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Site-specific intercalation at the triplex-duplex junction induces a conformational change which is detectable by hypersensitivity to diethylpyrocarbonate

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ABSTRACT

Using site-specific intercalation directed by intermolecular triplex formation, the conformation of an intercalation site in DNA was examined by footprinting with the purine-specific (A>>G) reagent diethylpyrocarbonate. Site specific intercalation was achieved by covalently linking an intercalator to the 5′ end of a homopyrimidine oligodeoxynucleotide, which bound to a homopurinehomopyrimidine stretch in a recombinant plasmid via intermolecular triplex formation. This directs intercalation to a single site in 3kb of DNA at the 5′ triplex-duplex junction. Footprinting with diethylpyrocarbonate and dimethylsulphate revealed strong protection from modification of adenine residues within the triple-helix in concordance with their Hoogsteen pairing with the third strand, and a strong hypersensitivity to diethylpyrocarbonate at the first adenine of the duplex. This result indicates that intercalation at this site induces a conformational change at the 5′ triplex-duplex junction. Furthermore, the same diethlypyrocarbonate hypersensitivity was observed with an unmodified triple-strand forming oligonucleotide and a range of intercalating molecules present in solution. Thus the 5′ triplex-duplex junction is a strong binding site for some intercalating molecules and the junction undergoes a conformational change which is sensitive to diethylpyrocarbonate upon insertion of the planar aromatic chromophore. This conformational change can be used to direct a single-strand cut in duplex DNA to a defined site.

INTRODUCTION

Certain bases in DNA are hypersensitive to chemical reagents in the presence of micromolar concentrations of the bis-intercalating quinoxaline antibiotics. This has been ascribed to local conformational changes induced upon binding of this ligand to DNA (1–5). Crystallographic studies indicate that bases adjacent to drug binding sites can adopt a Hoogsteen pairing arrangement (6) while NMR studies show that Hoogsteen pairing is possible but depends on the sequence of the intercalation site (7). Recent evidence using a DNA fragment in which Hoogsteen pairing is prevented indicates that chemical hypersensitivity induced by quinoxaline antibiotics may in fact result from exposure to chemical attack of bases adjacent to the intercalation site because of the large local unwinding induced upon binding of this antibiotic to DNA (5).

We investigated whether other intercalating molecules could also induce chemical hypersensitivity at adjacent bases. Chemical footprinting reagents are insufficiently sensitive to detect local conformational changes induced by intercalators which show little sequence specificity in binding to DNA. Thus, we have performed chemical footprinting studies on duplex DNA with the intercalators acridine and orthophenanthroline intercalated at a single site. Site-specific intercalation can be achieved by linking an intercalating molecule to an oligonucleotide (Figure 1) which is able to recognise double-stranded DNA sequence-specifically via intermolecular triplex formation (9). It is possible to form local triple helices at purine stretches of DNA by the binding of a second polypyrimidine strand in the major groove of the duplex, parallel to the purine strand (10–14) (Figure 2). Sequence-specific recognition of the helix is achieved by hydrogen-bonding of the third strand to available donor and acceptor atoms of the duplex bases in the major groove. Bound in this manner, a thymine and a protonated cytosine form two Hoogsteen hydrogen bonds with an A·T and a G·C Watson-Crick base-pair respectively.

If an intercalating agent is covalently attached to either the 3′- or 5′-end of the third strand of the triplex, it intercalates specifically at the triplex-duplex junction (9) (Figure 2). Random intercalation is prevented as a result of repulsion between the negatively charged oligodeoxynucleotide tail and duplex DNA. The presence of an intercalating agent also strongly stabilises triple helix formation (9). In addition, substitution of 5-methylcytosine for cytosine further stabilises triple helical interactions (15–17) resulting from a small pKa change and the hydrophobic effect of the helical spine of methyl groups present in the major groove.

