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The Charge Conduction Properties of DNA Holliday Junctions Depend Critically on the Identity of the Tethered Photooxidant

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Abstract: The mechanism for electrical charge conduction in DNA has been the subject of much recent interest and debate. Many of the measurements of DNA conductivity have been made in aqueous solution, with an aromatic photooxidant moiety such as anthraquinone or a rhodium(III) complex covalently tethered to the DNA. Such studies, however, have given discrepant results, for instance, regarding the relative ability of AT- and GC-rich sequences to conduct charge and the possibility of thymine cyclobutane dimer repair through the DNA from a distance. A recent paper on conduction in DNA immobile four-way junctions using the rhodium photooxidant reported conduction in all four helical arms, contrary to what is known about the three-dimensional structure and stacking of 4-way junctions. We have reexamined conduction in such junctions using rhodium $[Rh(phi)_2(byp^*)Cl_3]$ as well as the anthraquinone photooxidants, and find that although our rhodium data agree with the previously published work, the anthraquinone data reveal conduction in only two of the four helical arms, consistent with the known tertiary structure of four-way junctions. An electrophoretic investigation revealed the formation of intermolecular aggregates in the rhodium-derivatized junctions, but not in the anthraquinone-labeled junctions. Rhodium-specific aggregation was also observed with simple DNA duplexes under the same experimental conditions. A characteristic property of aggregation was that all participating DNA molecules required the rhodium derivatization, and underivatized molecules did not aggregate with the derivatized ones. It is conceivable that the results reported here will help reconcile the various discrepancies that have been reported from charge conduction experiments carried out on DNA utilizing different photooxidants.

Introduction

The first conjectures on the potential of DNA to conduct electrical charge date back to nearly forty years ago.[1−2] However, the technologies appropriate to testing such hypotheses have not been available until the past decade. The first studies reported divergent conclusions about the efficiency, distance dependence, and rates of observed charge transfer through DNA double helices. A number of studies reported that the aromatic base stacks of a DNA duplex act as an efficient electrical conductor;[3−4] however, other reports concluded that duplex DNA behaved essentially like an insulator.[5−6] Such conflicting observations have stimulated much further study of charge transfer through DNA. To date, the wealth of experimental data recorded and evaluated have made it evident that, likely, more than a single mechanism exists for charge transfer through DNA. One possible mechanism is a long-range charge "hopping", whereby an electron hole migrates through the duplex using the oxidizable guanine bases as "steps"[7] or by a polaron like hopping mechanism.[8] A second prevalent mechanism, for rapid, short-range charge transfer, is the single-step superexchange mechanism, where the DNA behaves essentially as a molecular wire having a continuous molecular orbital.[9−10]

Beyond the issue of the precise mechanism(s) of charge transfer, a consensus has emerged that continuous base-stacking throughout a DNA duplex is important. Efficiency of charge transfer is observed to be lower in duplexes containing either mismatches[11−13] or bulges.[14] However, not all perturbations to the helix have prevented charge transfer, as has been noted in helices containing abasic sites[15] and short, single-stranded overhangs.[16] Nevertheless, even these latter structures have been

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thought to base-stack continuously, which permits charge transfer through them. A large proportion of the studies on charge transfer through DNA have been carried out using DNA constructs containing covalently attached chemical groups for initiating charge transfer. However, a few direct measurements of charge transfer have also been made. Measurements made on ~600 nm long "ropes" of intertwined, desiccated DNA,[17] and later, through individual DNA duplexes,[18] confirmed the conductive ability of DNA.

To date, detailed (i.e., nucleotide-resolution) examinations of conduction through DNA still rely significantly on the use of covalently tethered photooxidant moieties, such as anthraquinone or rhodium- or ruthenium-containing organometallic complexes. Such strategies permit the use of custom-designed DNA sequences and the precise placement upon the DNA duplex of the photooxidant moiety, such that significant control can be exercised on where charge transfer is initiated. The different chemical groups that have been used as photooxidants, along with the interpretations obtained using such groups are summarized in various review articles.[19−20]

Fundamental examinations of the conductive properties of DNA have recently expanded into two related areas of interest. One is the use of DNA to template other conducting materials at the nanometer scale and the other is the examination of conduction through DNA structures more complex than the conventional duplex (double helix). Thus, DNA has been used to template the formation of silver wires[21] and for the assembly of gold colloids.[22] A novel metal-DNA complex, known as M-DNA, has also been reported to act as an efficient electrical conductor.[23] Exploration of charge transfer in other DNA structures has included studies on DNA/RNA heteroduplexes,[24−25] Z-form DNA,[26] DNA triplexes,[27−28] DNA guanine quartets,[29] DNA aptamers,[30] and immobile four-way DNA Holliday junctions (4W-junctions).[31−32]

In this paper, we have examined charge transfer in immobile DNA 4W-junctions ("single crossovers") using two different appended photooxidant moieties, and, on the basis of the results obtained, we offer a reevaluation of results published by Barton and colleagues.[32] Figure 1 illustrates schematically the structure of an immobile DNA 4W-junction. Four DNA oligonucleotides with precisely tailored base complementarities are used to assemble these junctions. Under low Mg^{2+} concentrations (<100 μM),[33] the 4W-junction adopts an open conformation, in which no significant stacking occurs between the four duplex stems (Figure 1A). Upon the addition of a sufficient divalent (or other highly charged) cation concentration, the conformation of the 4W-junction compacts to a folded "X"-like shape in which the duplex arms pair up to form coaxial helical stacks (Figure 1B−E). The identity of the preferred stacking conformer (among those shown in Figure 1B−E) is determined primarily by the identity of the base pairs located at the junction.[34] The thermodynamically favored conformers are typically "antiparallel" (Figure 1D, E), but if their formation is prevented, the "parallel" isomers (Figures 1B, C) can also form.[35] Once formed, the two coaxial stacks in these X-shaped structures retain no inter-stack base−base interactions, confirmed by a 4W-junction crystal structure.[36] Consequently, it might be expected that no significant charge transfer could occur in these X-shaped structures from one helical stack to the other. As a corollary, it might be expected that in the low-salt, "open" conformation (Figure 1A), all four duplex stems would be electronically insulated from one another, whereas in the salt-compacted, X-shaped 4W-junctions, the two coaxial (and, conducting) helical stacks would be insulated from one another. Naturally, such an assumption requires that the folded conformers of a 4W-junction do not interconvert on the charge-transfer time scale. In many 4W-junctions (including the ones used in this study), one of the two conformers (shown in Figure 1D, E) is heavily predominant. NMR studies have shown that interconversion between the two conformational states occurs on the 0.1−0.5 s time scale, with specific lifetimes being dependent upon the sequence composition of a particular junction.[37−39] These interconversion rates are significantly slower than those determined for charge-transfer rates.[40] It is therefore reasonable to expect that charge-transfer experiments should reflect the conformational bias of a given immobile 4W-junction ("immobile" in this context does not refer to the interconversion of conformers, but rather that the location of the junction within each construct is stable).

