7,8-Dihydro-8-oxoadenine as a Replacement for Cytosine in the Third Strand of Triple Helices. Triplex Formation without Hypochromicity

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ABSTRACT: Oligonucleotides containing thymine and cytosine (or 5-methylcytosine) bases are known to bind to specific homopurine sequences in double-stranded DNA by means of T·AT and C^+·GC base triplets. Cytosine in the third strand of such triple helices can be completely replaced by 7,8-dihydro-8-oxoadenine, a base which should not require protonation to form base triplets. Experiments using native PAGE and inhibition of triplex-directed photo-cross-linking demonstrate that triplexes with 7,8-dihydro-8-oxoadenine in the third strand are as stable at pH 6.0 as triplexes with 5-methylcytosine. The stability of triplexes with 7,8-dihydro-8-oxoadenine, unlike those with 5-methylcytosine, is not substantially diminished upon raising the pH to 7.4. Surprisingly, triplex formation with an oligonucleotide containing only thymine and 7,8-dihydro-8-oxoadenine was not associated with significant hypochromicity and could not be detected in conventional thermal denaturation experiments.

Recent advances in our understanding of triple-stranded nucleic acids permit the design of oligonucleotides which will bind in a sequence-specific manner to a homopurine·homopyrimidine tract of DNA (Moser & Dervan, 1987; Strobel et al., 1988; François et al., 1989; Perroualt et al., 1990). In the best characterized design motif (pyr-pur-pyr[1]), thymine and protonated cytosine in the third strand form Hoogsteen hydrogen bonds with adenine and guanine bases, respectively, in the duplex (de los Santos et al., 1989; Rajagopal & Feigon, 1989; Slkenar & Feigon, 1990). The resulting triple-helical structure has the third strand located in the major groove of the duplex DNA in a parallel orientation to the homopurine strand (Maher et al., 1989; Collier et al., 1991). In vitro, triplexes have been used to modify sequences of double-stranded DNA with extremely high specificity (Cooney et al., 1988; Young et al., 1991; Strobel et al., 1991; Duval-Valentin et al., 1992). Triplex formation has also been shown to inhibit sequence-specific DNA–protein interactions (Maher et al., 1989; Collier et al., 1991).

A limiting factor in using these pyr-pur-pyr triplexes to target duplex sites inside cells is the requirement that the N3 of cytosines in the third strand be protonated so that they can form hydrogen bonds to the N7 of guanines in the duplex. The stability of pyr-pur-pyr triplexes therefore greatly depends on the pH and the number of cytosines requiring protonation. These triplexes are usually much less stable at intracellular pH (7.2–7.3) than at the pH (6.0–6.6) typically employed in many in vitro studies. Replacement of the cytosine with 5-methylcytosine increases the overall stability of these triplexes (such that triplexes have been observed at intracellular pH), but does not eliminate the dramatic effect pH has on the stability of pyr-pur-pyr triplexes (Povsic & Dervan, 1989; Xodo et al., 1991).

To circumvent this sensitivity to pH, one strategy receiving attention is the use of another triplex design motif (pur-pur-pyr) in which the third strand is composed of purine bases and binds to the purine strand of the duplex by means of proposed G·GC and A·AT or T·AT base triplets (Postel et al., 1991; Durland et al., 1991; Chen, 1991; Beal & Dervan, 1991). The approach that we and others have taken, however, is to replace cytosine in the pyr-pur-pyr motif with a novel base that does not require protonation in order to form two hydrogen bonds to guanine. Pseudoisocytidine (Ono et al., 1991) and 3-methyl-5-amino-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (Koh & Dervan, 1992) can replace cytosine and form triplexes which are stable at intracellular pH. We have been studying the use of 7,8-dihydro-8-oxoadenine (8-oxoA) as a replacement for cytosine in the pyrimidine third strand of pyr-pur-pyr triplexes. At intracellular pH, this base has two hydrogens capable of forming hydrogen bonds to guanine (Figure 1B). The larger surface area of this base could also increase triplex stability through greater "stacking" of the bases in the third strand.