In the present study, we have performed chemical footprinting experiments on triplex-intercalator complexes, using the DNA-modifying reagent diethylpyrocarbonate (DEPC). These footprinting studies reveal strong protection from DEPC

modification of adenine residues within the triple helix which are involved in Hoogsteen pairing with the third strand. In addition, site specific intercalation of acridine at the 5′ triplex-duplex junction induces a strong DEPC hypersensitivity on an adenine residue immediately 5′ to the intercalated chromophore. This effect results from a conformational change at this adenine residue induced by intercalation, since an identical oligonucleotide without an attached acridine does not induce chemical hypersensitivity. Furthermore, the same oligodeoxynucleotide covalently attached to orthophenanthroline, which also intercalates into DNA (18), induces an identical DEPC hypersensitivity indicating that this conformational change does not depend on the chemical nature of the intercalating agent.

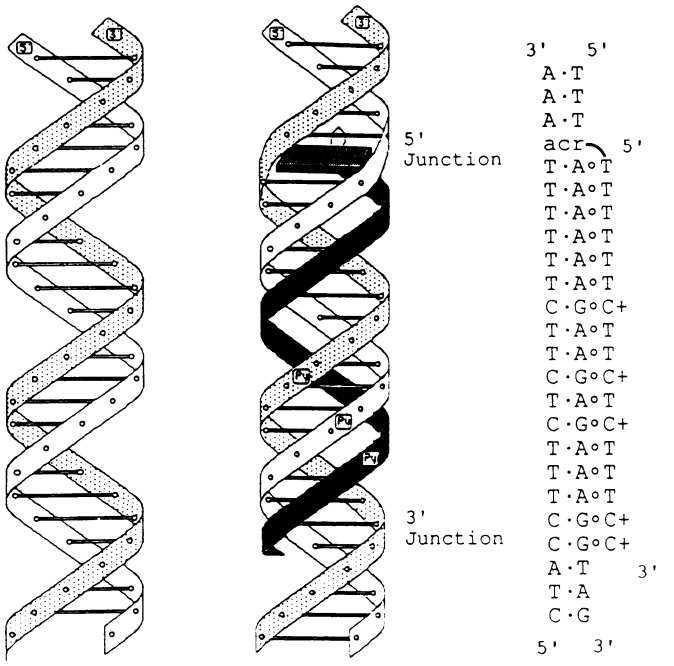

Fig. 1. Sequence and structures of the 17-mer oligodeoxynucleotides used in this study. All oligodeoxynucleotides were synthesised in the parallel orientation with respect to the homopurine stretch of the target duplex. The intercalating agent 2-methoxy-6-chloro-9-aminoacridine (acridine) is attached to the 5′ phosphate of the oligodeoxynucleotide by a pentamethylene linker, and the o-phenanthroline moiety is attached to the 5′ end of the oligodeoxynucleotide by a hexamethylene thiophosphate linkage. Asterisks indicate positions of 5-methylcytosine residues.

We also examined the effect of free intercalator molecules on chemical reactivity at the triplex-duplex junction. It has recently been reported that the 5′ triplex-duplex junction is a strong intercalative binding site for an ellipticine derivative as determined by photo-footprinting of ellipticine binding sites in a triple-helix complex (19). We report that intercalation of a range of planar chromophores at the 5′ triplex-helix junction induces DEPC hypersensitivity.

These results indicate that there is an altered conformation at the triplex-duplex junction which creates a strong binding site for planar aromatic chromophores, and upon intercalation at this site a further conformational change is induced which results in exposure of the N-7 of the adenine adjacent to the intercalated molecule. This is most consistent with a large local unwinding of the DNA induced by intercalation and thus exposure of bases in the duplex DNA to chemical attack, as has been proposed for quinoxaline antibiotics.

MATERIALS AND METHODS

Preparation of Oligonucleotides and Plasmid

Oligodeoxynucleotides which were either unmodified or which contained 5-methylcytosine (Figure 1) were synthesised on a Pharmacia automatic synthesiser by phosphoramidite chemistry. Oligodeoxynucleotides covalently attached to an intercalator at the 5′ end (Figure 1) were prepared by solid-phase synthesis using either coupling of the phosphoramidite derivative of 2-methoxy-6-chloro-9-(ω-hydroxypentlyamino) acridine (20) or reaction of 5-(ω-halogenoalkylamido)-1,10-phenanthroline with a 5′thiophosphate (18).

