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A Versatile Mismatch Recognition Agent: Specific Cleavage of a Plasmid DNA at a Single Base Mispair†

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ABSTRACT: $[Rh(bpy)_2(chrysi)]^{3+}$ is a novel, sterically bulky DNA intercalator that has been designed to bind specifically in the destabilized regions near DNA base mismatches and, upon photoactivation, to cleave the DNA backbone. Here the molecule is shown to be both a general and remarkably specific mismatch recognition agent. Specific DNA cleavage is observed at over 80% of mismatch sites in all the possible single base pair sequence contexts around the mispaired bases. Moreover, the complex is highly site-specific; it is shown to recognize and photocleave at a single base mismatch in a 2725 base pair linearized plasmid heteroduplex. Sterically demanding intercalators such as $[Rh(bpy)_2(chrysi)]^{3+}$ may have application both in mutation detection systems and as mismatch-specific chemotherapeutic agents.

Base mismatches arise naturally in the life cycle of a cell as a result of either polymerase errors or DNA damage. Under most circumstances the cell corrects these mispairings using a complex repair system to prevent mutations in the genetic code. Experimental systems for the recognition of mismatches are of particular interest in the cases where this repair system is not functioning efficiently and allows errors in the DNA sequence to persist. Persistent errors in the genetic code have been shown to be important in the mechanisms of many human diseases.

As the number of known diseases with such a genetic component continues to grow, techniques for molecular diagnosis at the genetic level become increasingly important. One method to detect the single base changes in a gene that may distinguish a healthy from a diseased individual involves the use of a DNA mismatch recognition system. When a known copy of a gene is mixed with a sample isolated from a patient, mutation sites will give rise to base mismatches in the resulting heteroduplex. Many procedures have been described to search such a duplex for mismatches using chemical, enzymatic, or denaturation methods (*1*, *2*). None of the known methods, however, are as selective, easy, inexpensive, and reliable as would be desirable for a widely used clinical testing procedure.

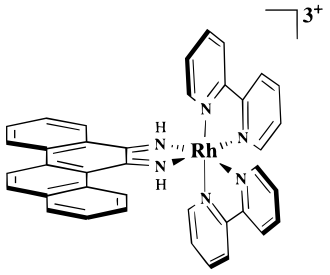

FIGURE 1: Structure of Δ-$[Rh(bpy)_2(chrysi)]^{3+}$

In addition to their usefulness in genetic diagnosis, mismatch-specific chemical agents could also have application in the design of novel chemotherapeutic drugs. The failure of mismatch repair (MMR) has been associated with

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several forms of cancer including hereditary nonpolyposis colorectal cancer (*3*, *4*). Since these tumor cells lack any mechanism to correct mispairs, they accumulate mismatches in their DNA. One could envision using the binding of a mismatch-specific drug molecule to these mispairs as a method to preferentially target cancerous tissue over nearby nondiseased cells. This prospect is of added importance as such MMR-deficient cancers are often resistant to many chemotherapeutic agents (*5*). As a result, compounds exploiting novel strategies for their treatment could have significant clinical utility.

Our laboratory has recently reported the construction of a novel mismatch recognition agent, bis(2,2′-bipyridyl)(5,6-chrysenequinone diimine)rhodium(III), $[Rh(bpy)_2(chrysi)]^{3+}$ (Figure 1), that binds at or near mismatch sites and, upon photoactivation, cleaves the DNA backbone (*6*). This molecule differs from other previously described chemical mismatch probes (*7*−*10*). Rather than reacting preferentially with exposed functionalities of mismatched bases, the selectivity of the rhodium complex is generated by preferential binding at mismatch sites. This recognition based on preferential binding is a significant advantage from the point of view of novel chemotherapeutic design; while it is doubtful that the small DNA-modifying molecules that form the basis of other chemical assays would retain mismatch specificity in the complex milieu of the cell, compounds that selectively bind to mispaired sites prior to any reactivity might continue to be therapeutically specific. Although other intercalators, like the phi complexes of rhodium which have been extensively studied in our research group, have been observed to bind at mismatch sites, such compounds also bind with high affinity to well-matched canonical duplex sites on DNA. In contrast, $[Rh(bpy)_2(chrysi)]^{3+}$ recognizes mismatch sites with much greater specificity. The source of this preferential binding is the sterically bulky chrysi intercalating ligand. This intercalator, which is too wide to intercalate readily into B-form DNA, binds in the destabilized regions at or near base mismatches. Preliminary results showed that the compound recognized base mismatches in oligonucleotide model systems and that the extent of its DNA cleavage roughly correlated with the thermodynamic destabilization at the mismatch sites (*6*).