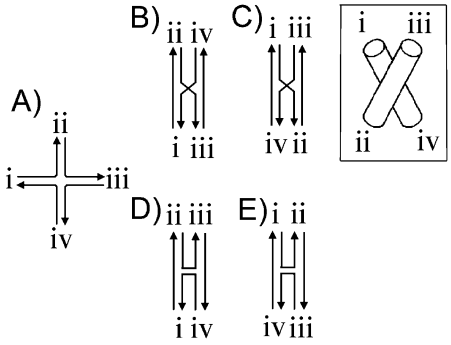

Figure 1. Models for the (A) unfolded and (B)−(E) folded states of immobile DNA 4W-junctions.

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The first report of charge transfer in a complex DNA structure was in fact in a DNA "double-crossover", which consists of two 4W-junctions fused end-to-end and contains, like the single-crossover 4W-junction, two separated helical stacks (double-crossover assemblies are reviewed in detail by Seeman[41]). In the double-crossover, the two coaxial stacks were found, predictably, to remain electronically insulated from one another.[31] However, a later report on charge transfer in isolated single-crossovers (4W-junctions) found what appeared to be charge transfer occurring down all four arms of a 4W-junction, in both the stacked and the unstacked conformations.[32] The data obtained in the presence of Mg^{2+} could be interpreted to indicate a slight preference for one of the two-stacked conformers; however, this slight preference was significantly lower than the much stronger preference for that conformer previously determined for this particular 4W-junction by NMR.[39] Thus, the charge transfer data appeared to contradict what was understood about the structure and dynamics (summarized above) of immobile 4W-junctions. The possibility that the charge transfer data reflected a very rapid conformational isomerization of this 4W-junction, on the time scale of charge transfer, appeared highly improbable, given that DNA conformational interconversion events are known to be very slow, as mentioned above, estimated to be in the millisecond to second time scale.[37−39,42]

We therefore undertook a reexamination of charge transfer through immobile DNA 4W-junctions, to obtain a clearer understanding of the process and to attempt to reconcile its apparent contradictions. To do this, we first examined whether the charge-transfer characteristics through DNA 4W-junctions depended on the *identity* of the photooxidant being employed. We therefore examined two distinct covalently tethered photooxidants: $Rh(phi)_2(byp^*)Cl_3$ [phi: 9,10 diaminophenanthrene; byp*: 4-(4-carboxybutyl)-4′-methyl-2,2′-bipyridine] (comparable to the rhodium complex in previous studies[32]), and, anthraquinone.

Materials and Methods

DNA Preparation. Unmodified DNA sequences were purchased from Sigma-Genosys and size-purified using PAGE before use. Sequences to be ^{32}P-end-labeled were pretreated with 10% piperidine (90 °C for 30 min, followed by lyophilization) prior to 5′-labeling with ^{32}P using standard kinasing protocols and PAGE purification. The pretreatment with hot aqueous piperidine cleaved those DNA molecules that contained base labile lesions created during chemical synthesis,[31] leading to lower background cleavages in photoirradiation experiments described in this paper.

DNA oligomers to be derivatized with anthraquinone (AQ) or the organometallic rhodium complex (Rh) were synthesized incorporating a commercially available 5′-C6-amino functionality, and were purchased from the University of Calgary Core DNA Services.

Prior to coupling, the DNA was treated to remove nitrogenous contaminants from the DNA synthesis procedures. The dried DNA samples were dissolved in 100 μL of ddH_2O, and were extracted three times with 100 μL of chloroform. The DNA remaining in the aqueous phase was then ethanol precipitated by the addition of 30 μL of 1M NaCl and 340 μL of 100% ethanol. Following mixing, the sample was chilled in dry ice for ∼10 min and then centrifuged for 20 min at 10 000 g to pellet the DNA. The pellet was washed once with 150 μL of 70% aqueous ethanol (v/v). Following air-drying, the pellet was dissolved in 100 μL of ddH_2O, and the DNA concentration of the solution was determined in a standard fashion using UV absorbance measurements (see below).

Modification of DNA with Anthraquinone. DNA with a 5′-amino modification was coupled to anthraquinone-2-carboxylic acid (Sigma-Aldrich). The N-hydroxysuccinimide (NHS) ester of anthraquinone-2-carboxylic (AQ-NHS) acid was prepared as previously reported.[43] The AQ-NHS ester was coupled to 5′amino-modified DNA oligomers, and the conjugates purified and quantified as previously described.[30]

Modification of DNA with $Rh(phi)_2(byp^*)Cl_3$. DNA with a 5′-amino modification was coupled to racemic $Rh(phi)_2(byp^*)Cl_3$ [phi: 9,10 diaminophenanthrene; byp*: 4-(4-carboxybutyl)-4′-methyl-2,2′-bipyridine]. The $RhCl_3$ was purchased from Pressure Chemicals and the phi ligand from Sigma Aldrich. The modified bipyridine ligand, byp*, was synthesized as mentioned below.

Synthesis of 4-(4-carboxybutyl)-4′-methyl-2,2′-bipyridine (byp*): The precursor to this compound, 4-(4-bromobutyl)-4′-methyl-2,2′-bypyridine, was synthesized as previously described.[44] This precursor was then converted to the ligand, byp*, as described by another report.[45]

Synthesis of $Rh(phi)_2(byp^*)Cl_3$ was accomplished in the same fashion as reported by Pyle et al.[46] for the synthesis of $Rh(phi)_2(byp)Cl_3$ [byp: bipyridine]. The purification protocol of $Rh(phi)_2(byp^*)Cl_3$ was modified somewhat from that described by Pyle et al.[46] Following synthesis of the $Rh(phi)_2(byp^*)Cl_3$ complex, the reaction mixture was dried under vacuum. The residual solid was then dissolved in a small volume of 1:1 water:acetonitrile and loaded onto a silica gel column. The column solvent was 1:1 200 mM NaCl:acetonitrile. Fractions containing the orange product were pooled and dried under vacuum. The resulting solid product and salt mixture were suspended in minimal water, and the product selectively precipitated by the addition of 10% (w/v) $NaBF_4$. The precipitate was collected by centrifugation then washed with water. The solid remaining was now dissolved in a minimal volume of acetone, and the product precipitated by the addition of 10% (w/v) tetraethylamine chloride (in acetone). The resultant product (a racemic mixture of the rhodium complex) was washed with acetone and dried. The product was assayed using electrospray MS, ^1H NMR, and absorption spectroscopy, to agreement with published data.[46] For coupling the $Rh(phi)_2(byp^*)Cl_3$ complex to DNA, stock solutions of the rhodium complex in ethanol were made and their concentrations determined by absorption spectroscopy, using extinction coefficients (ϵ) of 28 200 and 43 200 $M^{-1}cm^{-1}$ at pH 7.0 at 378 and 292 nm wavelengths, respectively.[46]