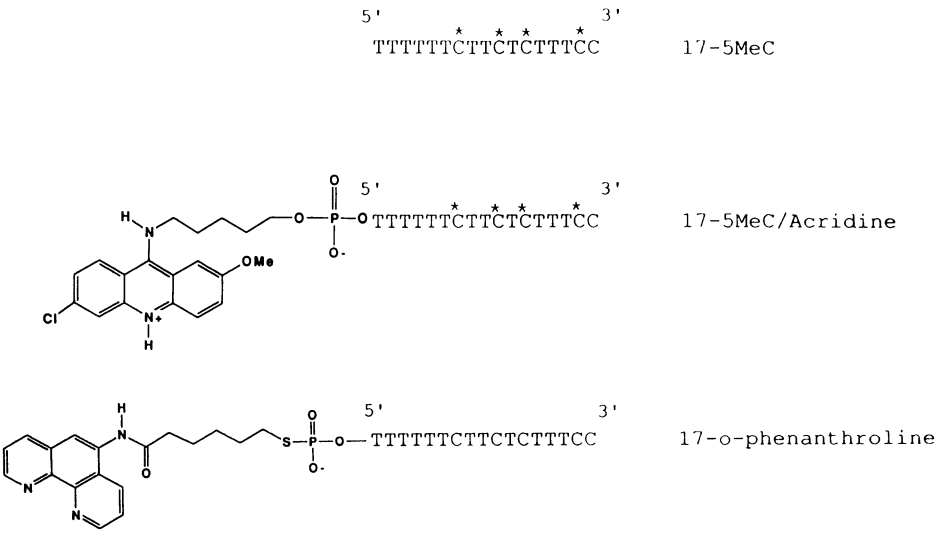

Fig. 2. Schematic diagram of triple helix formation showing duplex DNA (left) and the intercalator-triple helix complex (right) which has a covalently attached chromophore intercalated at the 5′ triplex-duplex junction. The sequence of the triple helix and flanking base-pairs are to scale and shown adjacent to the triple helix. C+ indicates protonated cytosines.

For the construction of pDC1, complementary oligodeoxynucleotides were synthesised so that when annealed, *Hin*dIII compatible ends were generated. The resulting double-stranded oligomer was then ligated into the unique *Hin*dIII site of a pUC19-derived vector, pBluescript KS−, and recombinant plasmid prepared by standard procedures (21). One *Hin*dIII site (at the 5′ of the pyrimidine strand) was lost by a single base-pair deletion during cloning procedures. The target sequence for triple helix formation is shown in figure 3.

UV Absorption

Thermal denaturation profiles were obtained on a Kontron Uvikon 820 spectrophotometer using 1cm optical path quartz cells. The temperature of the cell holder was regulated by circulating liquid and measured by thermoresistance. The temperature of the bath was changed at the rate of 0.11°C per minute, allowing complete thermal equilibration of the cells, with a start temperature of 2°C and a final temperature of 80°C. Profiles were different with decreasing temperature, showing that the rate constants for triplex formation and dissociation were very low.