During the course of our studies, Matteucci and co-workers demonstrated, by DNase I footprinting techniques, triplex formation with an oligonucleotide which contained N^6-methyl-8-oxoadenine substituted for cytosine (Young et al., 1991; Krawczyk et al., 1992). After our work was complete, Miller and co-workers (Miller et al., 1992) reported thermal denaturation and circular dichroism studies on triplexes with third strands containing one and three 8-oxoA bases. Herein, we present evidence that a third strand containing up to eight 8-oxoA bases forms stable triplexes. When fully substituted with 8-oxoA bases, these triplexes are nearly insensitive to changes in pH from pH 6.0 to 7.4. Surprisingly (and in contrast to data reported by Miller), triplex formation with the fully substituted oligonucleotide takes place with a negligible change in UV absorption (hypochromicity) and could not be detected by conventional thermal denaturation experiments.

MATERIALS AND METHODS

Synthesis of Oligodeoxynucleotides. Oligodeoxynucleotides were synthesized on an Applied Biosystems automated DNA synthesizer by solid-phase β-cyanoethyl phosphoramidite chemistry. The 5′-(dimethoxytrityl)-protected oligonucleotides were cleaved from the CPG (controlled-pore glass) support and the base protecting groups removed by treatment with concentrated ammonia for 4–16 h at 55 °C. The crude

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[1] Abbreviations: pyr-pur-pyr, pyrimidine–purine–pyrimidine; pur-pur-pyr, purine–purine–pyrimidine; 8-oxoA, 7,8-dihydro-8-oxoadenine; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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A)

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3'- GCGAATTCTTTCCCCTCCTTCTTAAGCG -5'    1
5'- CGCTTAAGAAAGGGGAGGAAGAATTCGC -3'    2

5'-      TTCTTTCCCCTCCTTCTT      -3'    3
5'-   Ps-TTCTTTCCCCTCCTTCTT      -3'    3-Ps
5'-      TTCTTTCaCCTCCTTCTT      -3'    3-1a
5'-      TTCTTTCACCTCCTTCTT      -3'    3-1A
5'-      TTaTTTCaCCTCCTTaTT      -3'    3-3a
5'-      TTaTTTaaaaTaaTTaTT      -3'    3-8a
5'-      TTcTTTccccTccTTcTT      -3'    3-MC
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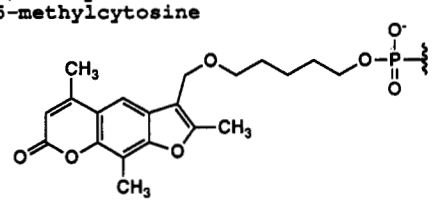
a = 7,8-dihydro-8-oxoadenine
c = 5-methylcytosine

Ps =



B)

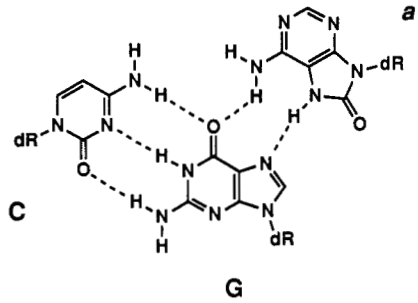


FIGURE 1: (A) Sequence of target duplex (**1** and **2**) and triplex-forming oligonucleotides used in this study. Ps indicates a trimethylpsoralen derivative is linked to the 5'-end. 1A refers to a single adenine mismatch. **3**-1a, **3**-3a, and **3**-8a refer to strands in which 7,8-dihydro-8-oxoadenine has replaced 1, 3, and 8 cytosines, respectively. **3**-MC refers to the strand in which all of the cytosines have been replaced by 5-methylcytosines. (B) Proposed base triplet formed between 7,8-dihydro-8-oxo-2'-deoxyadenosine and a G·C Watson–Crick pair.

oligonucleotides were purified by reversed-phase HPLC, eluting with a gradient of acetonitrile in aqueous triethylammonium bicarbonate (UV monitoring at 260 nm). The psoralen-derivatized oligonucleotide (**3**-PS) was synthesized by a method similar to that reported by Pieles and Englisch (1989). Denaturing PAGE analysis confirmed the purity of the oligonucleotides. Extinction coefficients (AU M^{-1} cm^{-1}) at 260 nm and 25 °C for strands **1**–**3** (245 000, 288 000 and 139 000, respectively) and **3**-1A (144 000) were calculated by the method of nearest-neighbor interactions (Fasman, 1976). The extinction coefficient for **3**-PS (157 000) was estimated by adding the extinction coefficient of the psoralen chromophore at 260 nm (18 000) to the extinction coefficient of **3**. The extinction coefficients (AU M^{-1} cm^{-1}) for strands **3**-1a (140 000), **3**-3a (142 000), **3**-8a (146 000), and **3**-MC (125 000) were calculated by UV analysis after enzymatic digestion (Eadie et al., 1987).