The procedure for covalently attaching the purified rhodium complex to the amino-modified DNA was as follows: To a 0.5 mL eppendorf tube was added 15 μL of 1 mM $Rh(phi)_2(byp^*)Cl_3$, 2.5 μL 1 M MES (pH 5.75), 2.5 μL *N*-hydroxysulfosuccinimide sodium salt (25 μg/μL in H_2O) and 2.5 μL of freshly dissolved 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (20 μg/μL in H_2O). The sample was incubated at room temperature for 10−15 min, and to it was then added 15 μg of the 5′-amino-DNA, as prepared above. Following the addition of DNA, a 2.5 μL portion of 50 mM $MgCl_2$ was added. The sample was then wrapped in aluminum foil and shaken overnight at room temperature, after which it was ethanol precipitated and then washed with 100% ethanol. The recovered pellet was dissolved in 25 μL of TE buffer (10 mM Tris, pH 7.8, 0.1 mM EDTA).

The derivatized DNA was now purified by reverse phase HPLC, using a C18 Deltapack column (Waters). The HPLC protocol was as follows: initial conditions were 100% solvent A [20:1 of 100 mM

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triethylamine acetate (pH 7.4):acetonitrile]. Over a 30 min period, with a linear gradient, the solvent was changed to 30% solvent B (acetonitrile). At all times, the column was heated to a temperature of 60 °C. After 30 min, the solvent was rapidly changed to 100% B and washed for 15 min to remove uncoupled $Rh(phi)_2(byp^*)Cl_3$ before reconditioning the column.

HPLC fractions containing the Rh-derivatized DNA were lyophilized, dissolved in 100 μL of 300 mM sodium acetate (pH 7.5) containing 10 μg of glycogen (as carrier), followed by ethanol precipitation. The recovered pellet was washed with 70% ethanol, dried, and finally dissolved in 75 μL TE buffer. The concentration of the sample was determined by measuring the aborbance at 390 nm. At this wavelength, the rhodium complex has an extinction coefficient of 19 000 $M^{-1}cm^{-1}$ when tethered to DNA.[47]

Duplex and 4W-Junction Assembly. The following steps were all carried out in the dark, whenever possible. A reaction solution (15 μL), containing each of the constituent DNA oligonucleotides (including an AQ- or Rh-derivatized oligonucleotide and a 5′ ^{32}P-labeled oligonucleotide) in 50 mM Tris-Cl (pH 8.0), 50 mM NaCl, and 0.1 mM EDTA, was heated to 85 °C for two min to denature the individual oligonucleotides. The solution was then allowed to cool slowly to room temperature. Following incubation at room temperature for 30 min, a 4 μL portion of a nondenaturing gel-loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 30% v/v glycerol in water) was added to the sample. The assembled 4W-junctions were then purified on a 7% nondenaturing polyacrylamide gel (containing and run in 50 mM Tris borate, pH 8.5, and 5 mM $MgCl_2$). The 4W-junction band(s) in the gel was visualized by autoradiography; the gel was excised and its DNA eluted into a solution of TNME buffer (50 mM Tris-Cl, pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM EDTA). The purified 4W-junction DNA was then concentrated using Microcon spin filters with a 10-kDa cutoff.

Photo-Irradiation and Guanine Oxidation Detection. DNA concentrations were adjusted to 0.3−0.5 μM DNA in TNME buffer. Thirty-μL aliquots were placed in siliconized 500 μL eppendorf tubes and placed under a UVP Black-Ray UVL-56 lamp (366 nm peak intensity, at 18 W) for 3.5 h. at a distance of 4 cm from the bulb. The temperature was maintained by having the samples tubes placed in a water bath set to the desired temperature. Various light sources (arc lamps and a nitrogen laser), as well as the use of band-pass filters (365 nm), were tested, but the best results were obtained with the abovementioned lamp, which gave identical results with or without the filter.

Following photoirradiation, samples were precipitated by the addition of 3.5 μL of 3 M sodium acetate (pH 7.5), 3 μL 100 mM EDTA, 2 μL glycogen (2 μg/μL), and 105 μL 100% ethanol. The resultant pellets were washed twice with 70% ice-cold ethanol, air-dried, and dissolved in 100 μL of 10% (v/v) piperidine. The solutions were then heated at 90 °C for 30 min, followed by lyophilization. The samples were then treated by two rounds of dissolving in 50 μL of water followed by drying under vacuum. Samples were finally dissolved in denaturing loading buffer and heated briefly to 95 °C prior to loading on a 12% sequencing gel [8.4 M urea, 50 mM Tris borate, pH 8.5, 0.1 mM EDTA (TBE)]. The gels were dried and DNA bands visualized using a Biorad GS-350 phosphorimager.

Direct Photolysis Experiments. Aliquots from stock solutions of gel-purified DNA samples (described above) were photoirradiated using a transilluminator with a peak wavelength of 312 nm. Samples were placed in a water bath during irradiation to maintain a constant temperature and a cutoff filter was used to remove light of $\lambda < 300$ nm. Samples were irradiated for 45 min, after which a 1/3 volume of denaturing loading buffer was added. The solution was mixed, and the samples were heated to 95 °C for 2 min prior to loading on a 12% sequencing gel (8.4 M urea, 0.5x TBE). Gel bands were visualized as above.

Figure 2. DNA constructs used in photoirradiation studies. All constructs utilized the same photooxidant-derivatized oligomer (1.2), which possess a 5′ amino functionality on a C6 linker. Roman numerals identify each individual arm of the immobile four-way junction (4W-junction).

Non-Denaturing Gel Electrophoresis to Examine DNA Aggregation. DNA samples where prepared as described as follows to test for DNA aggregation, using nondenaturing gel electrophoresis. Approximately equimolar amounts of each strand were combined in a 0.5 mL eppendorf tube; however, Rh− or AQ-modified strands were used at 5% lower concentrations relative to the concentrations of other strands to ensure that no free single strands with attached anthraquinone or rhodium complex functionalities persisted in the reaction mixture. Samples were made to final concentrations of 2 μM DNA (each strand) in 2× buffer (90 mM Tris-acetate, 2 mM EDTA, pH 7.5). The samples were heated to 90 °C for 2 min and then cooled to the target incubation temperature at a rate of 1 °C/min. After reaching the incubation temperature of 18 °C, the samples were diluted 2-fold with 10 mM $Mg(OAc)_2$, pH 7.5. The addition of the magnesium acetate was considered to be the initial time point for the reaction. At time points of 5 and 120 min, 6 μL aliquots of sample were removed and mixed with 1 μL of nondenaturing loading buffer. The samples were then immediately loaded onto an 8% nondenaturing polyacrylamide gels (0.5*x* TB, 2 mM $MgCl_2$, 0.1 mM EDTA) and run at 6 W (low enough to prevent any significant heating of the gel) at room temperature. Gels for these analyses were run under identical conditions, and for identical lengths of time, to control for variations in electrophoresis procedure.

