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Triplex formation by a psoralen-conjugated oligodeoxyribonucleotide containing the base analog 8-oxo-adenine

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ABSTRACT

Oligodeoxyribonucleotides containing thymidine and 8-oxo-2′-deoxyadenosine can form pyr·pur·pyr type triplexes with double-stranded DNA. Unlike triplexes whose third strands contain thymidine and deoxycytidine, the stability of these triplexes is independent of pH. We have prepared d-ps-TAAATAAATTTTTAT-L [I(A)], where A is 8-oxo-2′-deoxyadenosine, ps is 4′-hydroxymethyl-4,5′,8-trimethylpsoralen and L is a 6-amino-2-(hydroxymethyl)hexyl linker. The oligomer is designed to interact with a homopurine sequence in the promoter region of the human gene coding for the 92 kDa form of collagenase type IV. Oligomer I(A) and oligomer I(C), which contains 2′-deoxycytidine in place of 8-oxo-2′-deoxycytidine, both form stable triplexes at pH 6.2, but only I(A) forms a stable triplex with a model duplex DNA target at pH 7.5, as determined by UV melting experiments. Triplex formation is stabilized by the presence of the psoralen group. Upon irradiation both I(A) and I(C) form photoadducts with the DNA target at pH 6.2, but only I(A) forms a photoadduct at pH 7.5. In these photoreactions oligomer I(A) appears to selectively form a photoadduct with a C in the purine-rich strand of the duplex target. Although a T residue is present in the pyrimidine-rich strand of the target at the duplex/triplex junction, essentially no adduct formation takes place with this strand, nor is interstrand cross-linking observed. The extent of photoadduct formation decreases with increasing temperature, behavior which is consistent with the UV melting curve of the triplex. A tetramethylrhodamine derivative of I(A) was prepared and found to cross-link less extensively than I(A) itself. Oligomer I(A) is completely resistant to hydrolysis when incubated for 24 h in the presence of 10% fetal bovine serum at 37°C, although it is hydrolyzed by S_1 nuclease. The properties of oligomer I(A) suggest that 8-oxo-containing oligomers may find utility as antigene oligonucleotide reagents.

INTRODUCTION

Oligodeoxyribonucleotides can interact with purine rich sequences in double-stranded DNA to form triple-stranded complexes (for recent reviews see 1–4). These interactions may be useful in designing ‘antigene’ oligonucleotides which can specifically bind to DNA for the purposes of modifying or controlling gene expression (5–15). Two types of triplex ‘motifs’ are currently recognized, the pyr·pur·pyr motif and the pur·pur·pyr motif. In this notation the third strand is designated first. The third strand oligonucleotide in the pyr·pur·pyr motif is oriented parallel to the purine strand and usually consists of thymidines and deoxycytidines, which form T·A·T and C^+·G·C triads respectively with the A·T and G·C base pairs of the target duplex. The C of a C^+·G·C triad must be protonated in order to form two hydrogen bonds with G of the G·C base pair (16–20). This requirement limits the pH range over which triplex formation of this type can occur. Usually this range is below physiological pH, particularly when the third strand oligomer contains multiple contiguous C residues. Consequently this requirement could limit the use of antigene oligonucleotides of this type, especially in experiments involving living cells.

A number of base analogs have been designed to circumvent this problem. These include the pyrimidine analogs pseudoisocytosine (21,22), 6-amino-2′-*O*-methylcytidine (23) and 4-amino-5-methyl-2,6-pyrimidione (24) and the purine analogs 3-methyl-5-amino-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (19,25,26), 8-oxo-adenine (27–30) and 6-methyl-8-oxoadenine (31,32). Each of these analogs contains two hydrogen bond donor groups within its structure which can interact with the O-6 and N-7 of G. The 8-oxoadenine analogs are particularly interesting because they are easy to prepare and the nucleoside base adopts the *syn* conformation, a conformation which enables the analog to contact G with two hydrogen bonds.

Studies from our laboratory and others have shown that third strand oligomers containing thymidine and 8-oxo-2′-deoxyadenosine form stable triplexes with duplex DNA targets at physiological pH. To further explore the scope of these interactions we have prepared an 8-oxo-adenine-containing oligomer which is designed to bind to a homopurine tract found in the

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promoter region of the human gene for the 92 kDa form of collagenase type IV, a metalloproteinase implicated in a variety of disease processes, including tumor metastasis. This oligomer, which contains seven 8-oxo-adenine residues, is derivatized at its 5′-end with 4′-hydroxymethyl-4,5′,8-trimethylpsoralen and at its 3′-end with a 6-amino-2-(hydroxymethyl)hexyl linker. The 5′-trimethylpsoralen provides a photoreactive functional group which should enable the oligomer to form a photoadduct with the duplex target (33). By analogy with other 3′-modified oligonucleotides, the 3′-aminohexyl group should increase the resistance of the oligomer to degradation by exonucleases (34). The ability of this oligomer to interact and to form photoadducts with a model duplex DNA target has been evaluated and compared with oligomers lacking psoralen or containing C in place of 8-oxo-adenine.