The aim of the work described here was to assess how useful this recognition strategy might be in the design of novel mutation scanning systems or of mismatch-specific chemotherapeutic agents. To this end, we report both the complete examination of mismatch recognition by $[Rh(bpy)_2(chrysi)]^{3+}$ at all possible mismatches in all possible single base sequence contexts and the site-specific recognition of a single base mismatch in a 2725 base pair plasmid heteroduplex.

MATERIALS AND METHODS

Reagents and Instrumentation. DNA synthesis was performed either in-house on an ABI 392 DNA/RNA synthesizer (reagents from Glen Research, Sterling, VA) or at the Caltech Biopolymer Synthesis Facility. Oligonucleotides were purified on Poly-Pak II purification cartridges (Glen Research) according to the manufacturer's instructions. Photocleavage reactions were performed on an Oriel Hg/Xe arc lamp equipped with a monochromator, IR filter, and 300 nm cutoff filter. Enzymes were obtained from either New England Biolabs (Beverly, MA), USB (Cleveland, OH), or Boehringer Mannheim (Indianapolis, IN). Synthesis, purification, and enantiomeric resolution of $Rh(bpy)_2(chrysi)Cl_3$ are described elsewhere (*6*). Concentrations of the metal complex were determined spectrophotometrically (ϵ_{271}, 6.38×10^4 M^{-1} cm^{-1}). All reagents were obtained from VWR Scientific Products (West Chester, PA) unless otherwise noted and used without further purification.

Plasmid Construction. Derivatives of pUC19 were prepared by insertion of one of two oligonucleotide pairs (5′-CACTCAAGTGAGCCAAGGTAGGACCT-ACTTGAG-(C or G)CATCAAAGGGCCCTTTATT-3′ with appropriate 4 bp sticky ends) between the *Sal*I and *Hin*dIII sites in the pUC polycloning site. The two plasmids, $pVYA_C$ or $pVYA_G$ depending on which base was included in the variable position, were expressed in DH5α *E. coli* (Gibco BRL Life Technologies) according to the manufacturer's protocols. For each insert, a single transformed colony was selected and grown in a 500 mL culture (Terrific Broth, 200 μg/mL ampicillin), and the plasmid DNA was isolated using a Qiagen (Valencia, CA) plasmid isolation column according to the manufacturer's instructions.

Single Mismatch Detection in a 2725 bp Linearized Plasmid. Samples of $pVYA_C$ and $pVYA_G$ (26 μg of each) were linearized with *Sca*I and dephosphorylated with shrimp alkaline phosphatase. After ethanol precipitation, samples of each plasmid were combined (a total of 52 μg in 40 μL of 10 mM Tris) and denatured by addition of 10 M NaOH (2 μL) according to the method of Smith and Modrich (*11*). After incubation for 5 min, the samples were neutralized with acetic acid, sodium chloride was added (1 M, 3 μL), and they were buffered by addition of 1 M Tris, pH 8.5 (18 μL). The samples were then annealed by 30 min incubations at 65 and 37 °C. This mixture of heteroduplexes (some containing CC and GG mispairs) was desalted and exchanged into 10 mM Tris buffer (pH 8.5) on a Microcon 10 spin filter (Millipore, Bedford, MA). Similar base treatment and reannealing procedures were performed on samples of $pVYA_C$ and $pVYA_G$ separately for control purposes. These samples were combined before desalting to provide a sample with the same DNA composition as the heteroduplex sample but lacking base mismatches. Samples of both the heteroduplex and control plasmid samples were end-labeled (on both ends of the plasmid) with [^{32}P]ATP and polynucleotide kinase. Stocks of labeled plasmid for photocleavage were prepared from 2 μg of the labeled plasmid in 100 μL of 2× running buffer (100 mM Tris, 40 mM NaOAc, 36 mM NaCl). Aliqouts of the plasmid stocks (10 μL) were combined with an equal volume of a solution of Δ-$[Rh(bpy)_2(chrysi)]^{3+}$ (200, 20, 2, or 0.2 nM) in deionized water at least 20 min before irradiation for 10 min at 440 nm. Samples were combined with 1 volume of alkaline agarose loading dye (50 mM NaOH, 1 mM EDTA, 2.5% Ficoll, 0.25% bromocresol green) and run on an alkaline agarose gel (1% agarose, 50 mM NaOH, 1 mM EDTA) (*12*). The gel was fixed in 7% trichloroacetic acid, dried under vacuum, and visualized by Phosphorimagery. The lengths of the mismatch-specific photocleavage products (974 and 1751 bp) were verified by comparison with a *Sal*I digest of the labeled plasmid (producing fragments of 936 and 1789 bp.)