The synthesis of 2'-deoxy-7,8-dihydro-8-oxoadenosine phosphoramidite was carried out by the procedure of Guy et al. (1988). The phosphoramidite was used as a 0.1 M solution in acetonitrile with a standard oligonucleotide synthesis cycle. The free nucleoside (2'-deoxy-7,8-dihydro-8-oxoadenosine) exhibited an absorption maximum at 270 nm and a minimum at 232 nm in water. UV spectra of oligonucleotides containing 8-oxoadenosine bases had maxima near 260 nm and were uneventful except that (like the free nucleoside) the long wavelength tail of the absorption extends to slightly above 300 nm.

Thermal Denaturation Profiles. Thermal denaturation experiments were performed on a CARY 3 UV–vis spectrophotometer in 1 cm path length quartz cells fitted with Teflon stoppers. Buffer A (pH 5.8) contained 10 mM sodium cacodylate, 1 mM EDTA, and 0.1 M NaCl. Buffer B was the same as buffer A except the pH was 7.0 and 10 mM $MgCl_2$ was added. Each strand was present at a concentration of 1 μM. Samples (1 mL) were made from 100 μM stock solutions of individual strands and annealed by cooling from 90 to 0 °C at a rate of 0.2 °C/min. Absorbance at 260 nm was measured as the temperature was then increased from 0 to 90 °C at a rate of 0.2 °C/min. The melting temperatures (T_{max}) reported were the temperatures at the maxima of the first derivative of the absorbance vs temperature curves and were reproducible within 1 °C. Similar T_{max} values were obtained from denaturation and renaturation experiments.

Mixing Cell Experiments. Experiments were performed on a CARY 3 UV–vis spectrophotometer in 1 cm path length quartz mixing cells (Uvonic Instruments) fitted with Teflon stoppers. Each mixing cell is divided into two compartments that allow two solutions to be mixed together or to remain separated. Annealed duplex (1 nmol) in 500 μL of buffer A was placed in one compartment of the cell, and either strand **3**-MC or **3**-8a (1 nmol) in 500 μL of buffer A was placed in the other. After the spectrometer was zeroed, the sample cell was mixed and the reference cell was left unmixed. Before the spectrum was recorded, the sample was allowed to equilibrate at each temperature until the absorbance showed no change.

Photo-cross-linking Experiments. Strand **1** of the duplex was labeled with [γ-^{32}P]adenosine triphosphate using T4 polynucleotide kinase and annealed to strand **2**. Samples of duplex (~2 nM), strand **3**-Ps (1 μM), and competing strand **3** or **3**-8a were heated in 50 mM MES, 0.1 M NaCl, and 10 mM $MgCl_2$ at pH 6.0 for 15 min, cooled from 55 to 45 °C over 1 h, cooled to 37 °C, and left at 37 °C for 48 h. The samples were irradiated for 2 min at 37 °C in a Rayonet photochemical reactor equipped with 3500-Å tubes and a filter consisting of 1 cm of 20% (w/v) aqueous solution of cobalt(II) nitrate hexahydrate. Samples were analyzed by denaturing polyacrylamide gel electrophoresis on 16% gels [29:1 acrylamide/bis(acrylamide)] prepared and run in Tris–borate–EDTA (TBE) buffer, pH 8.3. Dried gels were visualized by autoradiography.

Gel Retardation Assay. Strand **1** was end-labeled with [γ-^{32}P]adenosine triphosphate using T4 polynucleotide kinase and annealed to strand **2**. Samples of duplex and strand **3**-MC or **3**-8a were equilibrated in either buffer C or buffer D overnight at 37 °C and loaded in a mixture of Ficoll, bromophenol blue, and xylene cyanol as tracking dyes. Buffer C was 0.1 M sodium acetate and 10 mM $MgCl_2$, pH 5.9, and buffer D was 10 mM Tris, 0.1 M sodium acetate, and 10 mM $MgCl_2$, pH 7.4. Samples were analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE) on 16% gels [29:1 acrylamide/bis(acrylamide)] prepared in either 5× buffer C or 5× buffer D. The gels were run at 4 °C at 95 V (30 mA) for approximately 18 h. Dried gels were visualized by autoradiography.

