Stabilization of Triple Helical DNA by a Benzopyridoquinoxaline Intercalator†

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ABSTRACT: Biophysical, footprinting, and chemical probing experiments are described which characterize the triple helix-stabilizing effects of a benzo[*f*]pyridoquinoxaline derivative B*f*PQ-4,3 structurally related to the previously reported benzo[*e*]pyridoindole compound BePI [Mergny et al. (1992) *Science 256*, 1681−1684]. Two parallel triple helix model systems have been investigated: one in which the third strand matched perfectly a 27 base pair purine−pyrimidine motif in the target DNA and another in which the third strand was one nucleotide longer, i.e., a 28-mer. In the latter system, the pairing of the $(Y)_{28}$ third strand to the $(Y{\cdot}R)_{27}$ target induces the formation of a bulge containing at least one unpaired base, which can be evidenced by chemical probing experiments with osmium tetroxide. BPQ, which unwinds a duplex DNA by 17° as judged by viscometric experiments and otherwise behaves as a typical nonspecific intercalating drug, promotes the formation of a Y·R·Y parallel triple helix containing both T·A·T and $C{\cdot}G{\cdot}C^+$ triplets. Both DNase I and MPE·Fe^{II} footprinting experiments concur that triplex formation with the target $(Y{\cdot}R)_{27}$ sequence can be detected in the presence of BPQ at about 10-fold lower oligonucleotide concentrations than are required to produce an equivalent footprint in the absence of the drug. In addition, BPQ will promote binding to the polypurine−polypyrimidine target sequence by the longer mismatched oligonucleotide, providing significant stabilization of the parallel bulge-containing $(Y{\cdot}R)_{27}{\cdot}(Y)_{28}$ triplex with nearly the same efficiency as the bulge-free $(Y{\cdot}R)_{27}{\cdot}(Y)_{27}$ triplex. Thus *in vivo* BPQ might enhance the formation of both undesired and desired DNA triplexes. By performing an MPE·Fe^{II} probing reaction with a 5′-^{32}P-labeled oligonucleotide third strand, we have obtained evidence that BPQ is actually bound to the triplex region and may distort it in a sequence-specific fashion.

The representation of DNA as a symmetrical, antiparallel double-stranded helix is no longer sacrosanct: nowadays nobody disputes the fact that it is a highly adaptable molecule which can exist within cells in a variety of conformations and structures not restricted to the canonical Watson−Crick B-form. Nucleic acid triple helices were first described in 1957, only shortly after the elucidation of the structure of double-stranded DNA (Felsenfeld et al., 1957; Felsenfeld & Miles, 1967). Yet triplex DNA remained little more than an object of curiosity until the late 1980s when the possibility of targeting a double-stranded DNA with an oligonucleotide was realized—one might say rediscovered (Moffat, 1991). The seeds for the concept were sown by the groups of Hélène and Dervan in 1987 (Le Doan et al., 1987; Moser & Dervan, 1987). Since then, studies on triple helical DNA have flourished, and the field of gene targeting using triple helix-forming oligonucleotides has moved very fast [reviewed in Hélène (1991), Hélène et al. (1992), and Radhakrishnan and Patel (1994)]. Within the space of a few years we have arrived at a relatively clear understanding of DNA sequence recognition by oligonucleotides, though gene targeting so far remains limited mostly to polypurine tracts. As data on triplex DNA accumulate, and a more detailed picture of the DNA−oligonucleotide recognition problem emerges at the molecular level, we increasingly encounter certain recurrent problems among which is the relatively low stability of the triplex structure. Several approaches aimed at enhancing triplex stability have been explored. For example, significant increases in stability can be gained by using DNA intercalating drugs either directly attached at the extremity of the third strand oligonucleotide or in the guise of triple helix-specific ligands, that is to say small molecules endowed with the rare capacity of preferentially stabilizing triple-stranded as opposed to double-stranded DNA structures (Thuong & Hélène, 1993).

The first drug shown to be capable of preferential binding to and stabilization of a triplex rather than a duplex was the benzo[*e*]pyridoindole derivative BePI (Figure 1) (Mergny et al., 1992; Pilch et al., 1993a; Duval-Valentin et al., 1995). More recently, a few other drugs have been shown to provide significant and selective stabilization of a triple helix: BgPI, a close analogue of BePI (Pilch et al., 1993b), coralyne which contains four fused aromatic rings (Lee et al., 1993), the acridine derivative quinacrine (Wilson et al., 1994), a naphthyl-substituted quinoline (Wilson et al., 1993; Cassidy et al., 1994; Chandler et al., 1995), the phenothiazinium dye methylene blue (Tuite & Nordén, 1995a), and certain 2,6-

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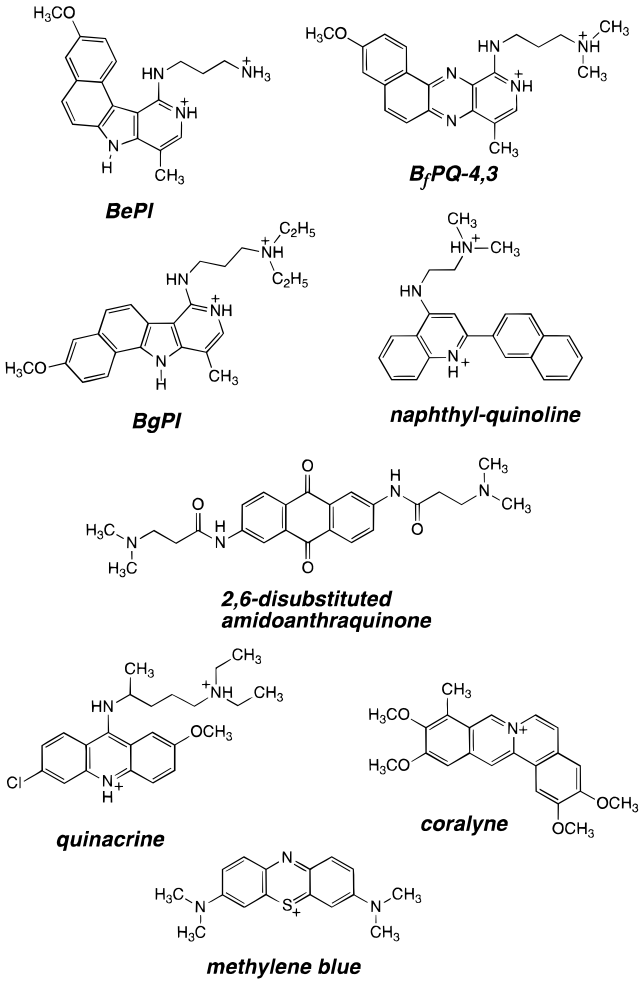

FIGURE 1: Structure of intercalating drugs which can stabilize DNA triple helices.

disubstituted amidoanthraquinones (Fox et al., 1995) (Figure 1). Recent thermal denaturation analyses using various series of benzopyridoindole derivatives have revealed that both the structure of the heterocyclic chromophore and the position of the positively charged side chain play a significant role in the triplex-stabilizing effect (Escudé et al., 1995). These observations have prompted the development of a new series of BePI-related drugs which can efficiently promote triple helix formation (Nguyen et al., 1995). In the present study we have sought to characterize the capacity of the benzopyridoquinoxaline derivative B*f*PQ-4,3[1] shown in Figure 1 to stabilize triplex structures. The investigation began with viscometric measurements aimed at securing evidence for the intercalative property of B*f*PQ-4,3 using double-stranded DNA. The interaction of B*f*PQ-4,3 with double-stranded DNA was further examined by MPE·Fe^{II} footprinting as recently applied to BePI and BgPI (Bailly et al., 1995). Then the capacity of the drug to stabilize triplex structures was explored using two model systems: one in which the third strand matched perfectly the 27 base pair purine−pyrimidine motif in the target DNA and another in which the third strand was one nucleotide longer, i.e., a 28-mer. In the latter system, the pairing of the $(Y)_{28}$ third strand to the $(Y{\cdot}R)_{27}$ target induces the formation of a bulge containing at least one unpaired base (Figure 2). Evidence for the formation of the bulge has been gained from chemical probing experiments with osmium tetroxide. The footprinting studies reported here establish unequivocally that B*f*PQ-4,3 can provide significant stabilization of parallel (Y·R·Y) triple helices irrespective of the presence of a bulge in the triplex motif.