Determination of [Rh(bpy)$_2$(chrysi)]$^{3+}$ Cleavage Site in pVYA$_{C/G}$ Heteroduplexes at High Resolution. A sample of pVYA$_C$ (26 μg) was digested with *Eco*RI, dephosphorylated with shrimp alkaline phosphatase, and 5′-end-labeled with polynucleotide kinase and [^{32}P]ATP. The labeled plasmid was then treated with *Pvu*II, yielding three restriction fragments. The fragment of interest (273 base pairs) was separated on a 6% nondenaturing polyacrylamide gel and isolated from the gel slice by extraction into NH_4OAc buffer and EtOH precipitation. Samples of the labeled restriction fragments were combined with a large excess (25 μg) of either pVYA$_G$ or pVYA$_C$ that had been treated with both *Eco*RI and *Pvu*II. This mixture of labeled 270mer and all three unlabeled fragments resulting from the double digests were alkaline-denatured and reannealed as described in the previous procedure. This treatment produced two samples, depending on whether the labeled material was combined with fragments of pVYA$_G$ or pVYA$_C$, one containing labeled CC mispairs (and CG base pairs) and the other containing only Watson−Crick DNA. Samples of these DNA solutions were diluted in 2× running buffer and mixed with an equal volume of Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$ (2 μM, 200 nm, or 20 nm in deionized water) solution 10 min before irradiation at 440 nm for 10 min. Samples were dried, resuspended in formamide loading dye, and dispersed on a 6% denaturing polyacrylamide gel. After drying, the gel was visualized by Phosphorimagery.

Sequence Context of Mismatch Recognition. Individual primer scale (0.04 μM) preparations of 29 base pair oligonucleotide hairpins containing one of the eight possible DNA mispairs in a unique single base sequence context were synthesized and purified by the Caltech Biopolymer Synthesis Facility. In total, 104 oligonucleotides were made which represent all the possible single base contexts around the 8 mispairs when rotational symmetry is taken into account. The oligonucleotides were 5′-end-labeled with ^{32}P and purified from unincorporated ATP on a 16% denaturing polyacrylamide gel. The labeled material was eluted into ammonium acetate buffer, ethanol-precipitated, and annealed in the presence of excess unlabeled DNA. Aliquots of each oligonucleotide solution (10 μL, 20 μM DNA polymers, 10 mM Tris, pH 8.0) were combined with an equal volume of Δ-Rh(bpy)$_2$(chrysi)Cl$_3$ solution (10 μL, 10 μM, H_2O) 30 min before irradiation for 30 min at 440 nm. Irradiation samples were dried under vacuum, resuspended in formamide loading dye, and electrophoresed through a 20% denaturing polyacrylamide gel. The cleavage products were visualized using a Molecular Dynamics Phosphorimager.

RESULTS AND DISCUSSION

Recognition of a Single Base Mismatch in a 2725 bp Linearized Plasmid. Although preliminary work with Δ-[Rh-