For experiments using gel-purified DNA constructs, samples of 0.5 μM DNA in TNME buffer were incubated overnight at 12 °C, and then loaded on 8% nondenaturing gels as described above.

Results

The three DNA constructs designed for charge-transfer experiments are depicted in Figure 2. These include a simple duplex (dsDNA), a duplex containing two adenines in an internal bulge (dsDNA-B), and an immobile four-way junction (4W-junction #1). This 4W-junction is a CCTT type, as determined by the classification rules outlined by Altona.[48] The schematic of the 4W-junction I (Figure 2A) depicts its preferred stacked orientation, with Stems I and IV stacking preferentially on each other, and Stems II and III pairing up likewise. This particular (A/D) pairing of helical stems of a CCTT class of 4W-junction has been previously determined by Duckett et al.[34] For the sake of consistent comparison, the oligonucleotide (strand 1.2) possessing the 5′-amino modification to which the photooxidant

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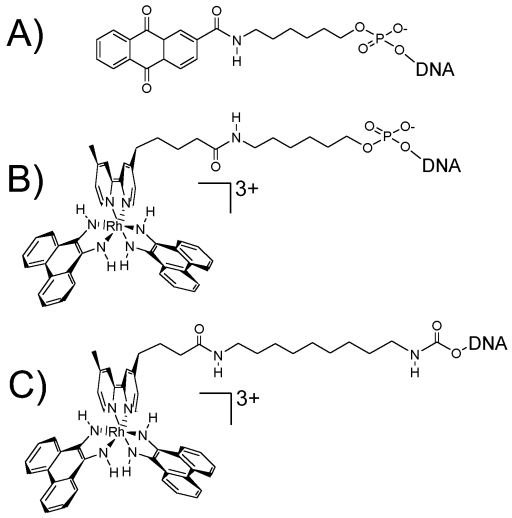

Figure 3. Photooxidants charge transport in the DNA constructs. (**A**) The anthraquinone modification. (**B**) The $Rh(phi)_2(byp^*)^{3+}$ modification. Only the Δ isomer is shown, but experimental protocols used a racemic mixture of both the Δ and Λ isomers. (**C**) The structure of an analogous rhodium complex used by Odom et al. (2001) in their report of charge transport through DNA immobile 4-way junctions.

was covalently tethered was used in all three DNA constructs. In all three constructs, too, pairs of contiguous guanines ("guanine doublets": 5′GG) were positioned strategically to aid in monitoring charge transfer from specific locations in the DNA. A sensitive way to monitor changes in electrical conductivity of DNA, at the level of individual nucleotides, is to use electrophoresis to monitor DNA strand cleavage resulting from base-labile oxidative damage suffered by individual guanines.[49] Guanine doublets allow a sensitive monitoring of charge transfer because of their higher reactivity (particularly that of the 5′ guanine of the doublet) in this respect compared to isolated guanines.[50−51]

All three DNA constructs were assembled with strand 1.2 being derivatized with one of two photooxidants. The first is an anthraquinone analogue (AQ—Figure 3A) and the second is a $Rh(phi)_2(byp^*)Cl_3$ (Rh—Figure 3B). For the sake of comparison, the rhodium complex ($Rh(phi)_2(byp')Cl_3$ [byp′: 4-(3-carboxypropyl)-4′- methyl-2,2′-bipyridine]) used in previous studies of charge transfer through immobile DNA junctions,[31−32] and for thymine dimer repair,[52−53] is also depicted (Figure 3C). The two rhodium complexes (Figure 3B,C) are very similar, with the main distinction being that our complex, used for this study (Figure 3B), had a tether that was 2 atoms shorter than that of the complex used by Barton and colleagues (Figure 3C). This reduction in tether length was not sufficiently different, however, to affect intercalation of our tethered Rh-complex into the end of the DNA duplex; a comparative study of tether lengths has shown that even a linker one atom shorter than the one used in this study is capable of intercalating comfortably into the end segment of the duplex.[54]

Anthraquinone- (AQ-) and rhodium complex- (Rh-) derivatized DNA constructs were assembled and gel purified as described in the Materials and Methods section. In the case of 4W-junctions, standard electrophoresis-based checks were carried out to ensure that they had assembled correctly.[56] Aliquots of the different constructs were photoirradiated for 3.5 h, at 10−12 °C, followed by treatment with hot piperidine (to cleave DNA strands at phosphodiesters immediately 3′ to photodamaged guanines[49]) and lyophilization, prior to analysis on 12% sequencing gels alongside nonirradiated, but piperidine-treated, control samples. Results of irradiation experiments on the Rh-derivatized DNA constructs are shown in Figure 4A and those on the AQ-derivatized constructs are shown in Figure 4B. Little difference was observed in the guanine-damage patterns of the double-helix (duplex) constructs, regardless of whether they were derivatized with Rh or AQ. Both duplexes exhibited significant cleavage at guanines throughout the duplex length (relative to damage at the same guanines in "dark", or nonirradiated, controls). This was an expected observation, given that both AQ and Rh had been shown previously to induce charge transfer within DNA duplexes at distances of >180 Å.[47,55] Our 30-base pair duplex only measured ∼100 Å from end to end, by comparison.

A second set of comparisons could to be made between the AQ- and Rh-derivatized *bulged* duplexes (labeled as "dsDNA-B" in Figure 4A,B). Guanine-damage patterns were somewhat different between the two: In the AQ-dsDNA-B, a reduction in cleavage levels was observed at the guanines distal (with respect to the site of AQ-modification) to the bulge (the position of the bulge is indicated by brackets in Figure 4. By contrast, the Rh-dsDNA-B duplexes exhibited a more uniform cleavage on either side of the bulge. In other words, the AQ-dsDNA-B appeared to be more responsive to the presence of the bulge as a disruption to base-stacking, and hence, to charge transfer.