RESULTS AND DISCUSSION

Triplex formation was studied using a synthetic 28 base pair duplex containing a homopurine target site which was 18 bases long (Figure 1A, strands **1** and **2**). The sequence of the duplex was chosen such that nearly one-half of the base triplets formed using a conventional pyr-pur-pyr triplex motif would

Table I: T_{max}'s of Triplexes in Buffer B[a]

third strand	T_{max} (°C)
3	18, 74
3-Ps	23, 74
3-MC	25, 74
3-1A	12, 74
3-1a	18, 74
3-3a	25, 74
3-8a	74[b]

[a] 100 mM Na^+, and 10 mM Mg^{2+}, pH 7.0. [b] Only one T_{max} was observed.

be pH-sensitive C^+·GC base triplets. In addition, a conventional third strand has four contiguous C's that presumably should be protonated in the triplex, a situation known to increase the pH sensitivity of triplexes (Kiessling et al., 1992). This target provides a rigorous test of the feasibility of a given protonated cytosine replacement. Third strands (18-mers) were designed parallel in orientation (5′–3′) to the purine-rich strand of the duplex target using C, 5-methylcytosine, or 8-oxoA to recognize G and T to recognize A (Figure 1A). A scheme showing how 8-oxoA could bind to the guanine of a CG base pair is shown in Figure 1B. Modeling predicts that the 2′-deoxy-8-oxoadenosine in triplexes will adopt the syn conformation. Fortunately, the syn conformation is the preferred conformation for 8-substituted purine nucleosides (Uesegi & Ikehara, 1977; Kouchakdjian et al., 1991), as confirmed by our own NMR evidence.

Triplex formation with the candidate third strands was initially probed by thermal denaturation studies. A number of workers have reported that thermally induced dissociation of a third strand from a duplex results in an increase of the absorbance at 260 nm (Pilch et al., 1990; Plum et al., 1990; Shea et al., 1990; Miller et al., 1992). UV melting experiments on equimolar mixtures of the duplex (strands **1** and **2**) and strands **3** or **3**-MC produced biphasic curves similar to those reported by others. The lower temperature transition was assigned to dissociation of the third strand, and the higher temperature transition was assigned to dissociation of the duplex (Table I). The melting temperature of the lower temperature transition decreased significantly with increasing pH. The presence of magnesium was required to detect a triplex transition for strand **3** at pH 7. Suprisingly, the hyperchromicity associated with third strand dissociation decreased with increasing pH (Figure 2A).

Strand **3**–1a, with a single substitution of 8-oxoA for cytosine, gave a UV melting profile essentially indistinguishable from the parent strand **3**. In contrast, when the same cytosine was replaced by a single mismatch adenine (strand **3**-1A), the melting temperature of the triplex decreased by 6 °C (Table I). This suggests that 8-oxoA is interacting with a guanine of the duplex in a stabilizing manner. The triply substituted strand, **3**-3a, showed a slight decrease in melting temperature at pH 5.8 (data not shown) and a slight increase in melting temperature at pH 7.0 (Figure 2B). Similar results were reported by Miller and co-workers (Miller et al., 1992) for third strands which contained only thymine and one or three 8-oxoA bases.

Given these results, the fully substituted strand was expected to form a stable triplex. However, mixtures of the target duplex and **3**-8a exhibited only one melting transition with temperature and hypochromicity consistent with duplex melting. (Figure 2B). 8-oxoA absorbs strongly at the wavelength used in these thermal denaturation experiments (260 nm), and there are no unusual features in the spectra of the free nucleoside or the oligonucleotides (see the Materials and Methods section) which might explain the missing transition. Four hypotheses were put forth to explain these results: (1) Strand **3**-8a forms a triplex, but the wavelength at which the thermal denaturation was monitored (260 nm) is at or near an isosbestic point for triplex dissociation. (2) Strand **3**-8a forms a triplex which is at least as stable as the target duplex, and therefore the triplex melting transition is "hidden" under the duplex melting transition. (3) Strand **3**-8a does not associate with the target duplex to form a triplex. (4) Association of the third strand, **3**-8a, with the duplex does not produce a significant change in the UV spectrum. A referee suggested a fifth hypothesis: The triplex or other complex formed by strand **3**-8a dissociates with low cooperativity. The melting transition produced under these circumstances might be too broad to detect.