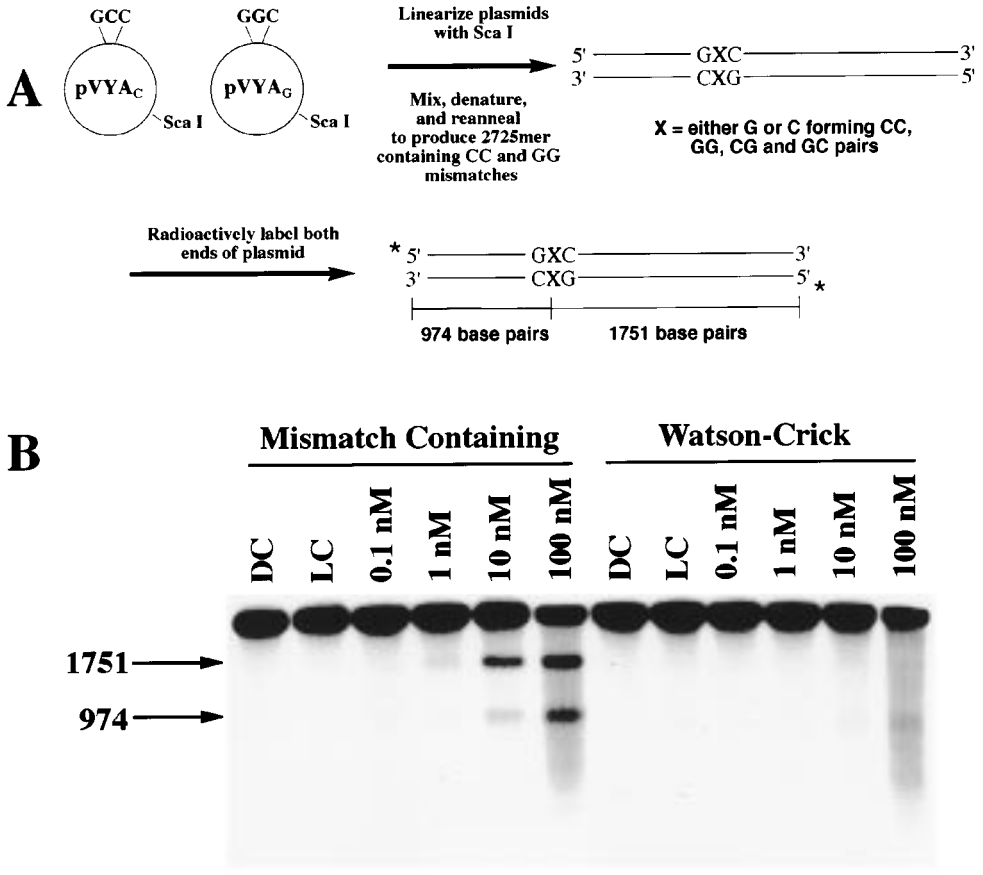


FIGURE 2: (A) Schematic of the application of plasmids pVYA$_C$ and pVYA$_G$, derivatives of pUC19 differing in a single base pair, to the generation of a 2725 base pair plasmid heteroduplex containing a single base mismatch. After linearization and denaturation/reannealing, a mixture of the two plasmids produces a statistical population of heteroduplexes, one-fourth of which contains CC mispairs. (B) Image of an alkaline agarose gel of the photolysis (440 nm, 10 min) products of the plasmid mixture described in (A) with varying amounts of Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$. As the concentration of rhodium complex is increased from 0.1 to 100 μM, conversion of the full-length plasmid to segments of lengths consistent with specific cleavage at the mismatch site is observed. Samples without mismatches (Watson−Crick), samples incubated with metal complex in the absence of light (DC), and samples irradiated without metal complex (LC) show no cleavage.