MATERIALS AND METHODS

Protected deoxyribonucleoside phosphoramidites, the trimethylpsoralen phosphoramidite and controlled pore glass supports were purchased from Glen Research. [γ-^{32}P]ATP was purchased from Amersham Inc. and T4 polynucleotide kinase was purchased from United States Biochemical Corp. Tetramethylrhodamine-5-isothiocyanate isomer G was purchased from Molecular Probes Inc. Polyacrylamide gel electrophoresis was carried out on 14 × 16 × 0.75 cm 15% or 20% polyacrylamide gels which contained 7 M urea (35). The TBE running buffer contained 89 mM Tris, 89 mM boric acid and 0.2 mM ethylenediamine tetraacetate buffered at pH 8.0. The gel loading buffer contained 90% formamide, 0.05% xylene cyanol and 0.05% bromphenol blue in TBE. Reversed phase HPLC was carried out on Microsorb C-18 columns purchased from Rainin Instruments.

Oligonucleotide syntheses

Oligodeoxyribonucleotides, whose sequences are shown in Table 1, were synthesized on nucleoside-derivatized or 3′-amino-modifier C-7 controlled pore glass supports using an Applied Biosystems Model 392 DNA/RNA synthesizer. The oligomers were prepared using commercially available protected 5′-*O*-dimethoxytrityldeoxyribonucleoside-3′-*O*-*N*,*N*-bis(diisopropylamino)-β-cyanoethyl phosphoroamidites following standard procedures (36). The synthesizer was programed to remove the last 5′-terminal dimethoxytrityl group from the protected oligomer. The psoralen-derivatized oligomers were prepared on the controlled pore glass support using 2-[4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen]hexyl-1-*O*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)] phosphoramidite in the final coupling reaction. The oligomers were deprotected by treatment with a solution of 50% concentrated ammonium hydroxide in pyridine at 55°C for 6 h. Oligomers containing 8-oxo-adenine were deprotected by treating the support-bound oligomer with a solution of ethylenediamine in 95% ethanol (1:1 v/v) at room temperature for 16 h. The deprotected oligomers were purified by C-18 reversed phase HPLC on a 1.0 × 25 cm Microsorb C-18 column using a linear gradient of 2–20% acetonitrile in 50 mM sodium phosphate buffer, pH 5.8. The column was eluted at a flow rate of 2.0 ml/min and monitored at 254 nm. The oligomers were desalted on a C-18 reverse phase cartridge. The oligomers migrated as single bands on 20% polyacrylamide gels. The extinction coefficients of oligomers **IV** and **V** were determined after digestion with snake venom phosphodiesterase as previously described (27). These extinction coefficients were also used for oligomers **I(A)** and **I(C)**.

Table 1. Triplex formation between oligonucleotides and duplex DNA targets

Oligomer		T_m (°C)[a]	
		pH 6.2	pH 7.5
d-ps-T**AAA**T**AAA**TTTTT**A**T-L	**I(A)**	28	27
d-ps-TCCCTCCCTTTTTCT-L	**I(C)**	33	<0
d-T**AAA**T**AAA**TTTTT**A**T	**IV**	n.d	16[b,c]
d-TCCCTCCCTTTTTCT	**V**	26[b,d]	n.d.

[a]Melts were carried out in a buffer containing 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM MOPS at the indicated pH. The target was duplex **II·III.** Oligomer strand concentration was 1.5 μM. n.d., not determined.
[b]The target was: d-AGGGAGGGAAAAAGA
TCCCTCCCTTTTTCT-d
[c]pH 7.0.
[d]pH 6.0.

Tetramethylrhodamine conjugate of oligomer I(A)

A solution of 5 mg tetramethylrhodamine-5-isothiocyanate in 0.6 ml 95% ethanol was added to a solution of 0.14 μmol oligomer **I(A)** in 1 ml 0.2 M bicine–sodium hydroxide buffer, pH 9.0, and the reaction mixture was stirred at room temperature in the dark for 18 h (37). The solvents were evaporated and the residue was dissolved in a minimal volume of 0.1 M sodium phosphate buffer, pH 5.8, containing 25% acetonitrile. The solution was loaded onto a 1.6 × 20 cm Sephadex G-15 column and the column was eluted with 25% acetonitrile in 0.1 M sodium phosphate buffer to free the oligonucleotide from unreacted dye. The rhodamine-conjugated oligomer, whose retention time was 13.2 min, was then separated from unreacted oligomer, whose retention time was 11.8 min, on a 0.46 mm × 15 cm C-18 reversed phase HPLC using a 30 ml linear gradient of 2–60% acetonitrile in 50 mM sodium phosphate buffer, pH 5.8, at a flow rate of 1.0 ml/min. A total of 0.07 μmol rhodamine-conjugated **I(A)** was obtained by this procedure.