[1] Abbreviations: BPQ, benzopyridoquinoxaline; B*f*PQ-4,3, 11-[[(dimethylamino)propyl]amino]-3-methoxy-8-methylbenzo[*f*]pyrido-[4,3-*b*]quinoxaline; MPE, methidiumpropyl-EDTA.

MATERIALS AND METHODS

Chemicals and Biochemicals. The synthesis of the benzopyridoquinoxaline derivative B*f*PQ-4,3 has been reported recently (Nguyen et al., 1995). Ammonium persulfate, Tris base, acrylamide, bis(acrylamide), ultrapure urea, boric acid, and tetramethylethylenediamine were purchased from BDH. Formic acid, piperidine, hydrazine, and formamide were from Aldrich. DEPC was from Sigma. Photographic requisites were Kodak products. Bromophenol blue and xylene cyanol were from Serva. Nucleoside triphosphates labeled with ^{32}P (α-dATP and γ-ATP) were obtained from NEN Dupont. Unlabeled dATP was Ultrapure grade from Pharmacia. The 27-mer and 28-mer oligonucleotides were obtained from the Laboratory of Molecular Biology, Medical Research Council, Cambridge. Restriction endonucleases *Bam*HI, *Hin*dIII, *Eco*RI, and *Pvu*II (Boehringer Mannheim, Germany) were used according to the supplier's recommended protocol in the activity buffer provided. Alkaline phosphatase, T4 polynucleotide kinase, and avian myeloblastosis virus reverse transcriptase were from Pharmacia. Bovine pancreatic deoxyribonuclease I (DNase I, EC 3.1.21.1, Sigma Chemical Co.) was stored as a 7200 unit/mL solution in 20 mM NaCl, and 2 mM $MgCl_2$, 2 mM $MnCl_2$, pH 7.3 at −20 °C, and was freshly diluted to the desired concentration immediately prior to use. Methidiumpropyl-EDTA (MPE) was a kind gift from Professor Peter Dervan (California Institute of Technology, Pasadena, CA). All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionized, Millipore-filtered water.

Viscosity measurements were carried out in a capillary viscometer submerged in a 45 L water bath which was maintained at 25 ± 0.1 °C. Flow times were measured at least in triplicate to an accuracy of ±0.1 s with a stopwatch, and the average time was calculated. The covalently closed-circular supercoiled pTLX plasmid DNA was isolated by a sodium dodecyl sulfate−sodium hydroxide lysis procedure and purified by banding twice in CsCl−ethidium bromide gradients. Aliquots (1−5 μL) of the test drug solution (1−2 mM) were titrated directly into the viscometer which contained 2 mL of a solution of the plasmid at various concentrations (125−750 μM in nucleotides). After each addition the solutions were carefully mixed with a small flow of air through the dilution bulb of the viscometer, and the flow times were measured. Experiments were conducted in buffer containing 10 mM Tris-HCl (pH 7.0) and 10 mM NaCl. Unwinding angles were estimated with reference to an unwinding angle of 26° for ethidium bromide used as a control (Wang, 1974; Waring & Henley, 1975).

DNA Purification and Labeling. To clone the polypurine−polypyrimidine 27 bp sequence (Figure 2), a synthetic 72-mer oligonucleotide and its complement were mixed at a 1:1 ratio, heated to 70 °C, and slowly cooled to form the duplex. The sequences of the oligonucleotides were designed so that they could be cloned between the *Hin*dIII and *Bam*HI

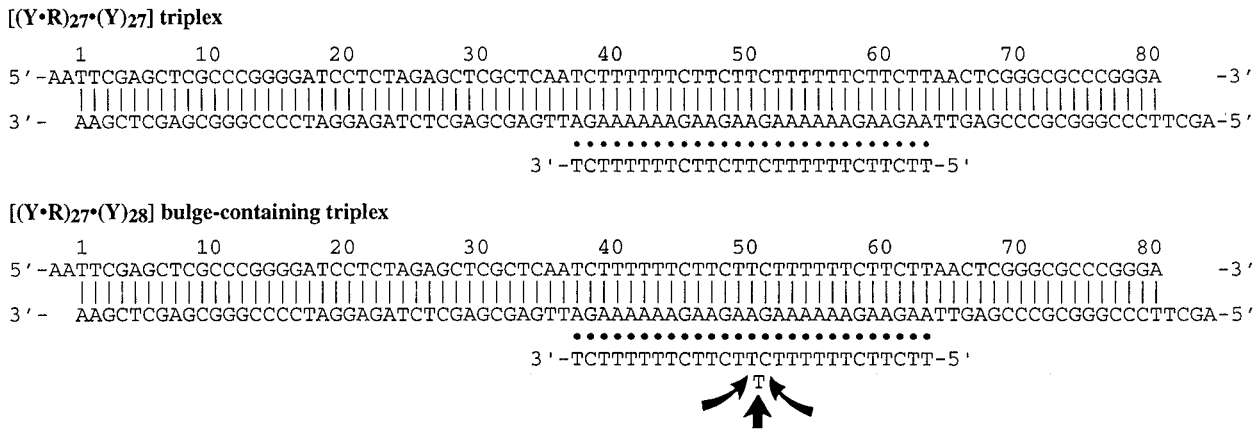

FIGURE 2: Sequences of the $[(Y\cdot R)_{27}\cdot(Y)_{27}]$ and $[(Y\cdot R)_{27}\cdot(Y)_{28}]$ parallel triplexes used in this study. For the bulge-containing $(Y\cdot R)_{27}\cdot(Y)_{28}$ triplex, the arrows point to the three nucleotide residues C13, T14, and T15 that are hyperreactive toward osmium tetroxide.

sites of the pUC12 vector. The vector plasmid pUC12 was double digested with *Bam*HI and *Hin*dIII prior to ligation with the insert. The resulting plasmid construct pTLX was transformed into *Escherichia coli* TG1 cells and isolated by a standard sodium dodecyl sulfate−sodium hydroxide lysis procedure and purified by banding in CsCl−ethidium bromide gradients. Ethidium was removed by several 2-propanol extractions followed by exhaustive dialysis against Tris−EDTA buffer. The purified plasmid was then precipitated and resuspended in appropriate buffer prior to digestion with the restriction enzymes. The 81 bp DNA fragment for footprinting was prepared by 3′-^{32}P-end labeling of the *Eco*RI−*Hin*dIII double digest of pTLX plasmid using [α-^{32}P]dATP (6000 Ci/mmol) and AMV reverse transcriptase or by 5′-^{32}P-end labeling of the *Eco*RI/alkaline phosphatase treated plasmid using [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase followed by treatment with *Hin*dIII. The digestion products were separated on an 10% polyacrylamide gel under nondenaturing conditions in TBE buffer (89 mM Tris−borate, pH 8.3, 1 mM EDTA). After autoradiography, the band corresponding to the 81 base pair fragment was excised, crushed, and soaked in elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate) overnight at 37 °C. This suspension was filtered through a Millipore 0.22 μm filter, and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the labeled DNA was resuspended in 2.5 mM cacodylate buffer adjusted to pH 6.5 containing 10 mM NaCl.