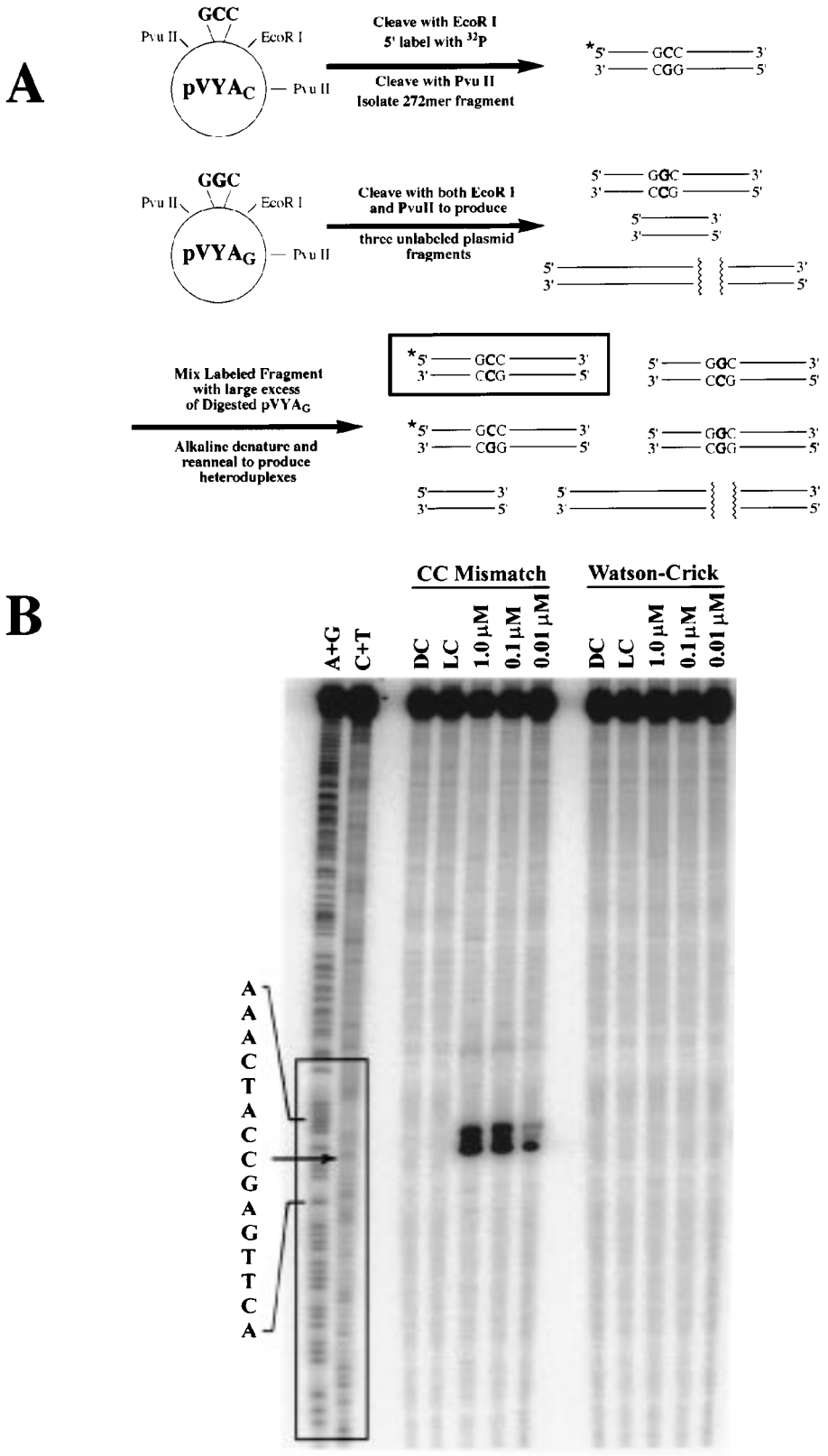


FIGURE 3: (A) Experimental procedure for the determination of the site of pVYA$_C$/pVYA$_G$ heteroduplex photocleavage with high resolution. A singly end-labeled pVYA$_C$ restriction fragment is combined with a doubly digested sample of pVYA$_G$ to produce heteroduplex samples with comparable DNA content to those described in Figure 2 but with the DNA in multiple fragments and only a single radioactive label. (B) Image of a 6% denaturing PAGE gel of photolysis (440 nm, 10 min) products of the restriction fragment heteroduplex mixture described in (A) with varying amounts of Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$. Specific recognition of the mismatch site with cleavage to the 3′ of the mismatch is observed at concentrations as low as 10 μM. Samples without mismatches, samples incubated with metal complex in the absence of light (DC), and samples irradiated without metal complex (LC) show no cleavage.

(bpy)$_2$(chrysi)]$^{3+}$ (Figure 1) has shown that the sterically bulky chrysene intercalating ligand provides binding specificity for mismatch sites on small oligonucleotide substrates (*6*), the ability to specifically recognize base mispairs on long

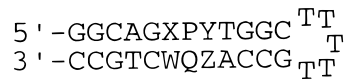


FIGURE 4: General schematic of mismatch sequence context hairpins. The base pairs XW and YZ flank the mismatch site PQ.

DNA polymers of biological origin is critical to its potential applicability and usefulness. It is only with high specificity that such a molecule could select its binding sites from the large excess of properly paired DNA that would exist in a cell or in any biologically derived nucleic acid sample. To determine if Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$ could be used for the specific recognition of base mismatches in such a DNA sample, an experiment on two plasmids was performed. Each plasmid had an oligonucleotide segment (57 bp) inserted between the *Sal*I and *Hin*dIII sites in the polycloning site of pUC19. The two inserts differed in only one base pair, producing plasmids with either a cytosine (pVYA$_C$) or a guanine (pVYA$_G$) at position 467 (as numbered from the first base pair of pUC 19). The plasmids were introduced into competent cells and milligram quantities produced by routine methods.

Samples of each plasmid were cut with *Sca*I, producing a linear 2725mer with the C/G variable site 975 base pairs from one end (see Figure 2A). Equal amounts of the C- and G-containing plasmids were combined and denatured under alkaline conditions (*11*). After reannealing, the resulting mixture should recombine statistically to produce correctly matched plasmids (half of the sample with either CG or GC pairs at site 467) and plasmids containing one base mismatch each (one-fourth each with either CC or GG mispairs at site 467). Each end of the 2725mer was radioactively labeled and was photolyzed at 440 nm for 10 min in the presence of increasing concentrations of Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$.

Because the plasmid was labeled at both ends, both segments of the plasmid produced by cleavage at the mismatch site are visible on the denaturing alkaline agarose gel (*12*) included as Figure 2B. The longer of the two products corresponds to cleavage on the "upper strand" in Figure 2A and the shorter to cleavage at the analogous site on the lower strand. The lengths of the two cleavage bands were confirmed by comparison with *Sal*I cleavage of the linear plasmid which produced two fragments of roughly similar lengths (data not shown). Irradiation of identically treated DNA not containing mismatches (samples of the two plasmids that were alkaline denatured and reannealed separately) shows no specific cleavage (Figure 2B). It is interesting to note that the molecule appears to cleave more readily at the site on the "upper strand" as its cleavage band grows in at a lower concentration of metal complex. This suggests that the geometry of this locally symmetrical site (5′-GA**GXC**AT-3′) is likely affected by the identity of the bases further away from the mismatch. This specific binding and recognition of a single mismatch site in a multi-kilobase DNA duplex by a small molecule is, to our knowledge, unprecedented.