Comparison of guanine cleavage in the 4W-junction constructs highlights significant differences, depending on whether the DNA is derivatized with Rh or with AQ. With strand 4.1 (see Figure 4D) ^{32}P-labeled at its 5′ end, cleavage was observed throughout the strand for both types of derivatizations (Figure 4A,B), whereas in experiments with strand 2.3 5′-labeled, high levels of cleavage were observed with the Rh-modified 4W-junction (Figure 4A), but not with the AQ-modified 4W-junction (where a low degree of strand cleavage, barely detectable over those in the "dark", nonirradiated controls were seen—Figure 4B).

To confirm that the charge migration in the AQ- modified 4W-junction remained localized to the coaxial stack of Stems I and IV, a second series of experiments were carried out. Photoirradiation experiments were conducted as described above, but with strand 3.4 5′-labeled with ^{32}P. This strand bridges Stems III and IV; therefore, it could be predicted that piperidine-dependent cleavage would be observed only in the 3′ half of this sequence (when AQ was the photooxidant). Figure 4C shows the results of photoirradiation experiments on Rh- and AQ-modified 4W-junctions when strand 3.4 was labeled. The Rh-modified construct exhibited cleavage at *all* guanines, whereas the AQ- modified sample only displayed cleavage at a guanine doublet located in Stem IV.

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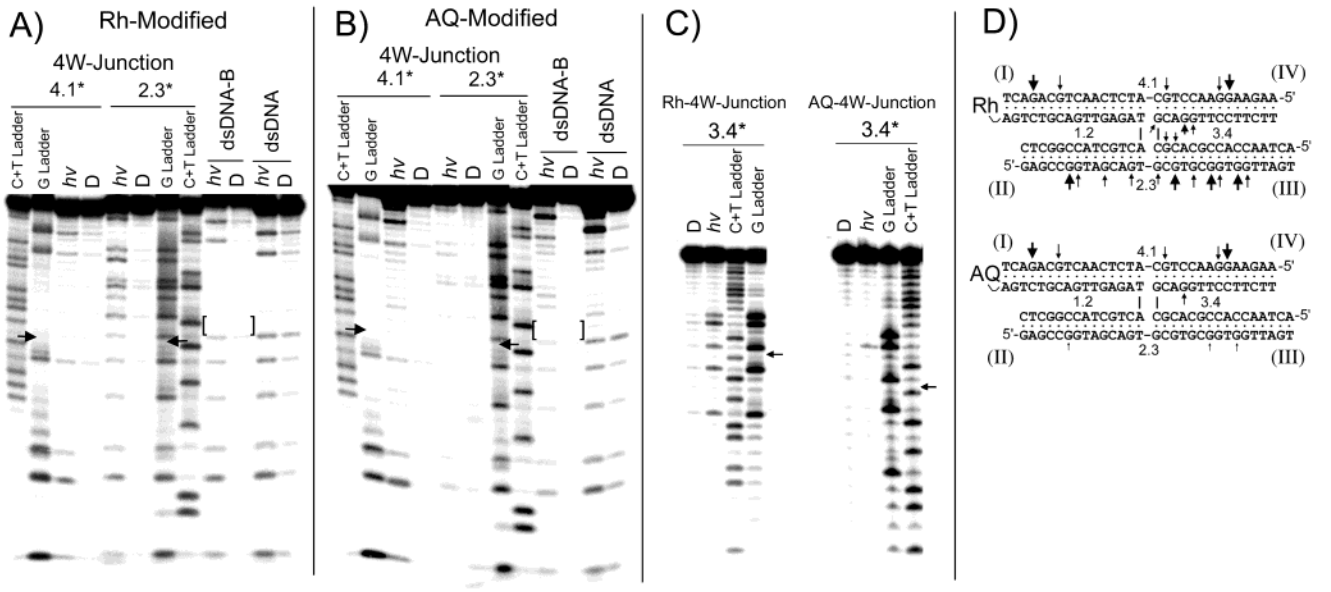

Figure 4. Photoirradiation of DNA assemblies. (**A**) Samples of gel-purified DNA constructs modified with $Rh(phi)_2(byp')^{3+}$ were either irradiated at 366 nm for 210 min (*hν* lanes) at 6−10 °C, or left in the dark (D lanes). Lanes 4.1* and 2.3* show 4-way junction samples with their 4.1 and 2.3 strand, respectively, 5′-labeled with ^{32}P. The dsDNA and dsDNA-B are samples, respectively, of duplex DNA and duplex DNA containing a bulge of two unpaired adenines. Following irradiation, all samples (including dark controls) where treated with hot piperidine (as described in the Materials and Methods), then loaded on a 12% sequencing gel along with the appropriate Maxam−Gilbert sequencing ladders. Arrows indicate the location of the junction on each sequence. (**B**) Identical experiments to that in (A), except that the DNA was modified with anthraquinone in place of $Rh(phi)_2(byp^*)^{3+}$. (**C**) 4W-junctions modified with either anthraquinone or $Rh(phi)_2(byp^*)^{3+}$, that were purified and treated as in (A) but possessed a ^{32}P label on the 5′ end of strand 3.4. (**D**) Mapped sites of cleavage on the $Rh(phi)_2(byp^*)^{3+}$ and anthraquinone modified 4-way junctions, respectively. Arrows indicate the relative intensities of piperidine-dependent cleavage at the indicated guanines.

Control experiments were carried out (under the same conditions as described above) with both the Rh- and the AQ-4W-junction samples, such that 4W-junctions possessing tethered photooxidant but not possessing a 5′-^{32}P label were photoirradiated mixed with ^{32}P labeled 4W-junctions *lacking* the tethered photooxidant. In both the Rh- and the AQ-experiments, no piperidine-dependent cleavage was observed upon photoirradiation (data not shown), suggesting that, at least under these conditions, intermolecular association of 4W-junctions (involving, possibly, crossintercalation of the photooxidant moiety) was not a significant occurrence.

The positions of cleavage and cleavage intensity on the Rh- and AQ-modified 4W-junctions are summarized in Figure 4D, with arrow sizes reflecting the relative magnitudes of cleavage at each site. This summary clearly shows that the cleavage pattern of the AQ-modified 4W-junction better fits the accepted structural model for an immobile 4W-junction, in which pairs of stems coaxially stack, each with one preferred partner. In such a structure, charge would be expected to flow to/from the AQ- (or Rh-) derivatized Stem I to the preferred stacking partner (Stem II) but not to Stems III or IV. In theory, some electron transfer might occur to Stem IV, onto which Stem I might stack to a lower degree of preference; however, no charge transfer should be expected to Stem III, with which Stem I can never stack in the magnesium-folded form of the immobile 4W-junction[56] From this perspective, the results obtained with the Rh-modified 4W-junctions did not easily fit what was known about the structure and dynamics of 4W-junctions.[56] Our Rh-dependent results agree with the results published by Odom et al.[32] on an $Rh(phi)_2(byp')^{3+}$-derivatized 4W-junction (of unrelated DNA sequence), where significant amount of piperidine dependent cleavage was observed in all four arms of the 4W-junction.