DNase I Footprinting. Reactions were conducted in a total volume of 10 μL. Samples (3 μL) of the labeled DNA fragment were incubated with 5 μL of a solution containing the desired concentration of third strand oligonucleotide and/or B*f*PQ-4,3. All reactions were performed in 5 mM cacodylate buffer, pH 6.5, containing 2.5 mM $MgCl_2$, 0.5 mM spermine, and 10 mM NaCl. To ensure equilibration of the binding reaction, samples were incubated for a minimum of 10 h (generally overnight) at room temperature. The digestion was initiated by the addition of 2 μL of the endonuclease solution whose concentration had been adjusted to limit the digestion to less than 30% of the starting material so as to minimize the incidence of multiple cuts in any strand ("single-hit" kinetic conditions). Optimal enzyme dilutions were established in preliminary calibration experiments. Typically, DNase I experiments included 7 unit/mL enzyme. At the end of the reaction time (routinely 6 min at room temperature), the digestion was stopped by adding 100 μL of ethanol. After precipitation each sample was washed once with 70% ethanol then dried prior to resuspending in 4 μL of an 80% formamide solution containing tracking dyes. Samples were heated at 90 °C for 4 min and chilled in ice for 4 min prior to electrophoresis.

MPE Footprinting. The labeled DNA (∼500 cps) was mixed with the oligonucleotide and/or the test ligand at the desired concentration (in 5 mM cacodylate buffer, pH 6.5, containing 2.5 mM $MgCl_2$, 0.5 mM spermine, and 10 mM NaCl), and the drug−DNA−oligonucleotide complex was left to equilibrate overnight at room temperature. Successively were added 1 μL of MPE (50 μM, aliquots stored at −20 °C), 1 μL of a freshly prepared 50 μM solution of Fe$(NH_4)_2(SO_4)_2\cdot 6H_2O$, and then 1 μL of 20 mM dithiothreitol to initiate the cleavage reaction. After 3 min incubation at room temperature, the reaction was quenched in ethanol (100 μL). The DNA sample was precipitated and washed once with 50 μL of 70% ethanol and then resuspended in 4 μL of formamide−TBE loading buffer, denatured at 90 °C for 4 min, and then chilled in ice for 4 min prior to loading onto the sequencing gel.

Osmium Tetroxide Modification. The procedure of McLean and Waring (1988) was closely followed. The substrate was the synthetic 28-mer oligonucleotide 5′-end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The reaction was conducted at 0 °C by mixing 35 μL of the preequilibrated drug−oligonucleotide−DNA solution (in the spermine-containing buffer indicated above) with 5 μL of a freshly prepared OsO_4−pyridine solution (4/1 v/v) to adjust the final OsO_4 concentration to 2.5 mM. After 15 min at room temperature, the reaction was stopped by extracting the reagent twice with diethyl ether, and the modified DNA was recovered by precipitation with ethanol. After two successive washes with 70% ethanol and vacuum drying, the DNA was subsequently cleaved at the modified residues by boiling for 15 min in 1 M piperidine. Piperidine was removed by repeated cycles of freezing and lyophilization. Samples were finally resuspended in the formamide-containing gel loading buffer.

Electrophoresis and Autoradiography. DNA cleavage products were resolved by denaturing polyacrylamide gel electrophoresis (about 2.5 h at 60 W, 1600 V, in TBE buffer, BRL sequencers model S2). Gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried

under vacuum at 80 °C, and subjected to autoradiography at −70 °C with an intensifying screen.

Densitometry and Data Reduction. A Molecular Dynamics 425E PhosphorImager was used to achieve an accurate and sensitive densitometric analysis of the footprinting data from storage screens exposed to dried gels overnight at room temperature. Base-line-corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Footprinting data are presented in the form ln (f_a) − ln (f_c) representing the differential cleavage at each bond relative to that in the control (f_a is the fractional cleavage at any bond in the presence of the drug, and f_c is the fractional cleavage of the same bond in the control). The results are displayed on a logarithmic scale for the sake of convenience; positive values indicate enhanced cleavage whereas negative values indicate blockage. Footprinting plots were constructed by plotting *R vs c*, where *c* is the ligand (e.g., the third strand oligonucleotide) concentration (Ward et al., 1987; Dabrowiak & Goodisman, 1989; Sayers & Waring, 1993; Bailly et al., 1996). The relative band intensity *R* corresponds to the ratio I_c/I_0, where I_c is the intensity of the band at a given concentration *c* and I_0 is the intensity of the same band in the control lane, i.e., in the absence of the oligonucleotide. Each resolved band on the phosphorimage was assigned to a particular bond within the DNA fragment by comparison of its position relative to sequencing standards (Maxam & Gilbert, 1980).

RESULTS

Interaction with Duplex DNA. Before attempting to characterize the triple helix stabilizing effect of B*f*PQ-4,3, we investigated the binding of the drug to double-stranded DNA using a combination of hydrodynamic and footprinting measurements. Viscometric titration of closed circular duplex DNA was undertaken in order to determine whether B*f*PQ-4,3 unwinds the double helix. The effect of the drug on the viscosity of closed circular duplex DNA containing the 81 bp triple helix-forming sequence is shown in Figure 3. B*f*PQ-4,3 decisively removes and reverses the supercoiling of the plasmid DNA. The maximum in the plot of flow time as a function of drug concentration occurs when the supercoils of the DNA are completely removed. The amount of BPQ required to reach that maximum (termed the equivalence point) is about 0.07 molecule per nucleotide, i.e., 1.7 times the amount of ethidium required to reach the maximum under the same experimental conditions (0.042; Bailly et al., 1994). We determined the position of the equivalence peak at a range of DNA concentrations in order to estimate the equivalence binding ratio precisely. The plot of B*f*PQ-4,3 concentration required to relax the supercoiling as a function of DNA concentration is a straight line (inset in Figure 3). Provided that a simple mass-action law is obeyed, the slope of this line (0.070) is equal to the equivalence binding ratio (Waring & Henley, 1975; Révet et al., 1971). On this basis we measured the unwinding angle for B*f*PQ-4,3 to be 17 ± 2°. This angle is well below the value of 26° taken for ethidium bromide (Wang, 1974) as well as the value of 21° calculated for the triplex-stabilizing ligands BePI, BgPI, and coralyne (Wilson et al., 1976; Pilch et al., 1993a,b) but is of the same order as the angles determined for typical intercalating drugs such as quinacrine and proflavin (Waring, 1970). The conventional viscometric

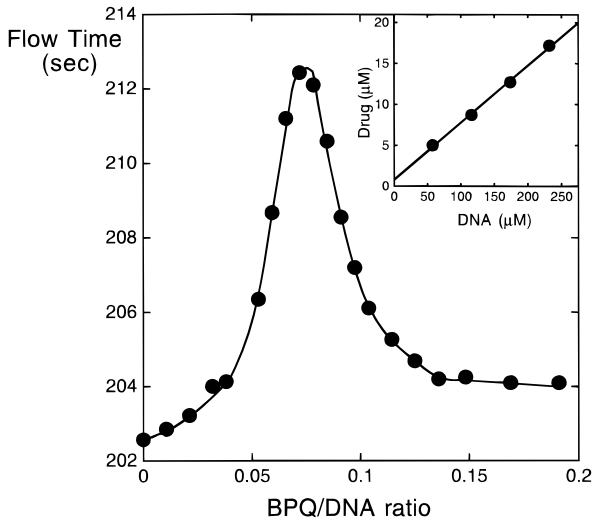

FIGURE 3: Viscometric titration of pTLX closed circular duplex DNA with B*f*PQ-4,3. The flow time is plotted as a function of the molar ratio of added drug to DNA nucleotides. Successive aliquots of a concentrated drug stock solution (0.5 or 2 mM) were added with a calibrated microliter Hamilton syringe into the viscometer containing 2.0 mL of DNA solution (400 μM). The results were corrected for slight changes in DNA concentration during the titration. The inset shows a plot of the concentration of DNA and B*f*PQ-4,3 at equivalence (the maximum in a plot of flow time *vs* drug/DNA where supercoiling is removed) from four viscometric titrations starting at different DNA concentrations.

curves illustrated in Figure 3 provide firm evidence for unwinding of the helix associated with the binding of B*f*PQ-4,3. In other words, B*f*PQ-4,3 fulfils the most stringent criterion for intercalation into DNA.