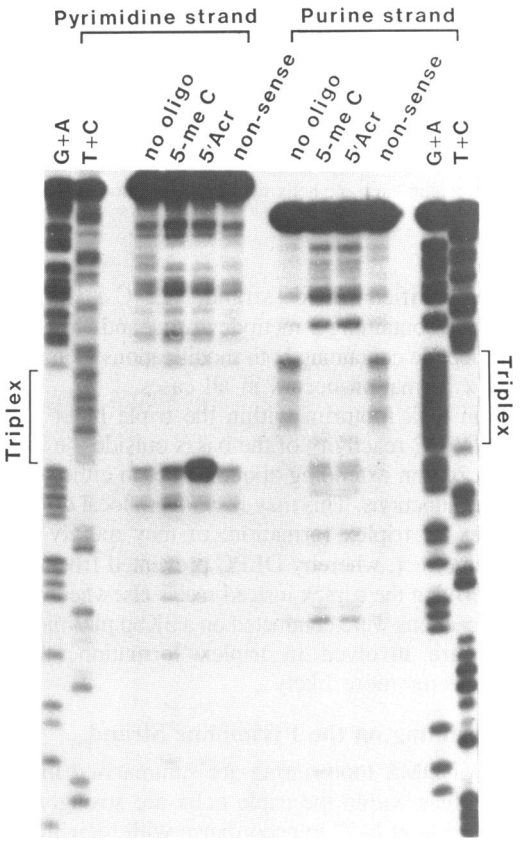

Fig. 3. DEPC footprinting of the site specific intercalation complex. Lanes marked 'no oligo' show the pattern of reactivity with DEPC of the fragment alone. Lanes marked '5-meC' show the pattern of reactivity in the presence of a complementary 17-nucleotide pyrimidine oligodeoxynucleotide (oligomer) containing 5-methylcytosine in place of cytosine, lanes marked '5′ Acr' show the pattern of DEPC reactivity in the presence of an oligomer containing 5-methylcytosine with an acridine residue covalently attached at the 5′ end, and lanes marked 'non-sense' show the pattern of reactivity in the presence of a non-complementary 11-base pyrimidine oligomer. The brackets marked 'Triplex' indicate the homopurine·homopyrimidine stretch which is the target for intermolecular triplex formation, and 'pyrimidine strand' and 'purine strand' indicate the strand labelled for footprinting. Corresponding lanes from the purine and pyrimidine strands are samples from the same footprinting reaction, which was divided in two prior to labelling of each strand to allow direct comparison of chemical reactivity.

Chemical Footprinting

DEPC and dimethylsulphate (DMS) modification reactions were performed as described (22). Briefly, supercoiled or linear plasmid DNA were incubated in 50μl of buffer (100mM Tris·acetate pH 6.5, 100mM NaCl and 100μM spermine) for 30 minutes at room temperature in the presence or absence of oligonucleotide. The mixture was then treated with 5μl of DEPC at room temperature (22°C) for 15 minutes or 1μl of DMS at 4°C for 1 minute. After termination of the reactions the DNA was divided into two aliquots and cleaved with either NotI and KpnI (to visualise the purine strand) or XhoI and SacI (pyrimidine strand). The resulting fragments were labelled with (α-^{32}P)dGTP by 'filling in' with Klenow fragment and cold dNTP's, purified, and treated with 1M piperidine for 20 minutes at 90°C to cleave at chemically modified sites. For footprinting of the triplex in the presence of drug molecules, a labelled XhoI-SacI fragment of pDC1 either with or without the triple helix strand was incubated for 30 minutes in the presence of drug molecules. DEPC footprinting was then performed as described above. Samples were applied to denaturing polyacrylamide gels in parallel with Maxam-Gilbert sequencing ladders (23).

RESULTS

I. Oligonucleotide-intercalator Conjugates

The triple helix formed by the 17-mer oligodeoxynucleotide-intercalator complex is shown in Figure 2, and the result of diethylpyrocarbonate footprinting on a restriction fragment of pDC1 containing the 17-bp homopurine·homopyrimidine target for triple helix formation is shown in Figure 3. Lanes marked 'no oligo' show the pattern of reactivity of DEPC with the plasmid alone. Lanes marked '5meC' show the pattern of reactivity in the presence of a complementary 17-nucleotide pyrimidine third strand containing 5-methylcytosine in place of cytosine, lanes marked '5′Acr' show the pattern of reactivity in the presence of a complementary 17-nucleotide pyrimidine strand containing 5-methylcytosine and an acridine derivative covalently attached at the 5′ end, and lanes marked 'non-sense' show the pattern of reactivity in the presence of a non-complementary 11-nucleotide pyrimidine strand with the sequence 5′ TTTCCT-CCTCT 3′. The results of both DEPC and DMS footprinting are shown in summary form in Figure 4.