We first wanted to confirm that the $Rh(phi)_2(byp^*)^{3+}$ complex covalently attached to a 4W-junction molecule in fact intercalated *only* into that duplex stem (i.e., Stem I) of the 4W junction to which it was covalently tethered (as opposed to bridging the gap and intercalating into Stem II of the same 4W-junction molecule- - which would explain our observation of guanine damage in all four helical stems). To test this we used direct photolysis mapping, whereby photoirradiation with shorter wavelength UV light (∼310 nm) causes direct DNA-strand cleavage at the site of intercalation of these types of rhodium complexes.[49] Samples of $Rh(phi)_2(byp^*)^{3+}$-modified 4W-junctions (and the appropriate double-stranded controls—dsDNA) were prepared such that only *one* of the strands 2.3, 3.4, and 4.1 was 5′-labeled at a time with ^{32}P. Double stranded controls (dsDNA) were also assembled in this way. Aliquots of each sample were photoirradiated at 310 nm for 45 min, and then immediately loaded onto a 10% sequencing gel alongside nonirradiated ("dark") controls of the same complexes. Only the 4W-junction constructs with label on strand 4.1 exhibited cleavage at the most 3′ end of this sequence (data not shown), suggesting that the tethered $Rh(phi)_2(byp^*)^{3+}$ complex was indeed only intercalating into Stem I. One caveat with the above experiment, using this particular 4W-junction (4W-junction I), was that any putative intercalation into Stem II would only be observed as cleavage of the ^{32}P-labeled strand 2.1, at its extreme 5′ end, generating 1−3 nucleotide pieces that in general are difficult to detect and to resolve using denaturing gel electrophoresis. To overcome this problem, another Rh-derivatized 4W-junction ("4W-junction II") was examined. Figure 5A shows the sequence and the helix-stacking partners of this new junction, as well as the sequence of the double-stranded control for this junction. In 4W-junction II, the preferred stacking partners are Stems I and II, to form one coaxial stack, and Stems III and IV

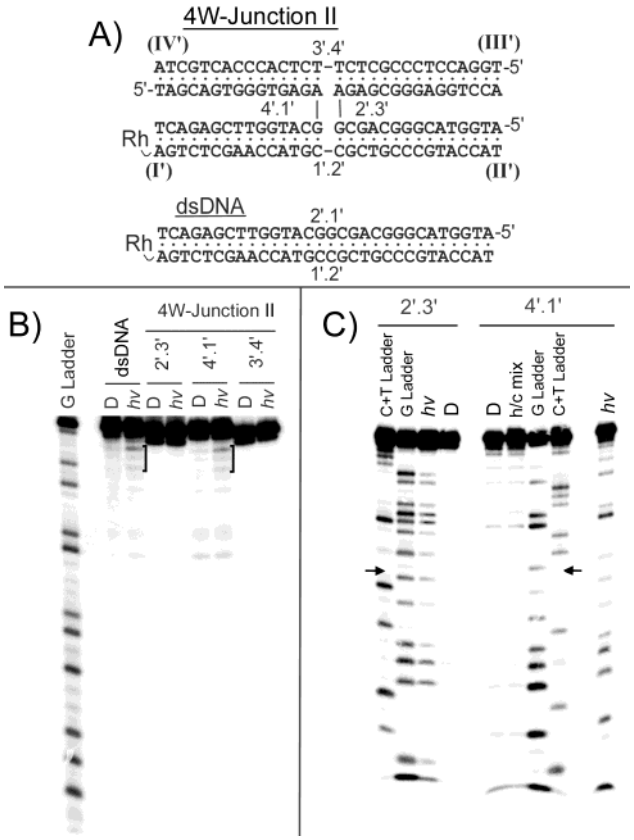

Figure 5. (**A**) Sequences of 4W-junction-II and of its double-stranded control. Duplex stems of the 4W-junction are numbered sequentially I′−IV′. (**B**) Direct photolysis experiment on the 4W-junction-II. Samples of gel-purified DNA constructs derivatized with $Rh(phi)_2(byp^*)^{3+}$ were either irradiated at 312 nm for 45 min (*hν* lanes) at 6−10 °C or left in the dark (D lanes). Samples were then loaded directly onto a 12% sequencing gel. Samples treated in this way include the duplex control as well as 4W-junction samples with strand 2′.3′, 4′.1′, or 3′.4′ individually 5′- ^{32}P-labeled. Brackets highlight areas of direct strand cleavage. (**C**) Charge-conduction related damage at the guanines of the 4W-junction-II, following irradiation at 366 nm and piperidine treatment. Samples of gel-purified DNA constructs modified with $Rh(phi)_2(byp')^{3+}$ were either irradiated for 210 min (*hν* lanes) at 6−10 °C or left in the dark (D lanes). Lanes indicated as 2′.3′ and 4′.1′, are 4-way junction samples with the 2′.3′ and 4′.1′ strand, respectively, being ^{32}P-end labeled. The lane indicated as h/c mix contains the unmodified ^{32}P-labeled 4-way junction irradiated in the presence of nonradiolabeled, Rh-modified 4-way junction. Following irradiation all samples (including dark controls) were treated with hot piperidine (as described in the Materials and methods), then loaded on a 12% sequencing gel alongside appropriate Maxam−Gilbert sequencing ladders. Arrows indicate the location of the junction upon each sequence.

pairing to form the other.[34] If it were possible for the tethered $Rh(phi)_2(byp^*)^{3+}$ moiety to span the separation between spatially adjacent stems (such as between Stems I and IV) of this 4W-junction, it might also intercalate into Stem IV, with consequent 310-nm induced direct photolysis both at the 5′ end of strand 4′.1′ and at the 3′ end of strand 3′.4′. However, the only direct cleavage observed with this 4W-junction construct was at the 3′ end of strand 4′.1′ (Figure 5B), indicating that, at least using this assay, the only substantial interactions of the Rh− moiety was with Stem I. However, when this 4W-junction, carrying the $Rh(phi)_2(byp^*)^{3+}$ modification, was tested for charge transfer, via irradiation with 366-nm light, piperidine-sensitive guanine damage was found in *all four* helical arms as shown in Figure 5C (as with 4W-junction I, above).

The above data report what appear to be contradictory observations on charge transfer through DNA 4W-junctions. On one hand, the anthraquinone (AQ-) modified 4W-junction yielded guanine-damage data consistent with our understanding of coaxial stacking in 4-W junctions (with the two coaxial stacks formed by a 4W-junction I electrically insulated from each other). On the other hand, experiments with two different $Rh(phi)_2(byp^*)^{3+}$-modified 4W-junctions suggested the opposite, that charge transfer could happen with equal probability down all four helical arms. This latter result was, however, consistent with the data of Odom et al. (2001), who examined 4W-junctions derivatized with a closely related Rh-complex (see Figure 2c).