DNase I and MPE·Fe^{II} footprinting experiments were performed to determine whether B*f*PQ-4,3 can selectively recognize certain nucleotide sequences in DNA. The results of the tests are entirely consistent with those recently reported for BePI and BgPI (Bailly et al., 1995): B*f*PQ-4,3 does not significantly modify the pattern of DNA cleavage by DNase I even at concentrations as high as 100 μM, but it does affect the cleavage by MPE·Fe^{II}. As shown in Figure 4, the effect is relatively weak but small variations in band intensity can be reproducibly detected by phosphorimaging at concentrations of BPQ down to 5 μM. They indicate a slightly diminished susceptibility to MPE·Fe^{II} digestion at the oligo(adenylic acid) tracts, though at a level which cannot be taken to signify the existence of any noteworthy sequence selectivity. It is, however, interesting to note that B*f* PQ-4,3 shares the property with BePI and BgPI that it can interact with $d(A)_n$·$d(T)_n$ tracts, a characteristic not generally encountered with classical intercalating drugs.

Stabilization of the $(Y{\cdot}R)_{27}{\cdot}Y_{27}$ Triple Helix. Figure 5 shows the results of DNase I footprinting experiments performed with the 27-mer oligonucleotide targeted at an 81 base pair DNA fragment containing the polypurine−polypyrimidine motif shown in Figure 2. A synthetic 72 base pair sequence comprising the $(R{\cdot}Y)_{27}$ motif was cloned between the *Hin*dIII and *Bam*HI restriction sites of the pUC12 polylinker, and the *Eco*RI−*Hin*dIII restriction fragment of the resulting plasmid construct was labeled at the *Eco*RI site: this was used as a substrate for the different footprinting studies. In the absence of the third strand (control lanes) the homopurine (double stranded) sequence is cleaved relatively well at the GpA steps and only weakly at the ApA steps. The binding of the 27-mer oligonucleotide

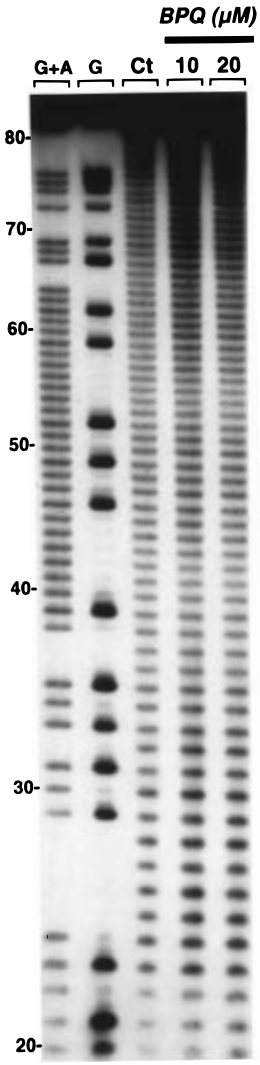

FIGURE 4: MPE·Fe^{II} footprinting of B*f*PQ-4,3 on the 81 base pair *Eco*RI−*Hin*dIII restriction fragment cut out from plasmid pTLX. The duplex DNA was 3′-end labeled at the *Eco*RI site with [α-^{32}P]-dATP in the presence of avian myeloblastosis virus reverse transcriptase. The cleavage products of the MPE·Fe^{II} digestion were resolved on a 10% polyacrylamide gel containing 8 M urea. The concentration (μM) of the drug is shown at the top of the appropriate gel lanes. The control tracks labeled Ct contained no drug. The tracks labeled G and G+A represent dimethyl sulfate− and formic acid−piperidine markers specific for guanine and purine residues, respectively. Numbers at the left side of the gel refer to the standard numbering scheme used in Figure 2.

to its $(R{\cdot}Y)_{27}$ receptor site is manifested on the sequencing gel by the strong inhibition of cleavage within the polypurine tract. In the absence of B*f*PQ-4,3, footprints are detected for oligonucleotide concentrations ≥ 5 μM whereas footprints develop at lower concentrations (down to 1 μM) in the presence of B*f*PQ-4,3. The presence of the drug strongly promotes cutting by the nuclease at the duplex−triplex junction. But the extent of enhanced cleavage at the 3′ end of the target sequence is apparently dependent on the concentration of the third strand rather than on the BPQ concentration. The BPQ-induced enhancement of DNase I cutting suggests that the local changes in DNA conformation which result from the formation of the triplex structure are more pronounced in the presence of B*f*PQ-4,3 than without drug. It is plausible that BPQ amplifies the structural distortions which are already known to exist at the duplex−triplex junction (Sun et al., 1991; Collier et al., 1991a,b). Band intensities in the gels in Figure 5 were measured so as to provide a more accurate picture of the effect of B*f*PQ-4,3. The differential cleavage plots in Figure 6 reveal clearly that B*f*PQ-4,3 strongly stabilizes the binding of the 27-mer to its target sequence in DNA. The plots also show that the footprint (negative values) is 3′ shifted by two to three base pairs beyond the actual binding site. No doubt this is connected with the mechanism of cleavage of the DNA by the nuclease (minor groove binding of the enzyme as opposed to major groove binding of the third strand). Quantitative analysis of the footprinting data yields definite evidence for the stabilizing effect of the BPQ derivative. The full concentration-dependence profile for all bands affected by the 27-mer oligonucleotide has been determined at all nucleotide positions of the $(R{\cdot}Y)_{27}$ motif. Three typical examples of footprinting plots obtained in the presence and absence of B*f*PQ-4,3 are illustrated in Figure 7. The effect of the drug is significant. For example, the footprinting at the ApG step at position 45 occurs with half-maximal effect at concentrations of about 7.5 μM in the absence of B*f*PQ-4,3 whereas it is about 1 μM in the presence of 20 μM B*f*PQ-4,3. Depending on the nucleotide position considered, the oligonucleotide concentration required to decrease the DNase I cleavage by 50% (C_{50}) is 3−12 times lower with B*f*PQ-4,3 than without drug. We estimate that the binding constant for the 27-mer at the $(Y{\cdot}R)_{27}$ sites is enhanced by at least a factor of 10 in the presence of B*f*PQ-4,3. Similar footprinting experiments were performed using MPE·Fe^{II} as DNA cleaving agent. Although with this reagent footprints were usually less pronounced than with DNase I, the stabilizing effect of B*f*PQ-4,3 was also clearly visible (data not shown).

Stabilization of the $(Y{\cdot}R)_{27}{\cdot}Y_{28}$ Triple Helix. The Bulge-Containing Triplex. In order to form a triplex between the $(Y{\cdot}R)_{27}$ target DNA sequence and the $(Y)_{28}$ oligonucleotide, at least one of the central thymine residues (T14, T15, or T16) has to be unpaired. In other words, a small bulge comprising a minimum of one pyrimidine should result from the formation of the triplex structure. We resorted to chemical probing experiments with the osmium tetroxide−pyridine complex in order to identify and locate precisely the bulge. The OsO_4−pyridine complex reacts almost exclusively with pyrimidine bases (T ≫ C) in DNA (it has been reported recently that OsO_4 can react specifically with guanine in the absence of pyridine and presence of $CaCl_2$; Dobi et al., 1994). In unmodified B-form DNA the 5,6 double bond of pyrimidine nucleotides is located in the major groove and is not easily accessible to the probe whereas it is readily accessible if the pyrimidine is not base paired. Osmium tetroxide has previously been used with marked success to characterize a variety of unusual DNA structures, including bulges and base mismatches (Glibin et al., 1984; Johnston & Rich, 1985; Galazka et al., 1986; Vojtiskova et al., 1988; Cotton & Campbell, 1989; Nielsen, 1990; Palecek, 1991), as well as to characterize the structural perturbations of DNA induced by drug binding (e.g., BePI and BgPI; Bailly et al., 1995). The gel in Figure 8a shows that the only three bases that are sensitive to OsO_4 attack are C13, T14, and T15. Although we cannot totally exclude the possibility that the three bands result from a mixture of three discrete bulges, it is much more likely that they are accounted for by a single bulge centered on base T14. The fact that the two residues flanking the unpaired thymine T14 are also sensitive to the osmium probe probably indicates that these two bases are