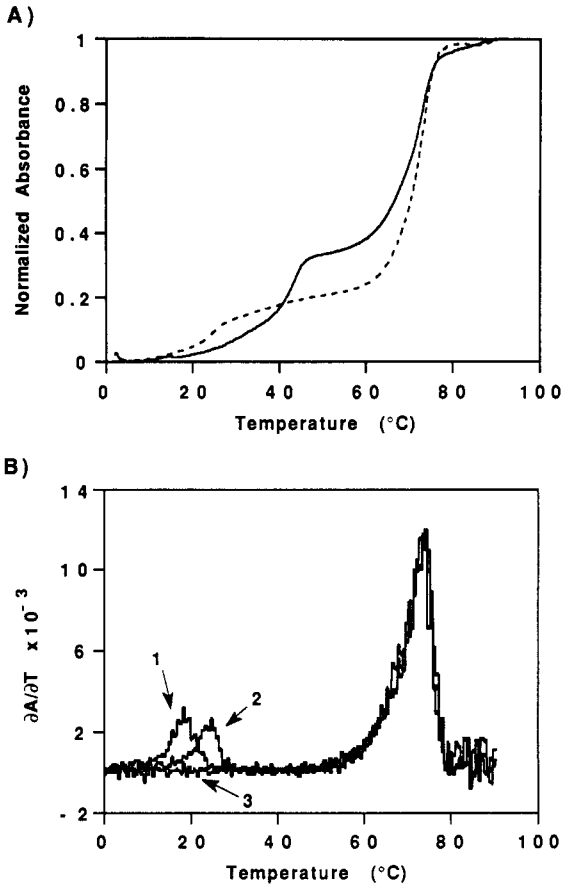


FIGURE 2: (A) Thermal denaturation of triplexes. Absorbance vs temperature plots for strands **1**, **2**, and **3**-MC in buffer B, pH 6.4 (—), or buffer B, pH 7.0 (- - -). (B) Derivative of absorbance vs temperature plots for strands **1**, **2**, and **3** (curve 1), **1**, **2**, and **3**-3a (curve 2), and **1**, **2**, and **3**-8a (curve 3) in buffer B, pH 7.0.

Thermal denaturation is not a very sensitive method for monitoring UV changes associated with triplex formation because the total absorbance change is relatively small and is superimposed on a background of instrument noise and changes in the absorbances of the isolated duplex and third strand. In order to study the UV changes associated with triplex formation and to test the first two hypotheses, experiments were performed in mixing or "split" cells. Duplex and third strand were loaded into separate compartments of the sample cell and then mixed. Changes in the absorbance with temperature were corrected by using an otherwise identical reference cell in which both components remained unmixed. In theory, the resulting difference spectrum reports only on UV absorbance changes that are due to the interaction of the third strand and duplex. When triplex formation