To dissect the reason for this apparent discrepancy, we first examined whether the different photooxidant-derivatized DNA constructs were well-behaved, "monomeric" samples, using nondenaturing (native) polyacrylamide gel electrophoresis. Native gels, especially when run in the appropriate buffer, maintain the structural integrity of macromolecular complexes such as 4W-junctions. To ensure that gel-running buffer conditions resembled as closely as possible the DNA irradiation conditions, gels were run in the presence of 2 mM Mg^{2+}. As seen in Figure 6A, a significant proportion of the Rh-derivatized duplex (lane 1), as well as 4W-junction (lane 5) migrate as retarded bands in the gel (shown with an asterisk), consistent with their having formed some kind of higher-order aggregates. By contrast, when ^{32}P-labeled DNA 4W-junction (Junction I) lacking the Rh-derivatization was co-incubated with nonradiolabeled but $Rh(phi)_2(byp^*)^{3+}$-derivatized 4W-junction, no slow-migrating band of presumed DNA aggregate was observed (lane 4). Controls with ^{32}P-labeled 4W-junctions lacking in any derivatization or AQ-modified 4W-junctions (lane 6), gave no sign of aggregate formation. Interestingly, the AQ-derivatized duplexes (lane 2) and 4W-junction (lane 6) did not show this apparent aggregation.

To ensure that this aggregation was not an isolated phenomenon with a particular batch of assembled DNA constructs (which, moreover, had been gel-purified after assembly), we again assembled our DNA constructs in conditions identical to those used by Odom et al.[32] in their published work on 4W-junctions. Following the cooling step to anneal the constituent DNA strands into the various constructs, an aliquot of each sample was removed for examination by nondenaturing electrophoresis, whose ^{32}P autroradiograph is shown (Figure 6B *upper*). The remainder of the samples were then incubated for 2 h at 18 °C, and examined in a similar fashion (Figure 6B *lower*). This incubation time is on the time scale of photoirradiations used to detect long-range charge transport in our assay (180 min) as well as in experiments reported in an earlier study on 4W-junctions (120 min).[32] In agreement with our earlier results, what was observed again was that in duplexes (lane 1) and in 4W-junctions (lane 5) that were derivatized with $Rh(phi)_2(byp^*)^{3+}$, a species of lower electrophoretic mobility (shown with an asterisk) emerged in time-dependent fashion. In these lanes, individual duplex and 4W-junction molecules were labeled with both $Rh(phi)_2(byp^*)^{3+}$ and ^{32}P, so that the DNA molecules visible by autoradiography necessarily also had the Rh-derivatization. By contrast, when ^{32}P-labeled DNA 4W-junction (Junction I) lacking the Rh-derivatization was co-incubated with nonradiolabeled but $Rh(phi)_2(byp^*)^{3+}$-derivatized 4W-junction, no slow-migrating band of presumed DNA aggregate was observed (lane 4). Again, when 4W-junctions lacked

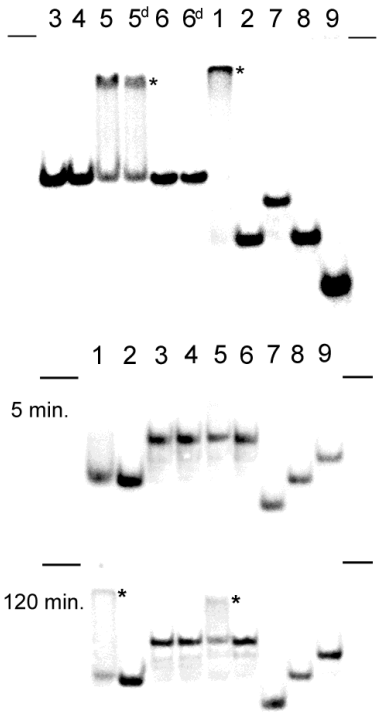

Figure 6. (**A**) Nondenaturing gel electrophoresis of derivatized DNA constructs. Lanes 1: Duplex DNA derivatized with $Rh(phi)_2(byp^*)^{3+}$ (Rh-); Lanes 2: Duplex DNA derivatized with anthraquinone (AQ-). Lanes 3: A nonderivatized 4-way junction; Lanes 4: ^{32}P-labeled, underivatized 4-way junction incubated with nonradiolabeled, Rh-derivatized 4-way junction. Lanes 5: Rh-modified 4-way junction; Lanes 6: AQ- modified 4-way junction. Lanes 7−9 show mixtures of the oligonucleotides used to assemble the 4-way junctions, showing one, two or three of the strands, respectively. All samples contained 0.5 μM total DNA, with the exception of lane 4, which contained 0.5 μM of each construct, and lanes 5^d an d 6^d, in which the DNA was diluted 2-fold in TNME buffer prior to incubation. Samples were incubated overnight at 12 °C before loading on an 8% nondenaturing gel (0.5× TB, 4 mM $MgCl_2$, 0.1 mM EDTA). Horizontal lines indicate the tops of the gels (bottom of the samples loading wells) and the asterisks indicate the rhodium-specific aggregates. (**B**) Identical to (A) but all samples contained 2 μM total DNA (with the exception of lane 4, which contained 2 μM of each construct). Samples were incubated in 50 mM Tris-acetate (pH 7.7), 1 mM EDTA, and 10 mM Mg-acetate for 5 *(upper gel)* or 120 *(lower gel)* minutes at 18 °C before loading. The gels were run under identical time and running conditions to each other.

any derivatization (lane 3) or were AQ-derivatized (lane 6), there were likewise no signs of aggregate formation. In other words, any DNA construct participating, under these conditions, in aggregate-formation necessarily had to possess the $Rh(phi)_2(byp^*)^{3+}$ derivatization. Conversely, the presence of the AQ-derivatization, or the lack of any such derivatization, did not support the formation of aggregates. One distinction between the results described above and what we observed in comparable experiments carried out with gel-purified constructs was that gel-purification gave rise to higher levels of aggregation, attributable to the long elution time of the DNA constructs from the gel. This high level of aggregation is likely the reason we observed no preference for apparent charge transfer through the different duplex stems of the 4W-junction constructs, whereas the previous work by Odom et al. did find some preference (despite significant cleavage observed in *all four* arms of their 4W-junction construct).