Melting experiments

Melting experiments were carried out in a buffer containing 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS). A solution containing 3.0 μM preformed duplex **II·III** or duplex **VI·VII** was mixed with an equal volume of 3.0 μM oligomer at room temperature. The solution was then incubated overnight at 4°C. The solution was loaded into a cuvette at 0°C and the absorbance versus temperature profile was measured using a Cary 3E UV/vis spectrophotometer fitted with a thermostatted cell block and temperature controller. The cell block was continuously purged with dry nitrogen at low temperature to prevent moisture condensation. The solution was heated at a rate of 0.5°C/min.

Photocross-linking experiments

Oligomers **II** and **III** were each phosphorylated with [γ-^{32}P]ATP in the presence of polynucleotide kinase and the phosphorylated oligomers were purified by polyacrylamide gel electrophoresis.

Solutions containing 0.8 µM [^{32}P]**II·III** or **II**·[^{32}P]**III** and 10 µM **I(A)** or **I(C)** in 10 µl 0.1 M sodium chloride, 20 mM magnesium chloride, 20 mM MOPS were prepared and stored overnight at 4°C. The solutions were pre-equilibrated at the temperature of the experiment for 20 min prior to irradiation. The solutions were then irradiated at 365 nm for 16 min as previously described (38). The solvents were evaporated on a Speed-Vac and the residue was dissolved in 10 µl gel loading buffer. The samples were electrophoresed on a 15% polyacrylamide gel run under denaturing conditions. The gels were dried and imaged by autoradiography and by a FujiX BAS 1000 phosphorimager.

Nuclease stability experiments

A 0.45 A_{260} U sample of oligomer **I(A)**, **I(C)**, **IV** or **V** was dissolved in 20 µl of Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum. The samples were incubated at 37°C. Aliquots of 2 µl were removed at 0, 1, 3, 6, 9, 12 and 24 h and diluted into 98 µl 2% acetonitrile in 50 mM sodium phosphate buffer, pH 5.8. The samples were analyzed by C-18 reversed phase HPLC.

Oligomers **I(A)** and **I(C)** were also treated with S_1 nuclease. A 0.45 A_{260} U sample of each oligomer was dissolved in 40 µl buffer containing 50 mM sodium acetate, 1 mM zinc chloride, 250 mM sodium chloride and 50 µg/ml bovine serum albumin. The oligomer solution was incubated with 0.4 or 2.0 U S_1 nuclease at 37°C. Aliquots of 4 µl were removed at 1, 2, 3, 4, 6 and 24 h, diluted into 96 µl 2% acetonitrile in 50 mM sodium phosphate buffer, pH 5.8, and analyzed by C-18 reversed phase HPLC.

RESULTS

Synthesis of psoralen-conjugated triplex-forming oligodeoxyribonucleotides

The general structure and the sequences of the psoralen-conjugated oligomers **I(X)** are shown in Figure 1. The oligomers are targeted to the purine-rich strand of duplex target **II·III**, whose sequence is also shown in Figure 1. The oligomers were synthesized on a commercially available support which allows introduction of a 6-amino-2-(hydroxymethyl)hexyl linker arm at the 3′-end of the oligomer. The 5′-end of the oligomer was derivatized with trimethylpsoralen using a commercially available phosphoramidite derivative of 2-[4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen]hexyl-1-ol. Control oligomers **IV** and **V**, whose sequences are shown in Table 1, were also prepared. Oligomers **I(C)** and **V** were deprotected by treatment with concentrated ammonium hydroxide in pyridine at elevated temperature, whereas oligomers **I(A)** and **IV** were deprotected by treatment with a solution of 50% ethylenediamine in ethanol at room temperature.

The 6-amino-2-(hydroxymethyl)hexyl linker arm of **I(A)** and **I(C)** was further derivatized. Reaction of **I(A)** with tetramethylrhodamine isothiocyanate gave the tetramethylrhodamine conjugate **Ir(A)** in ~50% yield. The hydroxyl group of the linker arm of **I(A)** could be phosphorylated using polynucleotide kinase and [γ-^{32}P]ATP. Similar reaction of the rhodamine derivative **Ir(A)** in the presence of [γ-^{32}P]ATP and polynucleotide kinase gave, after electrophoresis on a 20% polyacrylamide gel, a single radioactively labeled band which was also florescent when illuminated with long wavelength UV light (data not shown).