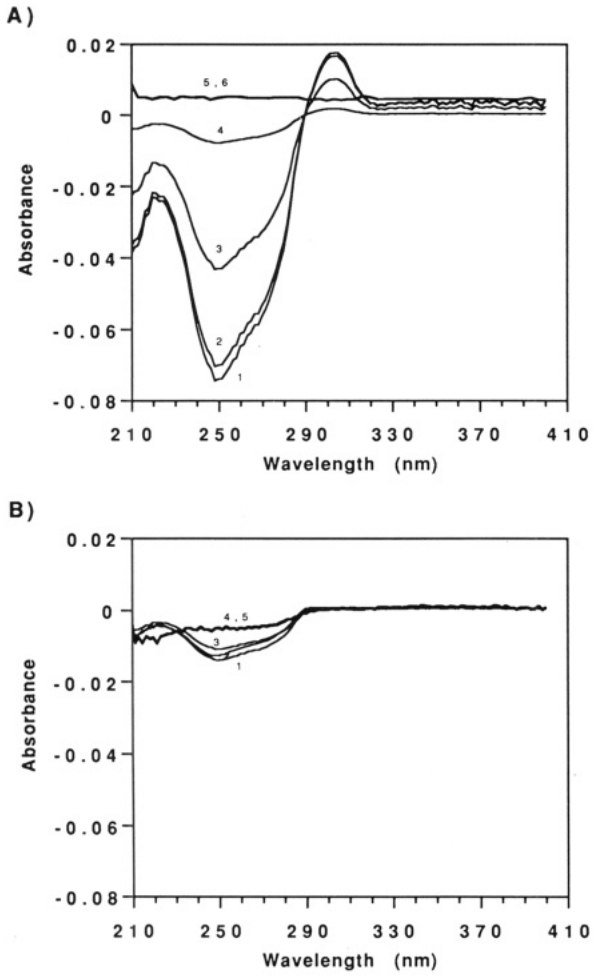


FIGURE 3: UV difference spectra from mixing cell experiments. (A) Duplex and strand **3**-MC, pH 5.8, at 10 (1), 30 (2), 50 (3), 60 (4), 70 (5), and 90 °C (6). (B) Duplex and strand **3**-8a at 10 (1), 30 (2), 50 (3), 70 (4), and 90 °C (5).

between duplex and **3**-MC was studied by this technique (Figure 3A), hypochromicity associated with triplex formation was observed primarily between 240 and 280 nm. Maximum hypochromicity (8%) was observed at 250 nm, while a smaller change (6.5%) was seen at 260 nm (the wavelength normally monitored in a thermal denaturation experiment).

In contrast to strand **3**-MC, mixing cell experiments with strand **3**-8a indicated that there was very little UV absorption change at any wavelength associated with mixing third strand with duplex (Figure 3B). The maximum hypochromicity was 1.4% (at 250 nm) or about one-fifth of that seen with **3**-MC. The small hypochromicity produced by mixing **3**-8a and duplex disappears between 50 and 70 °C, somewhat higher than with **3**-MC. The lack of a triplex melting transition in conventional thermal denaturation experiments is clearly not due to an isosbestic point or to obscuring by duplex melting. Since loss of hypochromicity occurs over a narrow temperature range in both cases, these transitions appear to exhibit normal (high) cooperativity. (However, the absorbance change which supports this conclusion is uncomfortably small for **3**-8a. If this apparent transition were an artifact, one could postulate that a broad transition with expected hypochromicity (8%) exists and extends below 10 °C. If this postulate were correct, these mixing experiments would demonstrate that relatively little triplex forms above 10 °C. This conclusion, however, is incompatible with results discussed below.) Thus, while they provided information consistent with conventional thermal denaturation studies, these mixing cell experiments also

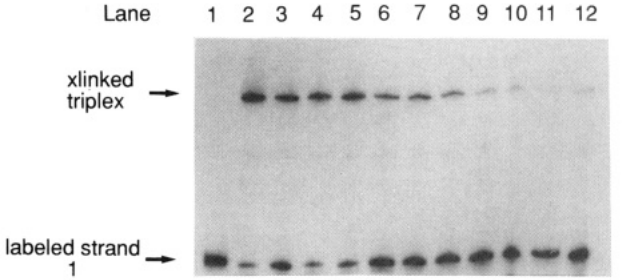


FIGURE 4: Autoradiogram showing inhibition of photo-cross-linking by competing third strands. Duplex (~2.5 nM, ^{32}P-end-labeled on strand **1**), strand **3**-Ps (1 μM), and competing strand were irradiated and analyzed on denaturing PAGE. Lane 3 contained 10 μM strand **3** as competing strand. Lanes 4–12 contained 1, 2, 4, 8, 10, 20, 40, 80, and 100 μM strand **3**-8a as the competing strand. Lanes 1 and 2 contained no competing strand. Lane 1 received no irradiation.

provided a more sensitive and detailed method for probing triplex formation. Unfortunately, in the absence of a satisfactory explanation for the greatly reduced hypochromicity, these experiments do not provide conclusive proof that strand **3**-8a and the duplex form a triplex.