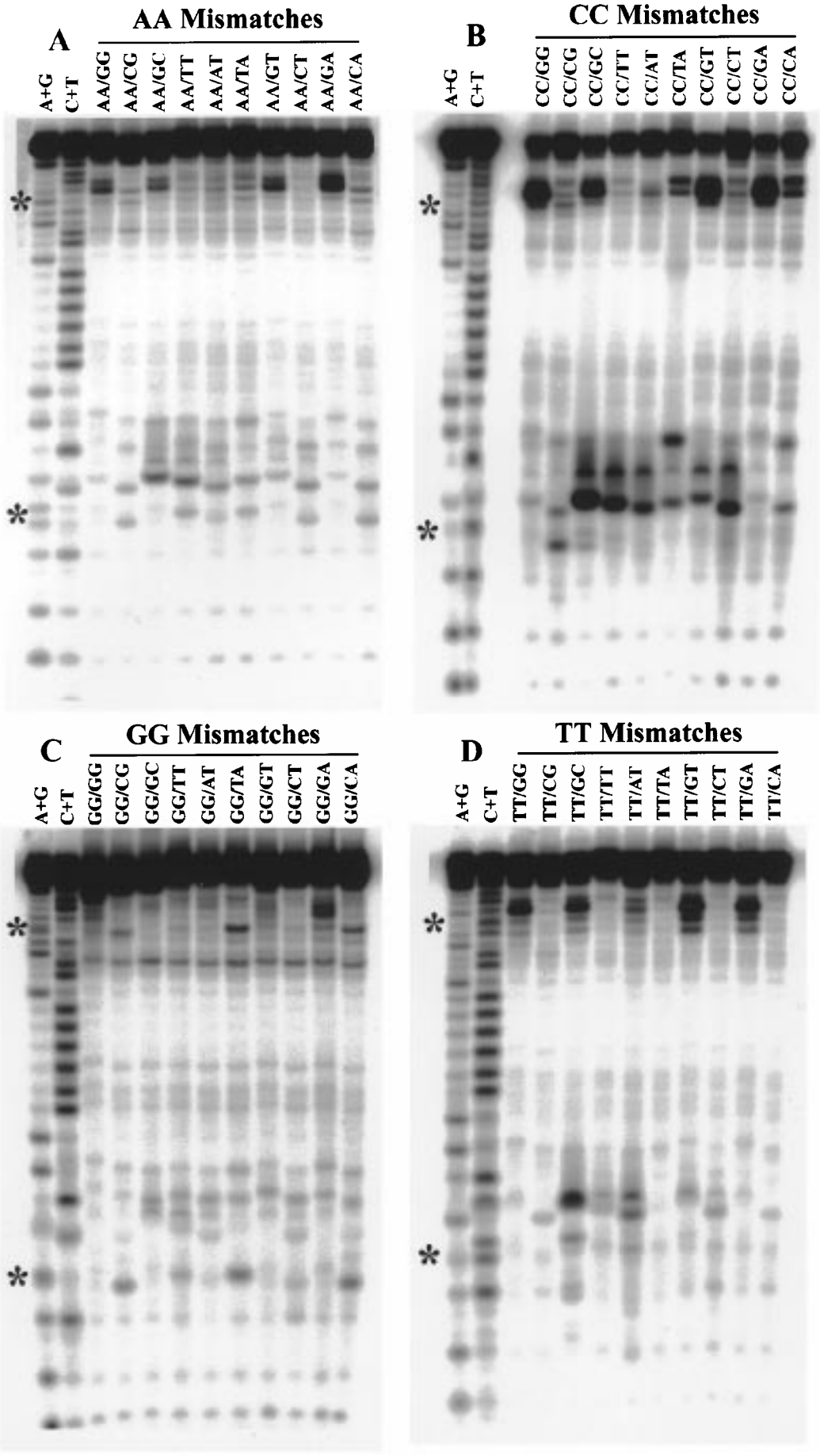
To identify the specific site of plasmid cleavage, an experiment analogous to that described above was performed on a combination of plasmid restriction fragments from pVYA$_C$ and pVYA$_G$. A 273 base pair, singly labeled fragment was obtained from pVYA$_C$ by sequential cleavage and phosphorylation of the plasmid with *Eco*RI, polynucleotide kinase, and *Pvu*II. The resulting radiolabeled restriction fragment containing site 467 was isolated on a nondenaturing gel. Samples of the fragment were combined with doubly digested samples of each plasmid and denatured/reannealed as described previously (*11*). This procedure produced DNA samples identical in DNA composition to those described above (i.e., containing the plasmid DNA in its entirety) but with the DNA cleaved into a number of fragments and labeled only at one end of the fragment of interest (Figure 3A). This mixture of DNA, which has the same ratios of mismatches to total plasmid base pairs as in the first experiment, was photolyzed in the presence of increasing amounts of Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$.