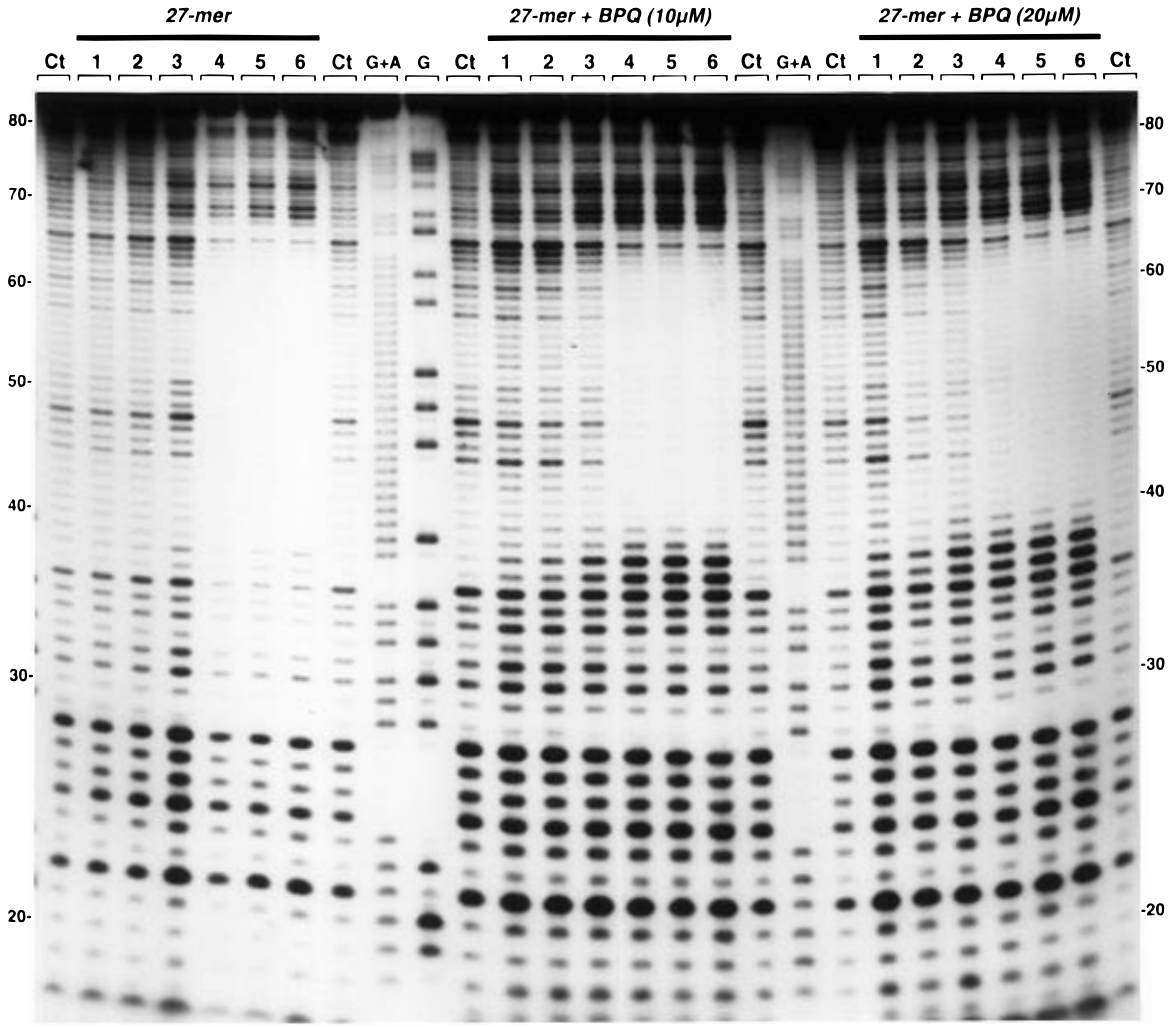

FIGURE 5: DNase I footprinting of the 27-mer oligonucleotide on the 81 base pair double-stranded DNA in the presence and absence of B*f*PQ-4,3. The control track (Ct) contained no oligonucleotide. Lanes 1−6 contained 0.5, 1, 2.5, 5, 7.5, and 10 μM oligonucleotide. Other details are as for Figure 4.

not deeply inserted into the major groove and may not be fully engaged in triplet formation with the target duplex. In the presence of 20 μM B*f*PQ-4,3 the same three bases remain susceptible to oxidation by the OsO_4−pyridine complex (Figure 8b). The drug apparently exerts little effect on the structure of the bulge.

The capacity of the benzopyridoquinoxaline derivative to stabilize the triplex formed between the 28-mer oligonucleotide and the $(R{\cdot}Y)_{27}$ target sequence was investigated by means of DNase I and MPE·Fe^{II} footprinting as reported above for the 27-mer oligonucleotide. The DNase I footprinting patterns observed in the presence and absence of B*f*PQ-4,3 for the 28-mer were little different from those observed with the 27-mer (data not shown). A clear-cut protection effect encompassing the entire $(Y{\cdot}R)_{27}$ motif was observed with the $(Y)_{28}$ oligomer. As we noticed with the $(Y)_{27}$ oligonucleotide, a strong enhancement of DNase I cleavage occurred at the duplex−triplex junctions (both 3′ and 5′) in the presence of B*f*PQ-4,3. The cleavage enhancement is presumed to reflect the triplex-stabilizing effect of the drug (Chandler et al., 1995). Experiments with MPE·Fe^{II} confirm that B*f*PQ-4,3 significantly stabilizes the $(Y{\cdot}R)_{27}{\cdot}(Y)_{28}$ triple helix. Both the gels (Figure 9) and the corresponding differential cleavage plots (Figure 10a) establish that the triplex formation is facilitated in the presence of the drug. The cleavage of the target $(Y{\cdot}R)_{27}$ duplex by hydroxyl radicals is only slightly reduced upon binding of the $(Y)_{28}$ oligomer alone while it is almost completely abolished in the presence of 50 μM B*f*PQ-4,3. Comparing the footprinting plots in Figure 7 and Figure 10b, it can be seen that B*f*PQ-4,3 promotes the formation of the bulge-containing $(Y{\cdot}R)_{27}{\cdot}(Y)_{28}$ triplex with nearly the same efficiency as the bulge-free $(Y{\cdot}R)_{27}{\cdot}(Y)_{27}$ triplex.

In many of the gels an additional, usually weak, region of protection against cleavage can be discerned around positions 29 and 30 of the target sequence. This does not correspond to an oligopurine−oligopyrimidine tract and bears little or no resemblance to sequences in the third strand oligonucleotides. Its origin is unknown, but it is most unlikely to represent a genuine footprint attributable to a tiny local region of triple helix formation. It might represent some kind of locally propagated structural distortion, for such effects have occasionally been noted by others (Collier et al., 1991a,b; Cassidy et al., 1994).