To test for any role of magnesium in promoting the aggregate formation, samples containing the Rh-dependent aggregate were also treated with a 5-fold excess (over magnesium) of EDTA (25 mM final) and incubated at room temperature for >20 min prior to loading on a nondenaturing gel. Such a treatment, however, led to only a modest decrease in the amount of aggregate formed, suggesting that these complexes were stable even in the absence of magnesium. Interestingly, prior work using the $Rh(phi)_2(byp^*)^{3+}$ photooxidant for long-range electron-transfer experiments had made the unusual observation of *higher* levels of damage at guanine doublets relatively distant from the tethered Rh-complex than at guanine doublets in closer proximity to the rhodium complex. This phenomenon, appeared, moreover, to occur independent of the ionic strength of the solution or of the presence or absence of Mg^{2+}.[57]

Discussion

Our reexamination of photoinduced charge transfer in immobile DNA 4W-junctions found discrepant results, which were dependent upon the identity of the photooxidant. We have identified the likely reason for this discrepancy as being the formation of higher order aggregates of DNA constructs containing the $Rh(phi)_2(byp^*)^{3+}$ modification. This was not an occurrence isolated to the 4W-junctions, for aggregation was also observed, under the same experimental conditions, with simple DNA duplexes derivatized with $Rh(phi)_2(byp^*)^{3+}$.

A key point about the formation of the aggregate structures was the requirement that *all* participating DNA construct molecules possess the $Rh(phi)_2(byp^*)^{3+}$ modification (i.e., 4W-junction molecules not derivatized with Rh did not participate in aggregate formation). This observation explains why control experiments that have traditionally been used[32] to show that intermolecular interactions were not occurring in these systems failed to detect aggregation. In such traditional control experiments, DNA constructs end-labeled with ^{32}P but *not* with a Rh complex were photoirradiated in a mixture with DNA constructs derivatized with the Rh complex but not with ^{32}P. Such experiments typically revealed an absence of piperidine-sensitive guanine damage in ^{32}P-labeled DNA strands, which was taken to mean that no significant intermolecular association between individual molecules of DNA construct was occurring. However, autoradiography revealed the fate only of the ^{32}P-labeled constructs, which neither possessed the Rh-derivatization themselves nor participated in aggregation (via association with the Rh moiety of a Rh-derivatized but nonradiolabeled construct). Our redesign of this important control, using DNA constructs in which individual molecules were *both* ^{32}P-labeled and Rh-derivatized, suggests that intermolecular association (aggregation) is an important feature of constructs carrying the rhodium complex.

The detailed structure and composition of the higher order aggregates that we have identified cannot be specified as yet, given that they may incorporate two, three, or more participating construct molecules. Figure 7 illustrates "minimal" models for these aggregates (in this figure, the duplex aggregates are hypothesized to contain more participating duplex molecules than those forming from the 4W-junction because the duplex aggregates—Figure 6 *lower*—exhibit even lower elecrophoretic mobility than the 4W-junction aggregates). In both the duplex and 4W-junction versions of the aggregation model, the loci of cross-interaction or -intercalation are arbitrarily placed within the DNA helices, on the presumption that preferred sites for

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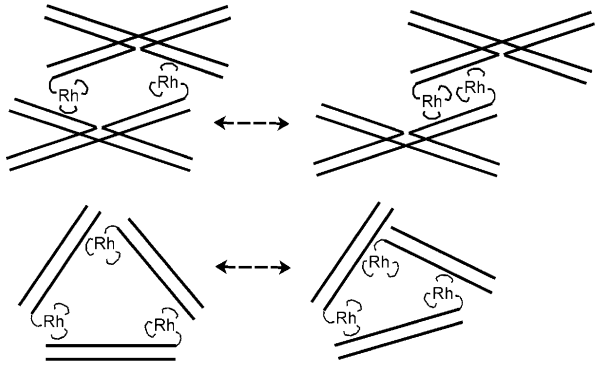

Figure 7. Minimal models of low gel mobility products (presumed DNA aggregates) formed from samples derivatized with $Rh(phi)_2(byp^*)^{3+}$. Cross intercalation by rhodium modifications are presumed to be random such that a multitude of individual species are obtained.

such cross-interaction may not exist. In fact, support for this supposition comes from data from an untethered rhodium complex ($Rh(phi)_2(byp)^{3+}$), which had previously been demonstrated to exhibit no sequence selectivity in its DNA binding.[58] A relatively random dispersement of such cross-intercalation sites would result in sufficiently low levels of direct, 313-nm induced cleavage at defined sites within the DNA to escape detection—as was found in our direct photolysis experiments, especially if one considers that not all of the constructs would be involved in such aggregate structures. We carried out a series of extended irradiation times for such experiments, to attempt to detect putatively preferred sites for such cross-intercalation, but extended irradiation at shorter wavelengths (313 nm) resulted in a variety of lesions and cross-links in the Rh-modified DNA constructs, that made it impossible to interpret the data (data not shown).

It is noteworthy in this respect that charge-transfer experiments carried out with 4W-junction double-crossovers, using a Rh photooxidant,[31] did report an insulation of the two sets of stacked helices from one another (such that charge transfer was restricted to that one helical stack to which the Rh-complex was attached and into which it was intramolecularly intercalated). The double-crossover construct is, however, a significantly more rigid structure than the simple 4W-junction (single crossovers), both in terms of its global structure (the spatial disposition of its helices) and its freedom of movement of helices away from the stack.[59] Owing to topological constraints, it may be expected that the central helical portions of double-crossover constructs are less likely to unwind (helical unwinding is necessary to permit intercalation within a helix). It is therefore quite conceivable that *inter*molecular aggregation involving double crossovers, mediated by inter-construct intercalation events, may be energetically disfavored, relative to such events occurring in single crossovers (i.e., 4W-junctions).

In summary, we offer here evidence and propose a testable hypothesis to explain the observed, photooxidant-specific, differences that have been reported in charge transfer-related processes in different DNA helical constructs. Further investigations into the salt- and temperature-dependence of aggregate formation by differently derivatized DNA constructs, as well as structural investigations thereof, will help to throw further light on the subject.

Conclusions. We provide experimental data that may help to reconcile the discrepancies observed in charge-transfer processes in DNA constructs when they utilize $Rh(phi)_2(byp^*)^{3+}$ versus anthraquinone as the covalently tethered photooxidant, under our experimental conditions. The formation of intermolecular aggregate structures, involving cross-construct intercalation of the photooxidant, likely results in guanine damage that is not a result of the anticipated intramolecular intercalation process. Experimental evidence from using anthraquinone as a photooxidant also suggest that immobile 4W-junctions do indeed form "insulated" coxial stacks, as one would predict from their known 3-dimensional structure, if charge transfer were proceeding strictly through the base stacks.

More broadly, the findings in this paper may provide a basis for understanding the discrepant and apparently irreconcilable results reported on the ability of different sequences within DNA to conduct charge.

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