DEPC Footprinting on the pyrimidine strand

In the pyrimidine strand of the duplex, no protection from DEPC reactivity is seen as there are no adenine residues involved in triple helix formation (Figure 3, pyrimidine strand). However when the oligo-acridine is used to form the triplex, a very strong DEPC hypersensitive site is induced at the first, but not the second and third adenine residues outside the triple helix at the 5′ junction (Figure 3, lane marked '5′ Acr' and Figure 4, upper strand). This hypersensitive adenine is adjacent to the intercalated acridine, and is indicative of a conformational change induced by intercalation at this site. Therefore, in contrast to the adenines within the triple helix which have their N-7 atoms protected from DEPC modification by Hoogsteen pairing, the N-7 atom of this adenine is very exposed. If the oligo-acridine is substituted by an oligonucleotide attached to an orthophenanthroline moiety at the 5′ end (Figure 1) for DEPC footprinting studies, an identical

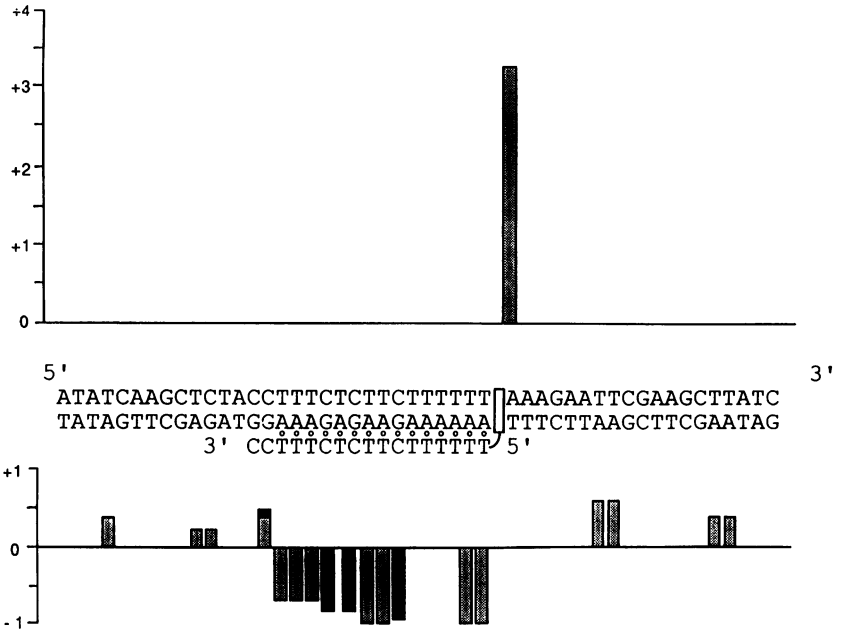

Fig. 4. Summary of chemical footprinting studies of the 17-mer-acridine complex with double-stranded target DNA. The pattern of DEPC reactivity is indicated with grey bars and the pattern of DMS reactivity is indicated with solid bars. The upper and lower plots refer to the upper and lower strands respectively. The values on the vertical scale are arbitrary and are taken from densitometric analysis of polyacrylamide gels by calculating the logarithmic value of the ratio between the density of bands, versus a control lane with no triplex formation. Open circles within the triplex indicate putative Hoogsteen base-pairs within the triplex, and the open rectangle within the sequence represents the position of the intercalated acridine. Adenines within the triple helix footprint which do not show protection from DEPC modification are also non-reactive in duplex DNA.

result is obtained (data not shown). Orthophenanthroline can also intercalate into DNA (18), and the induced hypersensitivity of the adenine residue towards DEPC indicates that the conformational change induced at this site is a result of intercalation itself, not because of specific interaction with the acridine residue. If identical reactions are conducted with the omission of only DEPC (data not shown), no chemical cleavage is observed, indicating that direct reaction of the chromophores with the DNA is not responsible for this observation.