The results, shown in Figure 3B, indicate that the complex cleaves to the 3′ side of the mismatch site as expected (*6*). Samples irradiated without metal complex, samples treated with metal complex in the absence of light, or nonmismatch containing samples irradiated with metal show no cleavage. The cleavage pattern on the plasmid restriction fragment, in addition to corresponding well to that observed on the oligonucleotide system described in the section below (see CC/GC in Figure 5B), shows at high resolution that cleavage of the plasmid occurs specifically at the mismatch site.

Defining the Generality of Mismatch Recognition by Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$. In addition to the ability to recognize mismatch sites in long DNA fragments with high specificity, for a mismatch-specific cleavage agent to be generally applicable, it must be able to recognize a broad range of the possible mismatch sites that can occur in DNA. This generality must include not only the bases involved in the mismatch itself, but also the different sequence contexts that can arise around individual mispaired sites. To test the breadth of recognition by [Rh(bpy)$_2$(chrysi)]$^{3+}$, a set of DNA hairpins of the general sequence shown in Figure 4, where WX and YZ represent the flanking base pairs in the single base context of the mismatch PQ, were made and purified by standard methods.

The use of DNA hairpins, in addition to minimizing problems associated with the reduced melting temperature of mismatch-containing duplexes, makes it possible to observe cleavage events that occur on both strands of the complex binding site. The family of hairpins includes 104 individual pieces of DNA, 10 for each homo-mismatch (CC, AA, etc.) and 16 for each hetero-mismatch. The group includes all possible single base sequence contexts around the mismatches when rotational symmetry about the homo-mismatches is considered. For example, the CC mismatch site 5′-G**C**G-3′ (CC/GG in the nomenclature of Figure 5) occupying the XPY sites (Figure 4) is equivalent to 5′-C**C**C-3′ (CC/CC) occupying those sites since they appear on the opposite strand of the same hairpin and can be interconverted by rotation. Consideration of the rotational symmetry allows study of the 128 possible mismatch sites (8 mismatches each of which have 16 possible single base contexts) on 104 individual DNA substrates.

When samples of each end-labeled DNA were photolyzed at 440 nm in the presence of metal complex, the resulting photocleavage products indicate that Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$ is a general mismatch recognition agent. It is important to note, however, that the intensity of DNA cleavage varies greatly as the sequence context around the mismatch site is changed (Figure 5). In addition, the exact position of cleavage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AA Mismatches
A+G
C+T
AA/GG
AA/CG
AA/GC
AA/TT
AA/AT
AA/TA
AA/GT
AA/CT
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B
CC Mismatches
A+G
C+T
CC/GG
CC/CG
CC/GC
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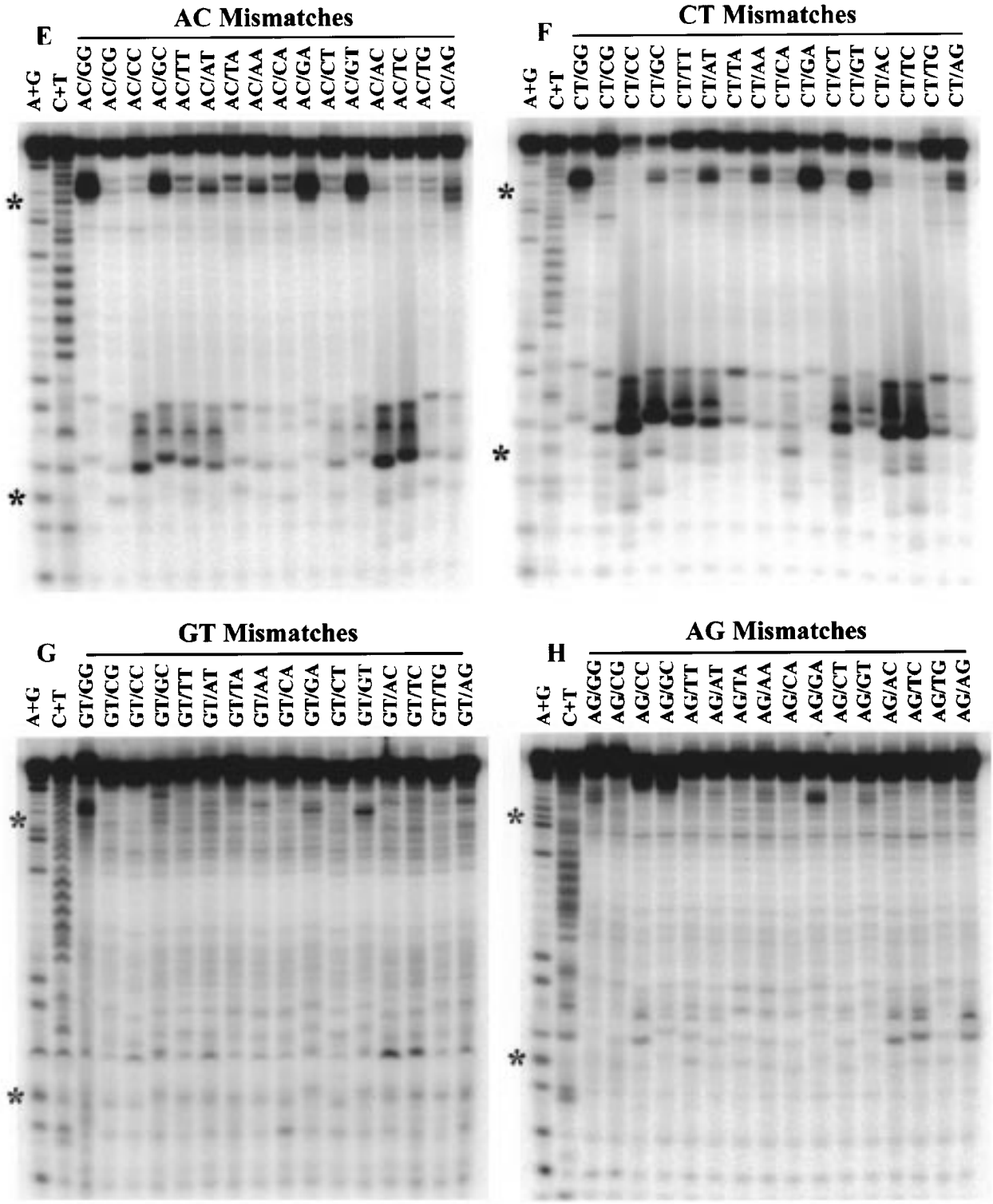