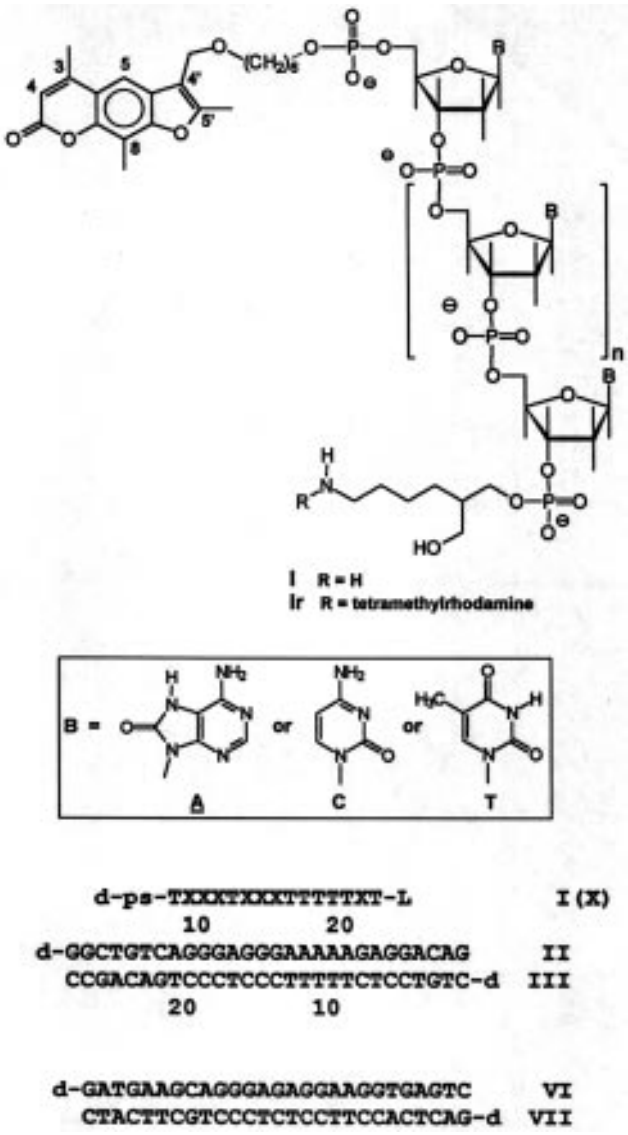

Figure 1. Structure of psoralen-conjugated oligodeoxyribonucleotide. The sequence of the triplex forming oligomer **I(X)**, where X is A or C, is shown at the bottom of the figure, as is the sequence of the target duplex **II·III**. Ps is the trimethylpsoralen linked via a hexamethylene linker to the 5′-end of the oligomer, L is the 6-amino-2-(hydroxymethyl)hexyl group at the 3′-end of the oligomer and R is H or tetramethylrhodamine.

Melting experiments

The interactions of psoralen-derivatized oligomers **I(A)** and **I(C)** with duplex DNA target **II·III** were studied by UV melting experiments. The absorbance versus temperature profiles for **I(A)·II·III** and **I(C)·II·III** at pH 7.5 are shown in Figure 2. The duplex target **II·III** shows a single transition with a T_m of 73°C. A similar melting curve with no additional hypochromicity was observed for **I(C)·II·III**, which indicates that **I(C)** does not interact with **II·III** at pH 7.5. In contrast to the behavior of **I(C)**, the melting profile for **I(A)·II·III** showed two transitions. The first transition, whose midpoint occurs at 27°C, corresponds to dissociation of **I(A)** from duplex **II·III**. Oligomer **I(C)** does form

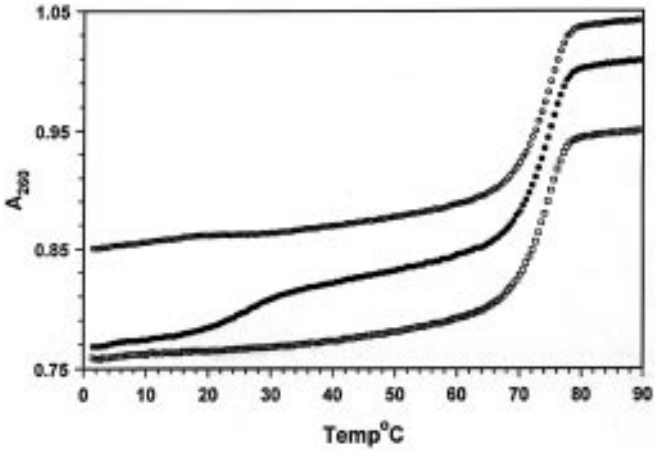

Figure 2. Absorbance versus temperature profiles of **II·III** (□), **I(C)·II·III** (○) and **I(A)·II·III** (●). The melts were carried out in a buffer containing 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM MOPS, pH 7.5. The concentration of each strand was 1.5 μM.

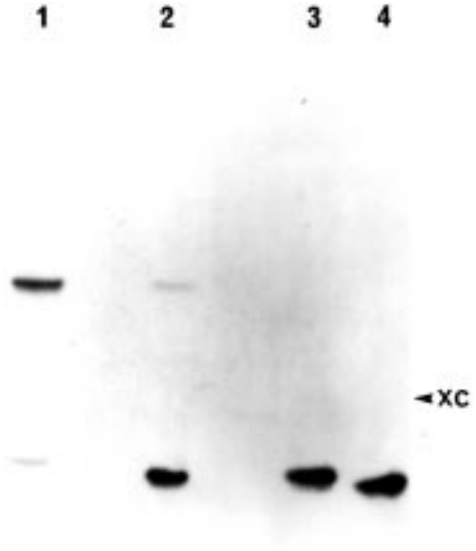

Figure 3. Photoadduct formation between **I(A)** and **II·III**. Reaction mixtures were irradiated with long wavelength UV light for 10 min at 0°C in a buffer containing 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM MOPS, pH 7.5, 10 μM **I(A)** and 0.8 μM **II·III**. The reactions were analyzed on a 15% denaturing polyacrylamide gel. Lane 1, **I(A)** and [^{32}P]**II·III**; lane 2, **I(A)** and **II**·[^{32}P]**III**; lane 3, [^{32}P]**II**; lane 4, [^{32}P]**III**. XC is xylene cyanol.