DEPC Footprinting on the Purine Strand

A strong protection of adenine residues from DEPC modification is found on the purine strand only when specific triplex formation occurs, shown in Figure 3 within the bracketed region of lanes marked 'purine strand', and in Figure 4, lower strand. DEPC carboxyethylates purines at N-7, provided they are exposed because they are in a *syn* conformation (24,25), and in intramolecular triplexes, where one half of the purine strand is unpaired (15, reviewed in 26). In intermolecular triplex formation, the N-7 of adenines in the Watson-Crick strand is involved in Hoogsteen pairing with the third strand which lies in the major groove. The N-7 of these adenines is rendered inaccessible to DEPC and this results in the strong protection from modification that we observe upon triplex formation. In contrast, the penultimate guanine residue at the 3′ end of the triple helix shows a slight reactivity to DEPC. This indicates that either the last two guanines do not participate in triplex formation, or that there is some transient dissociation of the triple helix at the 3′ end, probably resulting from the fact that the last cytosine is not methylated and thus Hoogsteen pairing is destabilised. Thermal denaturation profiles of the triplex-to-duplex transition (data not shown) indicate melting temperatures of 15°C ± 1°C for the unmodified third strand, 22°C ± 1°C for the oligonucleotide containing 5-methylcytosine and 35°C ± 1°C for the oligonucleotide containing both modifications, confirming that stable triplex formation occurs in all cases.

In addition to a footprint within the triple helix region, an increase in DEPC reactivity of the bases outside the triple helix is seen, in a region extending about 20-bp on either side of the triplex-duplex junctions. This may result from local destabilisation of the duplex by triplex formation, or may merely be a local concentration effect, whereby DEPC prevented from reacting in the region forming the triplex instead reacts elsewhere. However, since these reactions were conducted on a 3Kbp plasmid, of which only 17-bp are involved in triplex formation, the former explanation seems more likely.

DMS Footprinting on the Pyrimidine Strand

The results of DMS footprinting are summarised in Figure 4. Guanine residues within the triple helix are strongly protected from methylation at N-7, in accordance with their involvement in triple helix formation, with the exception of the penultimate guanine residue at the 3′ end for which an enhanced reactivity is seen. This is surprising since this guanine residue and its neighbouring guanine which has unchanged reactivity would be expected to be involved in Hoogsteen pairing with the third strand of the triple helix. It has previously been shown that the 3′ end of a triplex-duplex junction shows enhanced reactivity towards DMS (27). In our case, as for DEPC footprinting, this result indicates that the triplex-duplex junction is either two base-pairs within the pyrimidine stretch and that the last two residues at the 3′ end of the third strand are not involved in Hoogsteen pairing, or that there is transient dissociation of the third strand at the 3′ junction.