Since the above UV experiments were not conclusive, **3a**-8a was allowed to compete for the duplex with another third strand (**3**-Ps). We (Hobbs et al., 1990) and others (Hélène et al., 1991) have shown that a third strand can be photo-cross-linked to a target duplex when a psoralen is appropriately tethered at the 5′-end of the third strand. This reaction is very efficient (Figure 4, lane 2) and occurs only when a triplex is present. A mixture of strands **3**-8a and **3**-Ps was allowed to compete for a limited amount of ^{32}P-labeled duplex. Triplex formation by **3**-8a was assessed by the reduction in the amount of adduct formed by the photo-cross-linking between **3**-PS and the duplex. The extent of photo-cross-linking inhibition depended on the concentration of competing **3**-8a; 50% reduction in photo-cross-linking occurred at a **3**-8a concentration of ~3 μM. When strands **3** and **3**-MC were studied under similar conditions, 50% inhibition of cross-linking required ~30 and 1.5 μM, respectively (data not shown). Unless strand **3**-8a interacts in an unexpected way with strand **3**-Ps, these competition experiments indicate that **3**-8a forms a triplex at pH 6.0 with similar stability to the triplex formed by **3**-MC. Since the efficiency of photo-cross-linking by **3**-Ps above pH 7 is extremely low, this technique could not prove that triplex formation by **3**-8a occurs at intracellular pH.

A gel retardation assay was used to demonstrate that the triplex formed by **3**-8a was stable at intracellular pH. Oligonucleotide triplexes have been reported to migrate through a nondenaturing polyacrylamide gel without dissociating when the gel contains magnesium and is run at low temperature and voltage (Shea et al., 1990; Pilch et al., 1991). Strand **1** of the duplex was ^{32}P-end-labeled and a limiting amount of duplex was equilibrated with increasing amounts of either **3**-MC or **3**-8a at pH 5.9 or 7.4 for 16 h at 37 °C. The resulting mixtures were analyzed by nondenaturing PAGE using the equilibrating buffer as the electrophoresis buffer. Sharp bands and the lack of streaking between the duplex and triplex bands prove that negligible dissociation occurred during electrophoresis. At pH 5.9 (Figure 5A), both the **3a**-8a and the **3**-MC third strands form triplexes (upper band) and have very similar affinities for the target duplex. Roughly 0.1 μM third strand is required to convert 50% of the duplex to triplex (Figure 6). At pH 7.4 (Figure 5B), however, triplex formation is seen only with strand **3**-8a. Even 10 μM **3**-MC showed no evidence of triplex formation. (Samples equilibrated at pH 7.4 and run with pH 5.9 electrophoresis buffer gave the same

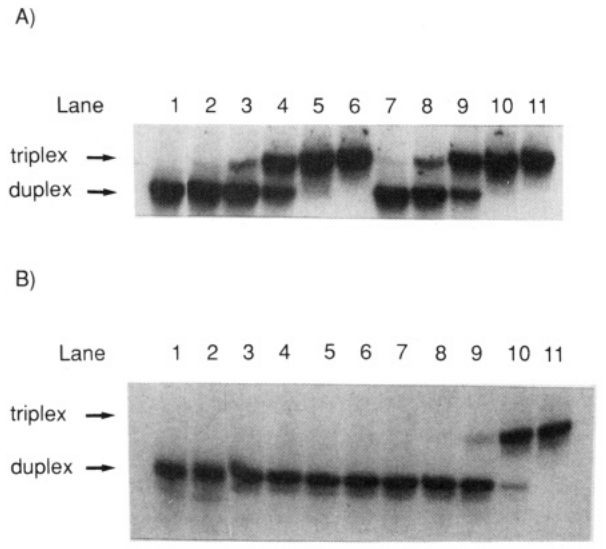


FIGURE 5: Autoradiogram of nondenaturing PAGE analysis of duplex/triplex equilibrium. Duplex (~1 nM, ^{32}P-end-labeled on strand **1**) and third strand were equilibrated and electrophoresed at pH 5.9 (A) or pH 7.4 (B). Lanes 2–6 contained 0.003, 0.01, 0.1, 1, and 10 μM strand **3**-MC; lanes 7–11 contained 0.003, 0.01, 0.1, 1, and 10 μM strand **3**-8a. Lane 1 contained no third strand.