a stable triplex with **II·III** at pH 6.2, as shown in Table 1. At this pH the stability of the third strand interaction of **I(C)·II·III** is greater than that of **I(A)·II·III**.

Oligomers **I(A)** and **I(C)**, which are derivatized with psoralen, form more stable triplexes than oligomers **IV** and **V**, which lack the psoralen group. Thus the triplexes formed by **IV** and **V** melt 11 and 7°C lower respectively than those formed by oligomers **I(A)** and **I(C)**. As indicated in Table 1, the duplex target for these oligomers is shorter than duplex **II·III**. We have found in other triplex systems that the third strand T_m of the triplex formed between an oligopyrimidine and a target duplex of the same chain length is 2–3°C higher than that of the triplex formed between the same oligopyrimidine and a longer duplex target. Thus the 7–11°C increase in the third strand T_m values observed for oligomers **I(A)** and **I(C)** is most likely due to the presence of the psoralen group and is not a consequence of the increased size of the target.

The interaction of **I(A)** with purine-rich target **VI·VII** (Fig. 1) was also investigated. This duplex contains a homopurine tract consisting of 13 G·C and 9 A·T base pairs which are interrupted by a single C·G and two T·A base pairs. A 1:1 mixture of **I(A)** and **VI·VII** gave a single melting transition at 72°C, which is the melting temperature of the duplex.

Photoadduct formation with duplex II·III

The ability of oligomer **I(A)** to form a photoadduct with duplex target **II·III** was determined. In these experiments one of the strands of the duplex was labeled with a [5′-^{32}P]phosphoryl group. Triplex **I(A)·II·III** was irradiated at 365 nm and the products of the reaction were analyzed on a denaturing polyacrylamide gel. As shown in Figure 3, when the photoreaction was carried out at pH 7.5 at 0°C ~75% of the radioactivity migrated as a band of reduced mobility when strand **II** of the duplex was end-labeled. Very little photoadduct was observed when strand **III** of the duplex was labeled. This result suggests that photoadduct formation occurs primarily with pyrimidine residues in strand **II**. The mobility of the photoadduct formed with labeled strand **II** and the lack of a concomitant amount of photoadduct when strand **III** was labeled indicates that interstrand cross-linking did not take place.

Oligomer **I(A)** formed photoadducts with strand **II** of duplex target **II·III** at both pH 7.5 and pH 6.2. Cross-linking increased with increasing oligomer concentration and reached a maximum at an oligomer concentration of 10 μM. As shown in Figure 4, photoadduct formation between **I(A)** and **II·III** decreases as the temperature of the reaction increases. These reactions were carried out at pH 7.5 in the presence of 10 μM oligomer. A sigmoidal curve was obtained whose midpoint was 25°C.

The rhodamine conjugate of **I(A)**, **Ir(A)**, also formed a photoadduct with strand **II** of the target duplex. However, the extent of cross-linking was <10% when the reaction was carried out at pH 7.5 at 20°C (data not shown).

In contrast to the behavior of oligomer **I(A)**, oligomer **I(C)** formed photoadducts only at pH 6.2. Adduct formation, which again occurred almost exclusively with strand **II** of the target duplex, reached a maximum level of 85% when the reaction was carried out in the presence of 10 μM **I(C)** at 0°C. No cross-linking was observed at pH 7.5, a result consistent with the inability of **I(C)** to form a triplex at this pH.

The ability of **I(A)** to form photoadducts with duplex **VI·VII** was also examined. These reactions were carried out at 0°C in the presence of 10 μM **I(A)**. Consistent with its lack of interaction with **VI·VII**, no photoadduct formation was observed between **I(A)** and either strand of the duplex.