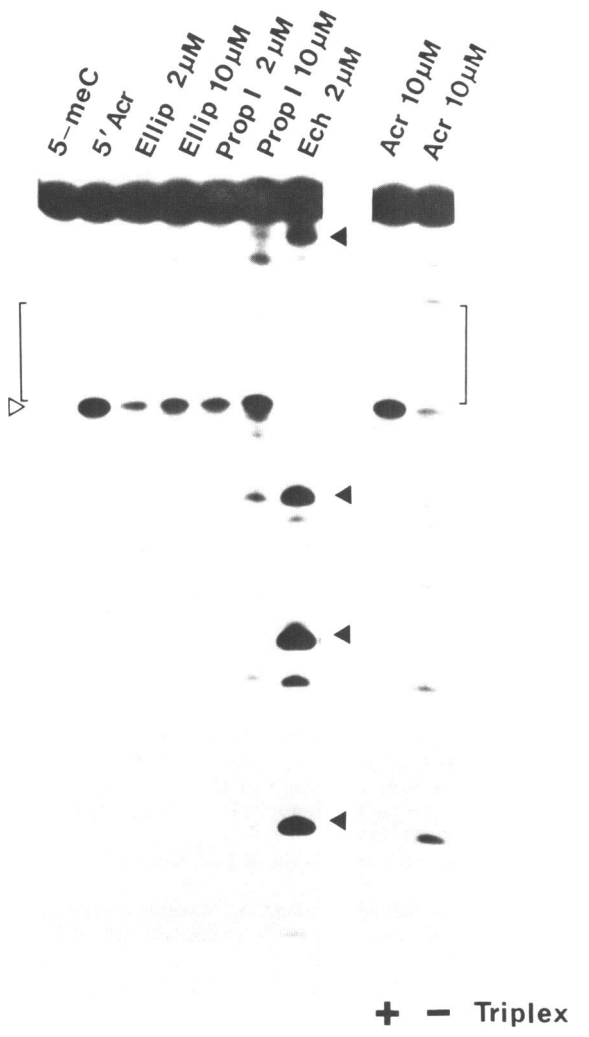

Fig. 5. Footprinting of the effect of free intercalators on the DEPC reactivity of the pyrimidine strand of the triple helix. The Lane marked '5-meC' shows the pattern of reactivity in the presence of the complementary 17-mer pyrimidine oligodeoxynucleotide with no attached chromophore, and the lane marked '5'Acr' shows the pattern of reactivity in the presence of the complementary 17-mer with an acridine covalently attached to the 5' end. The lanes marked 'Ellip', 'Prop I' 'Ech' and 'Acr' show the effect on DEPC reactivity of adding the specified concentration of free ellipticine, propidium iodide, echinomycin or acridine respectively to the restriction fragment containing the triple-helix. The lane marked 'Acr−triplex' shows the effect of adding free acridine (10μM) to the duplex in the absence of triple helix. All lanes left of and including the plus sign have a triple strand bound. The open triangle points to the 5' triplex-duplex junction, the closed triangles to the echinomycin induced DEPC hypersensitive sites, and the region of triple helix formation is indicated by a bracket. The gel reads 5' (top) to 3' (bottom).

II. Intercalation of Free Planar Chromophores at the Triplex-Duplex Junction

If DEPC footprinting reactions are performed using the triple helix-forming strand lacking the covalently attached acridine, but in the presence of free acridine, an identical hypersensitivity is observed (Fig 5, lane marked 'Acr'). This induced hypersensitivity is as strong as that observed for the acridine-oligo conjugate (lane marked '5' Acr'). Therefore, intercalation of free acridine at the 5' triplex-duplex junction in the presence of the triple helix induces a conformational change which can be detected by DEPC. Without triple helix formation, only a slight enhancement of DEPC reactivity was detected at this site (Figure 5 lane 'Acr−Triplex').

The 5' junction of the triple helix has already been determined to be a strong binding site for free ellipticine by photo-footprinting (19). Therefore we tested the ability of a range of free intercalating molecules in addition to the acridine derivative to bind to and induce DEPC hypersensitivity at the triplex-duplex junction. The results of these experiments are shown in figure 5. Lanes left of the plus sign all have the triple helix present. The intercalators acridine (lane marked 'Acr', + triplex) propidium iodide (lanes marked 'Prop I') and an ellipticine derivative (lanes marked 'Ellip') all induced DEPC hypersensitivity at the 5' triplex-duplex junction in a concentration dependent manner when present in solution. In contrast, echinomycin (lanes marked 'Ech'), actinomycin D and the minor groove binding drugs DAPI or Hoescht 33342 did not induce DEPC hypersensitivity at the triplex-duplex junction when present in solution (data not shown). Note the strong DEPC hypersensitivity induced by echinomycin binding at other sites in the restriction fragment. These echinomycin binding sites are not influenced by triple helix formation as identical results were obtained without the presence of the third strand. DEPC hypersensitivity at the 5' triplex-duplex junction was also induced by the intercalators dimethyldiazaperopyrenium (28) and daunomycin (data not shown). Thus a range of chemically unrelated intercalators are able to bind to the triplex-duplex junction and induce a conformational change which is revealed by DEPC hypersensitivity of a neighbouring adenine residue.