Binding Sites for BfPQ-4,3 within the Triple Helix. Previous studies have shown that drugs such as BePI, coralyne, and even ethidium bromide can intercalate into both duplex and triplex DNA (Pilch et al., 1995a; Lee et al., 1993; Sun et al., 1991; Scaria & Shafer, 1991; Mergny et al., 1991; Tuite & Nordén, 1995b). It is therefore likely that B*f*PQ-4,3, which evidently behaves like a typical intercalating drug with double-stranded DNA (Figure 3), also intercalates into

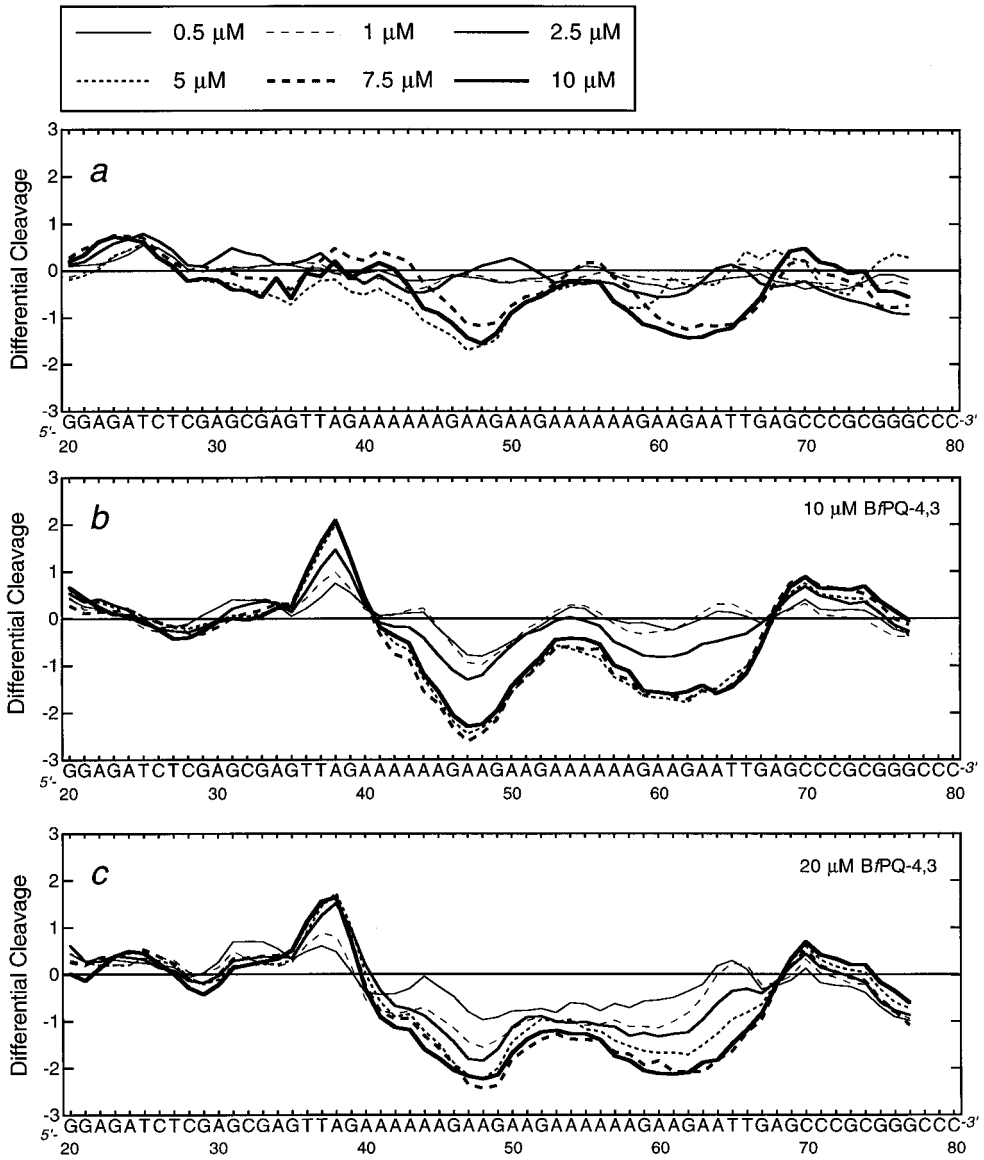

FIGURE 6: Differential cleavage plots comparing the susceptibility of the 81 base pair DNA fragment to DNase I cutting in the presence of increasing concentrations of the 27-mer oligonucleotide. In panel (a) no drug was present. In panels (b) and (c) B*f*PQ-4,3 was added at 10 or 20 μM, respectively. Vertical scales are in units of ln (f_a) − ln (f_c), where f_a is the fractional cleavage at any bond in the presence of the drug and f_c is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion. Negative values correspond to a ligand-protected site, and positive values represent enhanced cleavage. Each line drawn represents a partially smoothed curve obtained by taking a three-bond running average of individual data points, calculated by averaging the value of ln (f_a) − ln (f_c) at any bond with those of its two nearest neighbors. The results are displayed on a logarithmic scale for the sake of convenience. Only the region of the restriction fragment analyzed by densitometry is shown.

the triple helix, but at which sites within the triple-stranded DNA? None of the studies performed so far with triplex-stabilizing drugs have addressed this important question. We have attempted to investigate the issue using an original footprinting procedure. Site-specific binding of a drug to the triplex cannot be studied using conventional footprinting analysis because of the strong footprint at the $(Y{\cdot}R)_n$ motif generated by the binding of the third strand itself. However, despite the protection against cleavage afforded by the third strand, we reasoned that under appropriate conditions it might be possible to coax some reagent to cleave the third strand so as to identify drug binding sites within the triplex. For these experiments the $(Y)_{27}$ or $(Y)_{28}$ oligomer was 5′-end labeled, incubated overnight with an excess of the unlabeled 81 bp DNA containing the $(Y{\cdot}R)_{27}$ motif so as to form the triplex, and then subjected to attack by DNase I, EDTA·Fe^{II}, or MPE·Fe^{II}. DNase I proved useless since no cleavage of the third strand could be detected even with very high enzyme concentrations. This result is hardly surprising. The large size of the nuclease is unlikely to be compatible with the geometry of the triple helix, and the third strand inserted into the major groove of the duplex must be totally inaccessible to the enzyme. Experiments with EDTA·Fe^{II} resulted in very weak but measurable cleavage of the third strand. However, the pattern of cutting was identical in the presence and absence of BPQ, be it with the 27-mer or the 28-mer oligonucleotide (data not shown). By contrast, the use of MPE·Fe^{II} gave useful results (Figure 11). Hardly any cleavage of the third strand could be detected in the absence of the drug whereas cutting was substantially increased in the presence of B*f*PQ-4,3. Moreover, it is interesting to note that the bands showing the most pronounced enhancement of cleavage by MPE·Fe^{II} in the presence of B*f*PQ-4,3 always correspond to the 5′ thymine residue of a TTC triplet (see

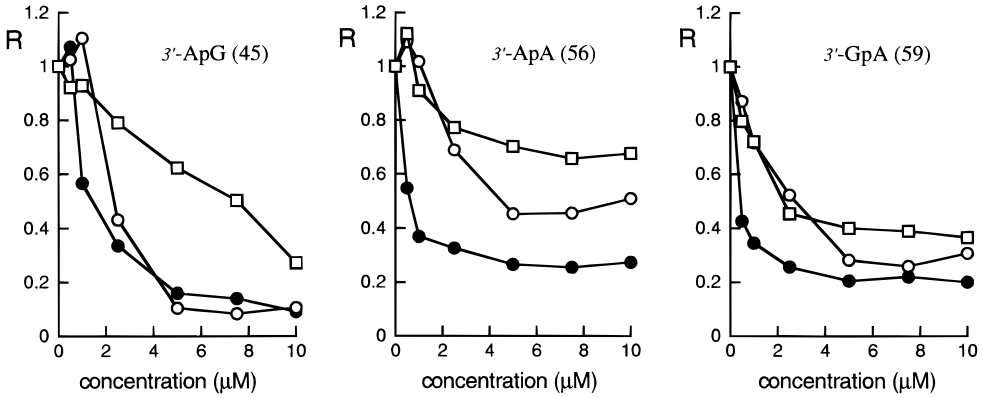

FIGURE 7: Footprinting plots for three selected bonds in the $[(Y{\cdot}R)_{27}{\cdot}(Y)_{27}]$ triple helix. The plots refer to the variation of the cleavage intensity in the absence (□) and presence of B*f*PQ-4,3 at 10 μM (○) or 20 μM (●). The relative band intensity R corresponds to the ratio I_c/I_0, where I_c is the intensity of the band at the oligonucleotide concentration c and I_0 is the intensity of the same band in the absence of the third strand. Data points were obtained from the gel shown in Figure 5.

