactions within the cell.

Methods and materials

Oligonucleotide preparation

Oligonucleotides were synthesized using phosphoramidite chemistry on an Applied Biosystems 394 DNA synthesizer with a dimethoxy trityl protective group on the 5′ end. Oligonucleotides were purified on a Rainin Dynamax C18 column by reversed-phase HPLC on a Waters HPLC (25 mM NH_4OAc, pH 7, 10–30% CH3CN over 30 min) and were deprotected by incubation in 80% acetic acid for 15 min. After deprotection, the oligonucleotides were purified again on the same C18 column by reversed-phase high performance liquid chromatography (HPLC) (25 mM NH_4OAc, pH 7, 5–20% CH_3CN over 30 min) and were quantitated by UV–visible absorption spectroscopy on a Beckman DU 7400 Spectrophotometer using the following extinction coefficients for single-stranded DNA: ε(260 nm, $M^{-1}cm^{-1}$) adenine (A) = 15,000; guanine (G) = 12,300; cytosine (C) = 7400; thymine (T) = 6700. Single strands were mixed with equimolar amounts of complementary strand and were annealed by gradual cooling from 95°C over 2 h. DNA strands containing a phosphate group at the 5′ end were prepared using the Chemical Phosphorylation Reagent (Glen Research) and were purified by HPLC without a dimethoxy trityl protective group.

Preparation of short metal-containing oligonucleotides

$Rh(phi)_2(bpy')^{3+}$ (bpy′ = 4-butyric acid-4′-methylbipyridine) and $Ru(phen)(bpy')(dppz)^{2+}$ were prepared according to published procedures [65,66]. Ruthenium and rhodium-tethered 17-bp oligonucleotides were prepared according to published procedures [34,67,68] and were purified on a Rainin Dynamax C4 column by reversed-phase HPLC on a Hewlett-Packard 1050 HPLC. The diastereomeric strands were isolated and numbered according to the order of elution, and absolute configuration around the metal center was determined by circular dichroism based on the stereochemistry of the metal center [69]. Purification of the rhodium-modified 28-mers was achieved by C18 reversed-phase HPLC at 65°C (25 mM NH_4OAc, pH 7, 0–15% CH_3CN over 30 min). A racemic mixture was used for all experiments with these constructs. The metal-conjugated oligonucleotides were quantitated using the following extinction coefficients: for Rh-modified oligonucleotides, ε(390 nm, $M^{-1}cm^{-1}$) = 19,000; for Ru-modified oligonucleotides, ε(440 nm, $M^{-1}cm^{-1}$) = 19,000.

Preparation of long metal-containing oligonucleotides by enzymatic ligation

Ligations were performed using high-concentration T4 DNA ligase (New England Biolabs) in T4 DNA ligase buffer specially prepared without DTT to maintain the coordination of the phi ligands. The metal-conjugated 63-mer was prepared by ligating a metal-conjugated 17-bp oligonucleotide to a 46-bp oligonucleotide bearing a phosphate on the 5′ end (Figure 4). First the 17-mer and the 46-mer were each annealed to their complementary oligonucleotides to create two duplexes with matching 6 bp sticky ends; then the two duplexes were mixed together with 10,000 units of high-concentration ligase and the solution was incubated at 14°C overnight. The final solution contained 5 nmoles of the metallated duplex and 6 nmoles of the phosphorylated duplex in a 350 μl volume. The metal-conjugated 63-mer was separated from the smaller pieces by denaturing polyacrylamide gel electrophoresis in a 14% polyacrylamide gel. The band containing the longest oligonucleotide was identified by UV shadowing, extracted from the gel, and ethanol precipitated. The metal-conjugated 46-mer was prepared in the same manner.

Irradiation of metal-containing oligonucleotides

Oligonucleotides were radioactively labeled by incubation with γ-^{32}P-ATP and T4 polynucleotide kinase [70]. The radiolabeled strands were purified by preparative gel electrophoresis in a 15% denaturing polyacrylamide gel, extracted and purified by elution from a Nensorb 20 cartridge. For the metal-conjugated 63-bp assemblies, the labeled strands were annealed with complementary unlabeled metallated single strands at a concentration of 2 μM (strands) in 75 mM Tris HCl (pH 8.0) as described above. For rhodium-containing 63-bp assemblies, 30 μl aliquots were irradiated at 365 nm for 80–90 min on a 1000 W Hanovia Hg-Xe arc lamp equipped with a monochromator. For ruthenium-containing 63-bp assemblies, 30 μl aliquots were irradiated at 436 nm for 8–12 min with 40 μM methyl viologen or for 1 h without quencher. Control aliquots were not irradiated. For the rhodium-conjugated 28-bp assemblies, the labeled strands were annealed with complementary cold metallated single strands at a concentration of 8 μM duplex in 15 mM sodium phosphate buffer (pH 7) and 50 mM NaCl. These 28-mer duplexes were annealed by gradual cooling from 90° to room temperature over 70 min. All samples were treated with 10% piperidine for 30 min at 90°C, lyophilized, then analyzed by denaturing PAGE in a 20% (28-mers) or 14% (63-mers) gel. Cleavage of the labeled strand was measured by phosphorimagery using ImageQuant, v3.3 (Molecular Dynamics). The level of oxidation at individual guanine bases was determined by measuring the intensity of the band corresponding to that base as a fraction of the intensity of the whole lane. The fractional intensity of the corresponding band in the control lane was subtracted out to account for background levels of damage.

Acknowledgements

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