Nuclease stability

The stability of oligomers **I(A)**, **I(C)**, **IV** and **V** to the nuclease activity found in fetal bovine serum was examined. The oligomers were incubated at 37°C in EMEM containing 10% fetal bovine serum. Aliquots of the reaction solution were

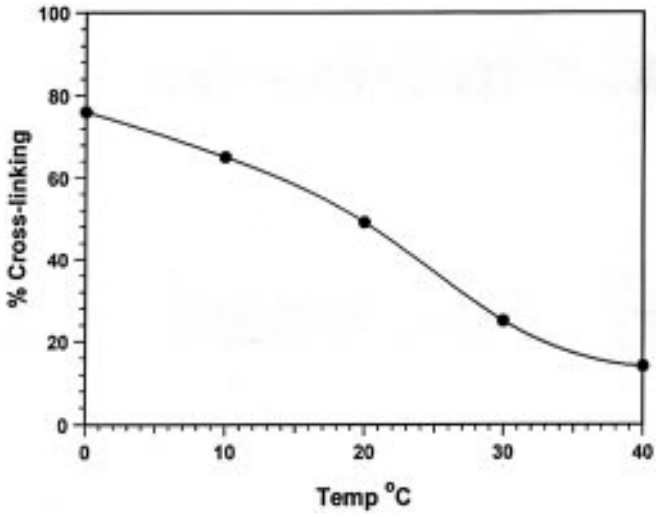

Figure 4. Effect of temperature on photoadduct formation between **I(A)** and **II·III**. Reaction mixtures containing 10 μM **I(A)** and 1 μM [^{32}P]**II·II** in 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM MOPS, pH 7.5, were irradiated for 10 min at the indicated temperature and the reaction products were analyzed by polyacrylamide gel electrophoresis.

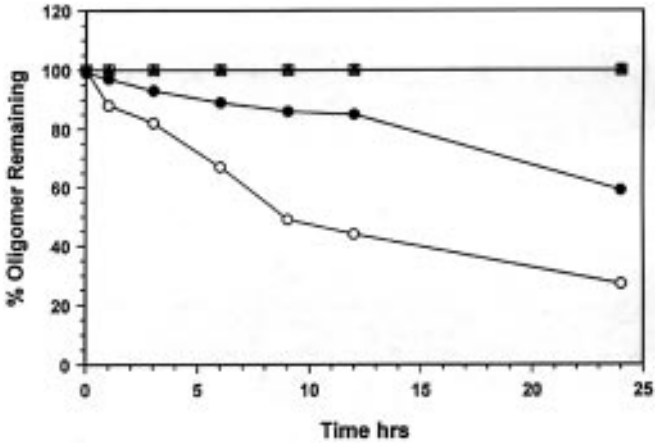

Figure 5. Stability of oligonucleotides in EMEM containing 10% fetal bovine serum. Oligonucleotides **I(A)** (▲), **I(C)** (□) **IV** (●) or **V** (○) were incubated at 37°C and analyzed by reversed phase HPLC.

removed at various times over a 24 h period and examined by reversed phase HPLC. The results are shown in Figure 5, where the percent of intact oligomer is plotted as a function of time.

Oligomers **I(A)** and **I(C)**, both of which contain the 3′-terminal 6-amino-2-(hydroxymethyl)hexyl linker and 5′-trimethylpsoralen, were completely resistant to nuclease hydrolysis for at least 24 h. In contrast, oligomers **IV** and **V**, whose 3′- and 5′-termini are not derivatized, were hydrolyzed. The rate of hydrolysis of **V**, which contains T and C nucleosides, was approximately twice that of oligomer **IV**, which contains T and 8-oxo-adenine nucleosides.

The stabilities of **I(A)** and **I(C)** toward hydrolysis by S_1 nuclease were also examined. Both oligomers were hydrolyzed within 1 h when incubated with 2 U of enzyme at 37°C. Less rapid hydrolysis was observed with deceasing amounts of enzyme, but there appeared to be no significant difference between the rates of hydrolysis of the two oligomers.

DISCUSSION

Oligonucleotide **I(A)** contains thymidine and 8-oxo-2′-deoxyadenosine and was designed to bind as a third strand to the purine-rich tract of strand **II** of duplex **II·III**. This purine sequence is found in the promoter region of the human gene which encodes the 92 kDa form of collagenase type IV (39). It is located at positions –514 to –500, which is 12 nt downstream of one of the putative TPA response elements found in this promoter.

The oligonucleotides used in this study were synthesized by standard solid phase phosphoramidite chemistry. Although deoxycytidine-containing oligomers **I(C)** and **V** were readily deprotected using the standard treatment with 50% concentrated ammonium hydroxide in pyridine, only partial deprotection of 8-oxo-2′-deoxyadenosine-containing oligomers **I(A)** and **IV** was achieved, as evidenced by the presence of multiple peaks in the HPLC of the deprotected oligomer. It appeared that the increased negative charge density on the oligomer caused by ionization of the multiple 8-oxo-adenine bases could account for the lack of complete deprotection. To overcome this problem the oligomer was treated with 50% ethylenediamine in 95% ethanol at room temperature. Under these conditions the benzoyl groups of the 8-oxo-adenine residues were completely removed.

