Conformational Diversity Versus Nucleic Acid Triplex Stability, a Combinatorial Study*

Eloy Bernal-Méndez and Christian J. Leumann‡

From the Departement für Chemie und Biochemie, Universität Bern, Freiestrasse 3, CH-3012 Bern, Switzerland

The stability of a triple helix formed between a DNA duplex and an incoming oligonucleotide strand strongly depends on the solvent conditions and on intrinsic chemical and conformational factors. Attempts to increase triple helix stability in the past included chemical modification of the backbone, sugar ring, and bases in the third strand. However, the predictive power of such modifications is still rather poor. We therefore developed a method that allows for rapid screening of conformationally diverse third strand oligonucleotides for triplex stability in the parallel pairing motif to a given DNA double helix sequence. Combinatorial libraries of oligonucleotides of the requisite (fixed) base composition and length that vary in their sugar unit (ribose or deoxyribose) at each position were generated. After affinity chromatography against their corresponding immobilized DNA target duplex, utilizing a temperature gradient as the selection criterion, the oligonucleotides forming the most stable triple helices were selected and characterized by physicochemical methods. Thus, a series of oligonucleotides were identified that allowed us to define basic rules for triple helix stability in this conformationally diverse system. It was found that ribocytidines in the third strand increase triplex stability relative to deoxyribocytidines independently of the neighboring bases and position along the strand. However, remarkable sequence-dependent differences in stability were found for (deoxy)thymidines and uridines.

Triple-stranded DNA and RNA structures were first discovered in the late fifties by Felsenfeld and Rich (1, 2). In 1986, two independent research groups demonstrated that triplex-forming oligonucleotides (TFOs)† can be used to specifically recognize a given sequence in a DNA double helix (3, 4), and thus paved the way for their potential use as therapeutics in the antigenic strategy and as tools in molecular biology. Polypyrimidine TFOs bind specifically to complementary poly(Pu)poly(Py) double-helix sequences by formation of Hoogsteen base pairs between thymines or protonated cytosines in the TFO and adenosines or guanines, respectively, in the poly(Pu) strand of the DNA double helix. The molecular recognition process is highly sensitive to base mismatches, so that a single duplex site can be targeted within megabase DNA (5–7).

It has been shown previously that tripleplexes not only form within the pure DNA or RNA backbone context but also within mixed RNA and DNA strands, although with distinct differences in stability within a given sequence context (8). Recent analyses by NMR and FT-IR have shown that D:DD and R:DD triple helices (where D stands for the DNA double helix, and D: or R: for the DNA or RNA TFO, respectively) have heterogeneous backbone conformations, leading to energetically compromised conformations for certain ribo- and deoxyribonucleotides in the three strands (9–15). Thermodynamic studies have shown that R:DD triple helices have higher thermal stability than D:DD ones (8, 15–18), whereas similar free energies and equilibrium constants have been found by isothermal titration calorimetry and EDTA cleavage (19, 20). Results in our laboratory and others, with third strand sequences containing a variable number of nucleotides with modified bases or backbones, show thus far understood differences in affinity as a function of the target sequence and the position of the modified nucleosides in the chain (21–23). Given these data, the question arises whether, upon binding with its target double helix, a TFO containing an intrinsically heterogeneous backbone conformation, allowing for a tailor-made structural fit to the target, could increase the triplex stability compared with a TFO with a homogeneous backbone.

With this in mind, we developed a general method based on a combinatorial approach. The method is based on the synthesis of a combinatorial library of TFOs containing either ribo- or deoxyribonucleotides with the requisite base at each position in the chain. Both types of nucleoside units intrinsically prefer different sugar conformations (3′-endo for ribonucleosides and 2′-endo for deoxyribonucleotides) and thus give rise to a large variety of backbone conformations in the corresponding TFOs. After affinity chromatography on the immobilized target double helix, utilizing a temperature gradient as the selection criterion, and subsequent deconvolution of the thus obtained fractions by chemical and analytical means, the molecular features of TFOs leading to enhanced triple helix stability were defined. Based on these features, single oligonucleotides were designed, synthesized, and characterized for proof of principle. The results obtained validate our combinatorial approach, showing distinct effects of the nature of the sugar on triplex stability, depending on the position within the TFO chain. This method lends itself for use in the assay of oligonucleotides containing new, chemically modified nucleoside analogues, as it does not rely on an enzymatic amplification and in vivo deconvolution step typically used in DNA and RNA selection protocols.