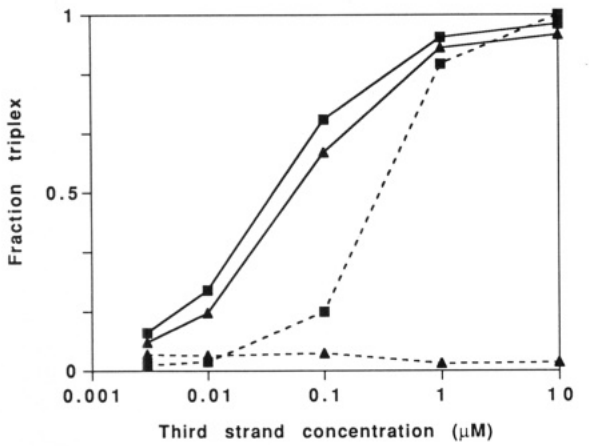


FIGURE 6: Graph showing the fraction of triplex formed by strands **1**, **2**, and **3**-MC (▲) or **3**-8a (■) as determined by gel retardation assay at pH 5.9 (—) on pH 7.4 (- - -).

result as those equilibrated and run at pH 7.4.) Third strand **3**-8a *appears* to bind about 4-fold less tightly at pH 7.4 than at pH 5.9 (Figure 6). If the stability of this triplex actually does decrease slightly with increasing pH, the acidity of the N7 hydrogen of 8-oxoA (pK_a = 8.7; Cho & Evans, 1991) might be responsible.

Significant hypochromicity is generally observed when two nucleic acid strands associate to form a duplex, when a third strand associates with a duplex to form a triplex, or when a single-stranded nucleic acid (such as poly(A)) adopts a helical conformation rather than a random coil (Rich & Tinoco, 1960; Breslauer et al., 1975). Triplex formation by third strands containing a few 8-oxoA bases (**3**-1a, **3**-3a, and those reported by Miller and co-workers (Miller et al., 1992)) takes place with significant hypochromicity. Thermal denaturation experiments and mixing experiments, however, show that the association of strand **3**-8a with its duplex target produces a minimal change in the UV absorption spectra. Likewise, the magnitude of the hypochromicity observed with strands **3** and **3**-MC decreases significantly with increasing pH (Figure 2A). In theory, hypochromicity arises mostly from parallel stacking of the electric dipole transition moments of neighboring bases (Tinoco, 1960). Although a host of geometrical factors can influence the magnitude of hypochromicity, hypochromicity is generally attributed to base stacking and helical structure, while lack of hypochromicity is generally attributed to the absence of these structural features.

In helical models constructed to fit fiber diffraction data from triple-stranded homopolymers, the bases of the third strand are stacked with the plane of the base nearly perpendicular to the helix axis (Arnott & Selsing, 1974; Arnott et al., 1976). NMR spectra of intrastrand triplexes have confirmed the general features of these models. As expected, thermal denaturation studies on conventional pyr-pur-pyr triplexes show significant hypochromicity. Therefore, if third strands containing 8-oxoA associate with a target duplex by means of the base triplet shown in Figure 1B, triplex formation presumably should be accompanied by an increase in base stacking and a decrease in UV absorption. The studies in this and other articles (Young et al., 1991; Krawczyk et al., 1992; Miller et al., 1992) clearly demonstrate that oligonucleotides with 8-oxoA bases form sequence-specific three-stranded structures and that CG·8-oxoA base triplets must help hold these structures together. While it is not currently possible to resolve this apparent dilemma, an answer may lie in one of two areas. First, the geometrical factors which influence the magnitude of hypochromicity could interact to reduce hypochromicity by a factor of 5 in the case of strand **3**-8a. If true, absence of significant hypochromicity should not generally be used to conclude that bases are not stacked. Second, while triplexes with 8-oxoA appear to be similar to conventional pyr-pur-pyr triplexes, 8-oxoA may not substitute for cytosine without consequential changes in some helix parameters. For example, the planes of the bases in triplexes with many 8-oxoA's might be tipped to an unusual extent. Whatever the reason for this observed lack of significant hypochromicity, the absence of hypochromicity or a UV melting transition should not be taken as conclusive proof that a triplex does not exist.

In contrast to the above theoretical speculations, our studies clearly demonstrate that strand **3**-8a, fully substituted with 8-oxoA bases, forms a triplex without a significant decrease in UV absorption. This triplex is approximately as stable at pH 5.9 as the analogous triplex with 5-methylcytosine in the third strand. Unlike the latter triplex, the triplex with 8-oxoA is nearly as stable at pH 7.4 as at pH 5.9. Most importantly, oligonucleotides with 7,8-dihydro-8-oxoadenine and thymine should be capable of forming stable triplexes inside cells.

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