The amino group of the 6-amino-2-(hydroxymethyl)hexyl linker arm provides a convenient ‘handle’ for preparing fluorescently tagged oligomers. This group could be readily derivatized with tetramethylrhodamine isothiocyanate to give the rhodamine-conjugated oligomer **Ir(A)**. In addition, we found that the linker arm hydroxyl groups of oligomers **I(A)** and **Ir(A)** could be phosphorylated enzymatically by polynucleotide kinase in the presence of [γ-^{32}P]ATP. The position of this primary hydroxyl group relative to the neighboring 3′-phosphodiester group is the same as that of the 5′-hydroxyl group relative to the 5′-terminal internucleotide linkage in a polynucleotide chain and this may account for the ability of the enzyme to carry out the reaction. This reaction thus allows the introduction of a radioactive label into the oligomer, which would otherwise not be possible due to the presence of the trimethylpsoralen group at the 5′-end of the oligomer.

Both oligomers **I(A)** and **I(C)** form stable triplexes with duplex target **II·III** at pH 6.2. The increased stability of **I(C)·II·III** versus **I(A)·II·III** may reflect the reduced charge repulsion between **I(C)** and duplex **II·III** as a consequence of the presence of the protonated cytosines in this oligomer. A similar reduction in the case of **I(A)** would not be expected because this oligomer lacks protonated bases. Consistent with this argument is the observation that a modified version of **I(A)**, d-ps-TAACTAACTTTTT-CT-L, in which three of the 8-oxo-adenine residues are replaced with 5-methyl-2′-deoxycytidine (C), forms a triplex whose T_m at pH 7.5 is 5°C higher than that of **I(A)·II·III**. At pH 7.5 triplex formation is only observed between **I(A)** and **II·III**. This is not unexpected, because this pH is above the range where oligopyrimidines containing multiple contiguous cystosines are normally capable of forming stable triple-stranded structures (19), whereas 8-oxo-adenine-containing oligomers can form triplexes whose stability is essentially independent of pH (27,28).

Triplex formation by oligomer **I(A)**, which contains seven 8-oxo-adenine residues, is clearly discernable in the absorbance versus temperature profiles shown in Figure 2. A similar profile was observed for the triplex formed by oligomer **IV** and its target duplex. This behavior is in marked contrast to that reported for an 18mer, d-TTATTTAAAATAATTATT, which contains eight 8-oxo-adenine residues (28). In this case the UV absorbance versus temperature profile for a 1:1 mixture of the 18mer and a 28mer duplex DNA target gave no indication of a third strand transition and no additional hypochromicity was observed. This oligomer did form a triplex, however, as evidenced by gel mobility shift experiments.

Derivatization of the oligomers with trimethylpsoralen appears to enhance triplex stability. This is demonstrated by the 7–11°C increase in the melting temperatures observed for third strand dissociation of triplexes composed of oligomers **I(A)** and **I(C)** relative to those formed by oligomers **IV** and **V**. This stabilization is most likely a consequence of intercalation of the tethered psoralen into the base pair/base triad which forms the duplex/triplex junction.

Irradiation of **I(A)·II·III** or **I(C)·II·III** with 365 nm UV light at pH 6.2 resulted in formation of a photoadduct with strand **II** of the duplex target. A very low percentage of photoadduct formation with strand **III** was observed and no interstrand cross-linking was observed. This behavior is in contrast to that seen by Takasugi *et al.* (33), who observed significant levels of interstrand cross-linking in a pyr·pur·pyr triplex by a third strand 5′-(5-hydroxypsoralen)-conjugated oligopyrimidine. In this system the psoralen ring was attached at its 5 position to a hexamethylene linker. High levels of interstrand cross-linking were observed in a pyr·pur·pyr triplex in which trimethylpsoralen was attached at the 4′-methylene position via a 2, 5 or 6 carbon linker arm to the 5′-end of the oligopyrimidine third strand (40). We have also prepared an oligopyrimidine, d-ps-TTTTCTTTTCTTCTT, and have shown that irradiation of the triplex formed by this oligomer gave high levels of interstrand cross-linked photoproduct. In each of these systems the sequence at the duplex/triplex junction, which appears to be the preferred site for intercalation of the psoralen (33,40,41), is 5′-TA-3′/5′-AT-3′. This sequence is also the sequence preferred when free psoralen binds to duplex DNA (42).

Molecular models suggest that psoralen intercalates at the duplex/triplex junction in such a manner that the furan ring is positioned nearest the base in the purine-rich strand and the pyrone ring is positioned nearest the base of the pyrimidine-rich strand of the target (40). This binding mode is supported by cross-linking experiments (33,40,41). However, the photoproducts which are formed are very much dependent upon the nucleotide sequence at the duplex/triplex junction (40).

In our system, where photoadduct formation occurs almost exclusively with strand **II**, intercalation of the psoralen between the 5′-CA-3′ (strand **II**)/5′-TG-3′ (strand **III**) sequence at the duplex/triplex junction would position the furan ring on the 3′-side of C-7 of strand **II** and the pyrone ring on the 3′-side of T-21 of strand **III**. Irradiation would then lead to photoadduct formation between the furan ring and C-7 of strand **II**. It is unlikely that a photoadduct is formed with T-6 of strand **II**. Treatment of the isolated photoadduct with 1 M aqueous piperidine at 90°C for 30 min failed to modify the photoadduct. This treatment is known to hydrolyze the cyclobutane ring of furan–thymidine photoadducts (33, 43, 44) and to cause strand cleavage at the site of pyrone–thymidine photoadducts (45). In addition, recent studies by Bates *et al.* (40) demonstrated that photoadduct formation occurs with a T residue in the lower strand of the 5′-TAA-3′ (upper)/5′-TTA-3′ (lower) sequence at a duplex/triplex junction. Photoadduct formation was not observed with T of the upper strand, even though the 5′-TA-3′ sequence adjacent to the junction is a preferred cross-link site for psoralen.