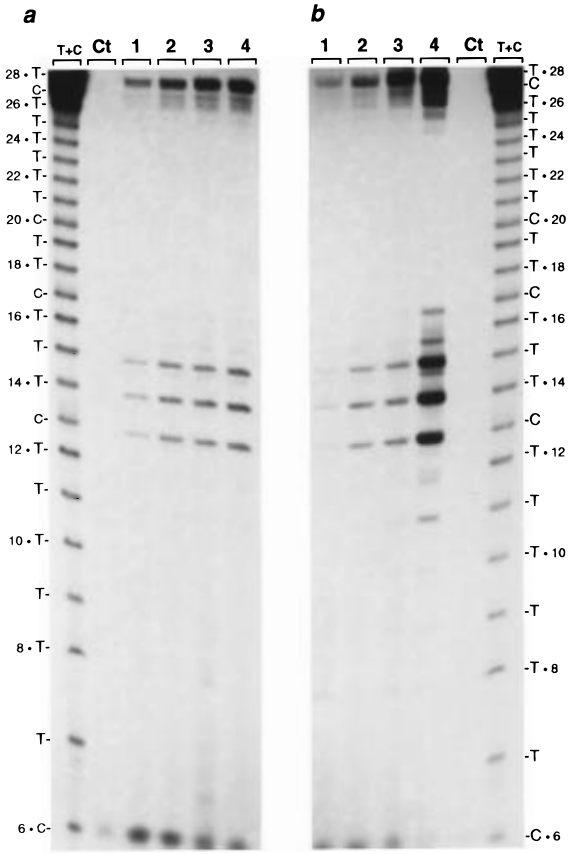

FIGURE 8: Reaction of the triple helix formed between the 81 base pair double-stranded target DNA and the 5′-labeled 28-mer oligonucleotide with osmium tetroxide (OsO_4) in the absence (a) and presence (b) of 20 μM B*f*PQ-4,3. Lanes marked Ct refer to the reaction of OsO_4 with the labeled single-strand oligonucleotide in the absence of the 81 base pair target; reaction is essentially complete and no macromolecular products are visible. In lanes 1−4 the target duplex was added at 1, 2.5, 5, and 10 μM. The cleavage products were resolved on a 15% polyacrylamide gel containing 8 M urea. Pyrimidine-specific sequence markers obtained by treatment of the 5′-labeled 28-mer oligonucleotide with hydrazine followed by piperidine were run in lanes marked T+C. Other details are as for Figure 4.

Ts at positions 11, 15, 18, and 25). This peculiarity was consistently observed with both the 28-mer (Figure 11) and the 27-mer oligonucleotide in the presence of B*f*PQ-4,3 as well as with the related compound BePI (not shown). The weak cleavage of the third strand in the control is most likely accounted for by the small size and diffusible nature of the hydroxyl radicals generated by the MPE·Fe^{II} complex. The unstacking of base triplets required to accommodate intercalation of the benzopyridoquinoxaline chromophore no doubt facilitates access of hydroxyl radicals to the deoxyribose residues, thereby promoting cleavage of the third strand. At first sight it is tempting to postulate that the hypersensitivity of the 5′-TTC sequences reflects the actual binding sites for B*f*PQ-4,3 within the triple helix. However, it could possibly be the result of an indirect effect deriving from BPQ-induced structural distortions outside the triplex motif [the duplex−triplex junction provides suitable intercalation sites (Perrouault et al., 1990; Sun et al., 1991; Collier et al., 1991a,b)] which are propagated into the structure. The fact that the hypersensitivity at 5′-TTC sites was detected using MPE·Fe^{II} but not with EDTA·Fe^{II} suggests that the intercalating moiety of MPE (the methidium group) somehow contributes to the observed phenomenon. Whether B*f*PQ-4,3 does indeed preferentially intercalate at 5′-TTC sites remains an intriguing question which warrants further investigation. It can probably be solved by application and further development of the third-strand labeling procedure introduced here.

DISCUSSION

The results presented above clearly indicate that compound B*f*PQ-4,3, which behaves as a typical nonspecific DNA intercalating drug, can promote the formation of a Y·R·Y parallel triple helix containing both T·A·T and $C{\cdot}G{\cdot}C^+$ triplets. The DNase I and MPE·Fe^{II} footprinting experiments concur that footprints on the target $(Y{\cdot}R)_{27}$ sequence can be detected in the presence of B*f*PQ-4,3 at about 10-fold lower oligonucleotide concentration than is required to produce a similarly intense footprint in the absence of the drug. As such, the BPQ derivative acts like its analogues BePI and BgPI, showing that the structure of the drug can be varied significantly while retaining similar properties. It seems to be the surface and shape of the chromophore rather than its chemical character *per se* that are important for the interaction with the triple helix. The list of compounds capable of binding to and stabilizing triple helices is growing rapidly, and one can predict that in the near future many more compounds will be found effective in the same respect. DNA intercalating drugs bearing both a large (and preferably curved) aromatic system for stacking with the base triplets

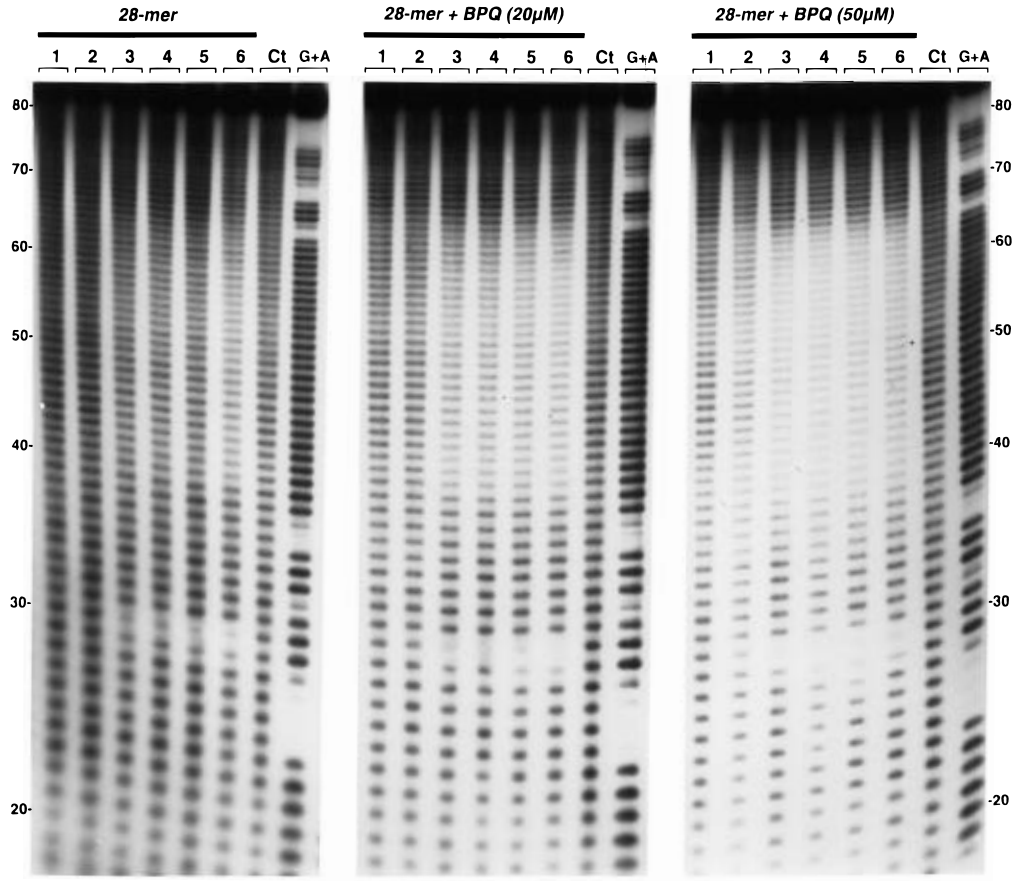

FIGURE 9: MPE·Fe^{II} footprinting of the 28-mer oligonucleotide on the 81 base pair double-stranded target DNA in the presence and absence of B*f*PQ-4,3. Lanes 1−6 contained 0.5, 1, 2.5, 5, 7.5, and 10 μM oligonucleotide. Other details are as for Figure 4.