DISCUSSION

In this study, we have demonstrated that intercalation of a series of planar chromophores at the 5' junction of an intermolecular triplex induces a conformational change at the intercalation site which is revealed by DEPC hypersensitivity. DEPC hypersensitivity is a property of intercalation into this site *per se*, as a range of chemically unrelated intercalators induce a conformational change, but groove binding drugs do not. It appears that the 5' triplex-duplex junction has an altered conformation prior to intercalation, as shown by a strongly enhanced binding of an ellipticine derivative to this site (19). This altered conformation creates a strong intercalation site which is not reactive to DEPC in the absence of an inserted planar chromophore. There is evidence that the 5' triplex-duplex junction has a kinked conformation (D.A.Collier, unpublished observation), which may tilt base-pairs at the junction towards the minor groove, thus creating a site which is receptive to ligand binding. Intercalation then results in a *further* conformational change which makes the N-7 of the first adenine of the duplex at the junction very exposed to chemical attack. This conformational change may depend not only on the presence of a triplex-duplex junction, but on local sequence context. Further experiments will be required to determine this.

We have considered four possibilities for this induced hypersensitivity, all of which involve a conformational change of the adenine residue in the intercalation site. Firstly, this base could be in a Hoogsteen conformation, as has been demonstrated for adenines adjacent to echinomycin binding sites (6,7) in which the N-1 and N-3 become exposed. However, studies with the base analogue tubercidin indicate that accessibility of N-3 or N-1 of adenines does not induce DEPC hypersensitivity (4). Furthermore osmium tetroxide footprinting studies on the binding

of echinomycin to a DNA fragment in which Hoogsteen pairing is prevented indicate that this conformation is not the source of chemical hypersensitivity (5). Secondly, the adenine could be in a *syn* conformation. This is analogous with the hypersensitivity of purines towards DEPC in left-handed Z-DNA (24,25). However, molecular modelling studies on the 5′ triplex-duplex junction indicate that this is unlikely because of energetic considerations (Sun, J-S, unpublished observations). Thirdly, widening of the major groove as a result of local unwinding induced by intercalation may result in exposure of the N-7 of this adenine, as proposed for echinomycin binding sites (5). This may be enhanced by a charge effect, in which the intercalated residue creates an electrophilic and hydrophobic pocket adjacent to the reactive N-7. Lastly, the adenine may no longer be part of the duplex at all but instead 'single-stranded'. However, this also seems unlikely because the strong base-chromophore stacking interactions involved in the intercalation complex would be lost.

In addition to the intercalator induced DEPC hypersensitivity at the 5′ triplex-duplex junction, duplex DNA adjacent to the triple helix displayed enhanced reactivity towards DEPC in a region extending for more than 20 base-pairs either side of the triplex-duplex junction, independently of the presence of intercalating molecules. This effect has also been observed for DNaseI footprinting of the triple helix[3]. Although the level of enhanced reactivity towards DEPC is substantially less than that observed for the 5′ junction-intercalator complex, this result indicates that triplex formation may induce longer range conformational effects, such as an unwinding of adjacent duplex DNA.

In conclusion, DEPC footprinting is a simple and rapid technique for investigating intermolecular triplex formation. This probe has the advantage of being unreactive to pyrimidines and thus does not disrupt the third strand from binding to the duplex, thus making it a sensitive probe for pyr·pur·pyr triple helix formation and conformation.

Whatever the precise nature of the conformation induced at the 5′ triplex-duplex junction by intercalation, we have demonstrated that chemical modification by DEPC can be directed predominantly to a single base in a long fragment of double-stranded DNA. Thus the potential exists for a simple system, using free intercalators and unmodified oligodeoxynucleotides, to perform site directed mutagenesis or chromophore induced cleavage of DNA adjacent to polypurine stretches.

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