FIGURE 5: Recognition of the eight possible base mismatches in all possible single base pair sequence contexts by Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$. Samples of oligonucleotides (20 μL total volume, 10 μM DNA strands) of the general structure shown in Figure 4 were photolyzed at 440 nm for 30 min in the presence of metal complex (5 μM). The labels above individual gel lanes indicate both the mismatch involved and the bases X and Y of the sequence context (PQ/XY). The position of the mismatch on each side of the hairpin oligonucleotide is indicated by the star. Although Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$ does recognize most of the mismatch sites, it is clear that the sequence context, either by changing the structure or by destabilization at the site, has a strong effect on the intensity of observed DNA photocleavage.

relative to the mismatch varies with both sequence context and mispair identity, likely reflecting differences in the structure of the DNA at and near the different mismatch sites. The most readily recognized mismatches, CC, TT, CT, AC, and AA, show cleavage in all the mismatch sequence context combinations but to varying extents. Of these cases, the pyrimidine−pyrimidine mismatches (CC, CT, and TT) and the purine−pyrimidine mismatch (AC) show the most intense cleavage. Of the remaining cases, the AG, GG, and GT mispairs all show cleavage in a few sequence contexts, but all cleavage is much less intense than that observed with the other mismatches. This weaker recognition of these

mainly purine-containing mispairs is understandable given that these mismatches are generally the least destabilizing of the DNA duplex. In all cases, DNA irradiated in the absence of metal complex or DNA incubated with metal complex in the absence of light showed no strand cleavage (data not shown). When all the experiments in Figure 5 are considered, the compound shows detectable DNA cleavage at approximately 80% (83 of 104) of the mismatch/sequence context combinations shown and, when symmetry is taken into account, at 82% (105 of 128) of the total possible mismatch sites.

It is interesting to note that, in some individual lanes on the gels included in Figure 5 (see CT/CC and CT/TC in particular), most of the full-length DNA strand has been cleaved at the mismatch site. This level of cleavage is unexpected given the ratio of Rh complex to DNA polymers in the irradiation sample is 1:2. In other experiments (data not shown), similar nonstoichiometric cleavage behavior has been observed; in a sample where the Rh:DNA ratio was 1:10, 80% of the total DNA was site-specifically cleaved.

CONCLUSIONS

These results clearly show not only that Δ-[Rh(bpy)$_2$(chrysi)]$^{3+}$ is a versatile mismatch recognition agent that produces detectable DNA cleavage at over 80% of possible mispaired sites but also that it possesses the necessary specificity to recognize a single mismatch in a 2725 base pair linear DNA. This level of specificity and versatility indicates that such *binding-selective* agents are applicable to mutation detection and genetic diagnosis. Furthermore, these kinds of compounds may represent a novel route for the design of mismatch-specific chemotherapeutic agents for the treatment of MMR deficient cancers.

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