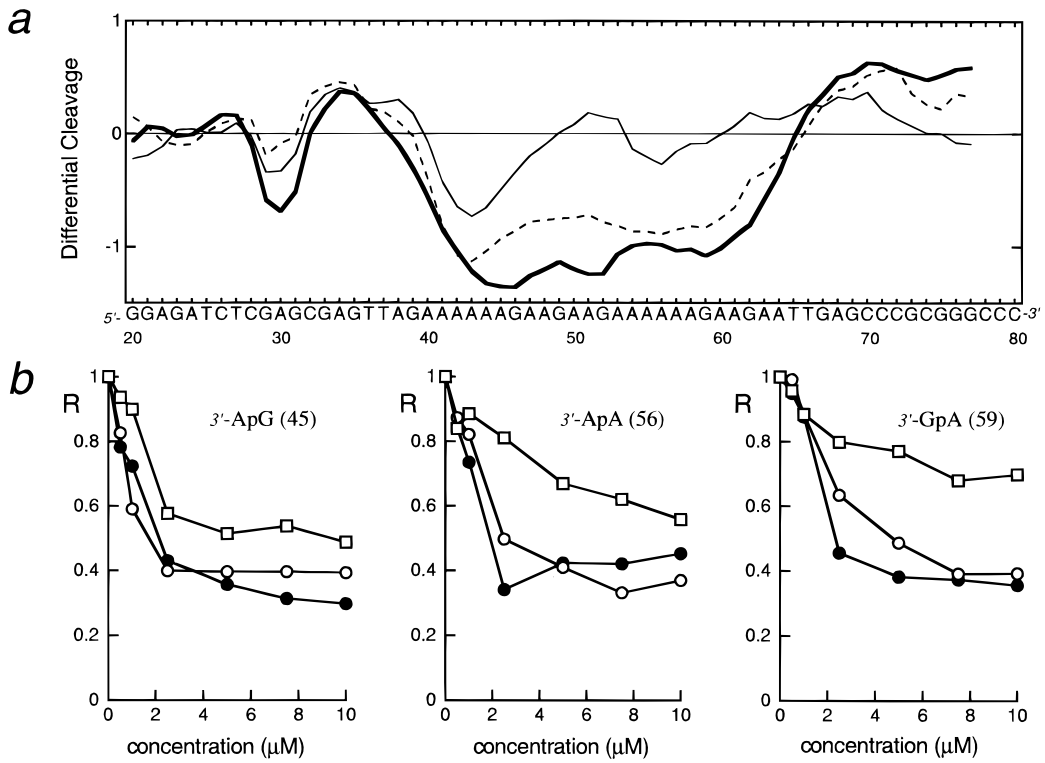

FIGURE 10: (a) Differential cleavage plots comparing the susceptibility of the 81 base pair DNA fragment complexed with the 28-mer oligonucleotide (5 μM) to MPE·Fe^{II} cutting in the absence and presence of B*f*PQ-4,3 (10 or 20 μM). Other details are as for Figure 6. (b) Footprinting plots for three selected bonds present in the $[(Y{\cdot}R)_{27}{\cdot}(Y)_{28}]$ triple helix. The plots refer to the variation of the cleavage intensity in the absence (□) and presence of B*f*PQ-4,3 at 10 μM (○) or 20 μM (●). Other details are as for Figure 7.

and a cationic side chain for providing favorable interaction within the groove of DNA make good candidates for triple helix-stabilizing agents (antigene enhancers). However, the extent of stabilization depends critically on the position of

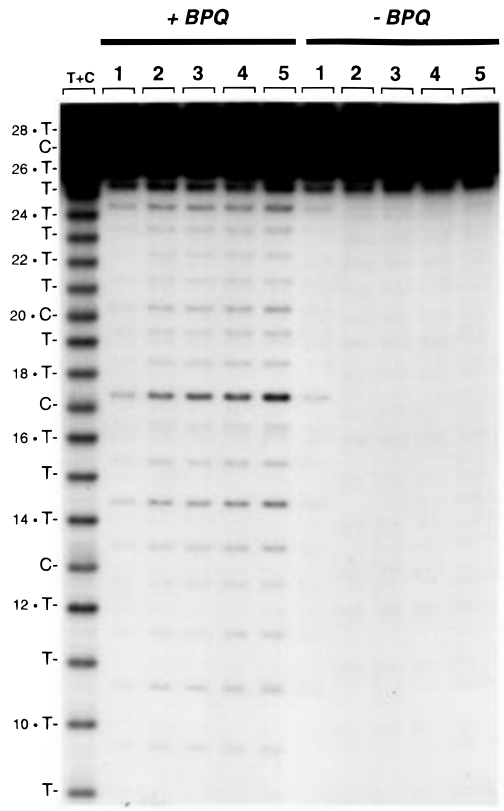

FIGURE 11: MPE•Fe^{II} cleavage of the 5′-end ^{32}P-labeled 28-mer oligonucleotide bound to the 81 base pair double-stranded target DNA in the presence (+BPQ) and absence (−BPQ) of the drug. Lanes 1−5 contained 1, 2, 5, 10, and 15 μM 81 bp double-stranded DNA.

the side chain on the aromatic nucleus (Fox et al., 1995; Escudé et al., 1995).

The experiments employing a third-strand oligonucleotide which is one base longer than the double-stranded target sequence have yielded two essential pieces of information. First, they emphasize the idea that an oligonucleotide is structurally very adaptable. Base bulges have been well characterized in both double-stranded DNA and RNA. They constitute an important feature in the repertoire of folding elements (Lilley et al., 1995). In some cases, extra unpaired bases in duplex segments of nucleic acids are implicated in site-specific recognition by proteins (Turner, 1992). A duplex with a bulged base is also the proposed intermediate in the process of frame shift mutagenesis (Streisinger, 1966; Turner, 1992). Here we show that a bulge can also form within a triple helical DNA. It is not impossible that such a bulge-containing triplex structure might exist *in vivo* and serve as a receptor for specific proteins. Just as there is considerable structural and functional diversity within double-stranded DNA molecules, so will there be diversity among triple helices. Moreover, this type of experiment reveals that a given oligonucleotide can bind reasonably well to sites in DNA which are imperfectly homologous. The extrapolation to an *in vivo* situation suggests that within the genome an antigene oligonucleotide [$(Y)_n$] might not bind exclusively to its complementary target sequence [$(Y\cdot R)_n$] but might also recognize shorter sequences [$(Y)_{n-1,n-2}$] as potential secondary binding sites.

Second, the footprinting experiments reveal that B*f*PQ-4,3 has the potential to promote binding to the polypurine−polypyrimidine double-stranded sequence by both an oligonucleotide strictly complementary to the target site and an oligonucleotide which is one base longer. Given the structural and functional analogy between benzopyridoquinoxaline and benzopyridoindole drugs (Pilch et al., 1993b), it is a reasonable supposition that the effect we have evidenced with B*f*PQ-4,3 would occur similarly with drugs of the BePI/BgPI family. There are two reports that bulges in double-stranded DNA can be stabilized by intercalating drugs such as ethidium (Nelson & Tinoco, 1985; Williams & Goldberg, 1988). It should not therefore be surprising that a bulge in a triple helical DNA can be stabilized by a triplex-stabilizing agent. If drugs such as B*f*PQ-4,3 are to be employed for therapeutic applications involving oligonucleotide-directed triplex formation, it needs to be borne in mind that the drugs might enhance the formation of both undesired as well as desired DNA triplexes.

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