The apparent selective reaction with C-7 of strand **II** and the lack of reaction with T-21 of strand **II** are unexpected, because free psoralen reacts more efficiently with T than with C in duplex DNA (46). However, our results appear to be consistent with and to extend the observations of Bates *et al.* (40), who found that photoadduct formation does not occur with either strand of a triplex containing a 5′-GA-3′/5′-TC-3′ sequence at its duplex/triplex junction. In this case the furan ring is positioned on the 3′-side of G, a base which cannot undergo photocycloaddition reactions. The psoralen pyrone ring would be positioned next to the T and could form a photoadduct, but does not. The sequence of the duplex/triplex junction of **II·III**, 5′-CA-3′/5′-TG-3′, is similar to that of the Bates and co-workers triplex, 5′-GA-3′/5′-TC-3′, except that C can undergo photocycloaddition reactions with psoralen. As in the case of the 5′-GA-3′/5′-TC-3′ junction, photoadduct formation does not take place with T.

In agreement with the melting data, oligomer **I(A)** also formed a photoadduct with strand **II** of duplex **II·III** when the triplex was irradiated at pH 7.5. In contrast, oligomer **I(C)** showed no evidence of photoadduct formation even when irradiation was carried out at 0°C in the presence of 100 µM oligomer. This result demonstrates that photoadduct formation is a consequence of triplex formation and is not driven by non-selective binding of the tethered psoralen to the duplex target.

Previous experiments have demonstrated that a single 8-oxo-adenine specifically interacts with a G·C base pair of a duplex target (27). Thus it is not surprising that oligomer **I(A)** showed no evidence of interaction with duplex target **VI·VII**. Although this target, whose sequence corresponds to nucleotides –554 to –528 of the 92 kDa form of the human collagenase IV gene, contains a long homopurine tract, the sequence of this tract is not expected to support binding by **I(A)**. No photoadduct formation between **I(A)** and either strand of the target was observed, even at low temperature. This result further demonstrates that binding between the psoralen-derivatized oligomer and a duplex target is determined by interactions between the bases of the oligomer and the target.

The effect of temperature on cross-linking between **I(A)** and **II·III** parallels the melting behavior of the **I(A)·II·III** triplex. As expected, increasing the temperature decreases the amount of photoadduct formation observed. This decrease appears to occur in a cooperative manner and is consistent with dissociation of **I(A)** from the triplex. The temperature at which photoadduct formation is reduced by 50% is approximately the same as the melting temperature for the third strand of the triplex.

Conjugation of **I(A)** with tetramethylrhodamine resulted in a >90% reduction in photoadduct formation. This result suggests that either the rhodamine interferes with triplex formation or that the rhodamine interferes with the photochemical reaction. The latter possibility appears unlikely, because we have previously observed that a 3′-rhodamine-conjugated 5′-psoralen-derivatized oligonucleoside methylphosphonate undergoes extensive cross-linking with a single-stranded RNA target (37). It appears then

that the presence of tetramethylrhodamine at the 3′-end of **Ir(A)** interferes in some way with formation of a stable triplex with **II·III**.

Previous reports have demonstrated that oligodeoxyribonucleotides which are derivatized at their 3′-ends with aminoalkyl groups are protected from the action of 3′-exonucleases (34). Thus it is not surprising that oligomers **I(A)** and **I(C)** are completely resistant to degradation when incubated with 10% fetal bovine serum, medium which contains 3′-exonuclease activity. What was unexpected was the increased resistance of the non-derivatized oligomer **IV** to nuclease hydrolysis. The presence of 8-oxo-adenine, which should exist primarily in the *syn* conformation (47), may confer protection to this oligomer. Similar protection was not observed in the presence of S_1 nuclease, a single-strand-specific endonuclease. Thus when **I(A)** and **I(C)** were incubated with S_1 nuclease both oligomers were hydrolyzed at approximately the same rates. It is not clear if this susceptibility extends to other endonucleases present in the intracellular environment. However, we have recovered intact **I(A)** from human fibrosarcoma HT1080 cells treated with this oligomer, whereas much lower amounts of intact **I(C)** were recovered in similar experiments.

Our results demonstrate that a psoralen-conjugated 8-oxo-adenine-containing oligomer can bind to and selectively photocrosslink with a duplex DNA target at physiological pH. It may be possible to design oligomers of this type to serve as antigene agents. Experiments to test this possibility are currently in progress.

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