MATERIALS AND METHODS

Synthesis of Oligonucleotides and TFO Libraries—Single oligonucleotides were synthesized on an Amersham Pharmacia Biotech Gene-
Assembler special DNA synthesizer using standard β-cyanoethyl phosphoramidite chemistry and the manufacturer’s protocols (24). All TFOs were synthesized on a 1.0-μmol scale. For the introduction of ribonucleotides, the coupling time was proportional to the target triple helix. After final detritylation, the TFOs were puriﬁed by IP-RP-HPLC followed by PAGE under denaturing conditions (24% nol (3:1), at 55 °C). All the members of the library from the non-tritylated, truncated separation was performed with the aim to facilitate the separation of one column and the corresponding deoxyribonucleotide in the other resin. Samples were mixed together and separated again in two equal parts. New coupling was then performed using a Cary 3E UV-visible spectrophotometer (Varian).

The molar extinction coefficient of each oligonucleotide (or AC fraction) was calculated using nearest neighbor approximation (28). 2 nmol of the corresponding oligonucleotides were lyophilized to dryness and redissolved in 1 ml of a 0.1 M NaCl, 1 mM EDTA, 10 mM citrate/phosphate buffer (pH 4.4–6.5). To ensure the correct formation of the triple helix, the solution was heated to 85 °C and allowed to slowly cool down to room temperature, followed by storage at 4 °C overnight. The pH of the solution was veriﬁed using a microelectrode. Prior to thermal experiments, the UV-visible spectra of the samples (210–350 nm) were measured at around 1 °C. Absorbance versus temperature proﬁles were measured at two wavelengths (260 and 300 nm), using a linear gradient between 5.5 °C/min and a heating-cooling-heating cycle between 0 and 90 °C. Data were analyzed using Origin50 software.

**RESULTS**

The TFO base sequence chosen in this investigation (see Fig. IA) was designed to contain a T-rich part, a part of equal distribution of T and C, and a C-rich part, allowing for maximum sequence variability in the TFO and in the corresponding target double helix. Its length was chosen in order to stay within the limits of its detectability by UV melting measurements (see Table I, and to comply with the permissive temperature range of the afﬁnity chromatography matrix.

**First Round of Selection**

A ﬁrst round of selection with the TFO library M, containing the complete set of 2048 individual oligonucleotides, was performed utilizing a temperature gradient as indicated under “Materials and Methods.” The results are shown in Fig. 2. Five fractions (M05 to M45) at ﬁve different temperatures, containing individuals with increasing afﬁnity to the double helix, were isolated and subsequently deconvoluted by a series of analytical steps explained in detail below.

**UV Melting Experiments**—These experiments were performed in order to verify that oligonucleotides were eluted exclusively as a function of the thermal stability of the corresponding triple helices. As expected, hysteresis was observed between heating and cooling proﬁles due to slow kinetics of triplex formation (29–32). The melting temperatures for third strand dissociation (Tm) were determined at the maximum of the ﬁrst derivative of the heating curves. They are taken as indicative values for the stability of the triple helices, and not as thermodynamic parameters. The observed Tm of the triple helices conﬁrmed the non-binding of the M05 fraction and the stability order of the triple helices formed by the other fractions under the conditions applied (Table I).
instead of a dT in the oligonucleotide leads to an increase of only two mass units (ΔH11001 /H11032 = 14 from 5-Me). Due to the fact that the spectrum is complicated by the presence of isotopic peaks that overlap with those corresponding to different rU contents in each group of constant rC, it was not possible to resolve the families of constant rC into their different rU-containing subfamilies, so that we could only estimate the rU/dT ratio by comparing the maximum values of the peaks obtained for each family. The mass spectrum of M is depicted in Fig. 3B together with the calculated one (Fig. 3B, inset). The detection of all signals near to the expected relative intensities assesses the quality of the library. This mass spectrum was thus used as a control for the study of the corresponding fractions.

The rC content in each fraction was defined from the quantification of the signals corresponding to different rC families, and compared with the mass spectrum of the whole library M. It clearly emerges (Fig. 3C) that there is a preference for more rC over dC units when moving toward the more stable AC fractions. The first eluting fractions have a slightly lower rC content than the average, while M35 contains a higher rC content. The AC fraction containing the strongest binding TFOs is populated by rC-rich species, suggesting that the increase in triplex stability goes parallel with the increase in the number of rC residues. In order to correct for the different sizes of the rC families and the different sizes of the fractions, each mass signal was normalized, and the values obtained for the same mass peak in different fractions were compared in order to obtain a rough idea on the number of species that they represent. For this calculation, the assumptions were made that every TFO of the library is only present in one fraction, and that each TFO represents an equal part of the library. The values obtained are shown in the table in Fig. 3D. It can be seen that the proportion of each rC family, which is present in the M45 fraction, doubles at each single increase in rC content, corroborating that individuals with more rC units have a higher probability of being found in the more stable TFO fractions.

The average rU content in each rC family was estimated from the maximum value of the corresponding mass peak. We found values ranking from 2.6 to 4.6, with higher rU content in the more retained AC fractions for every rC family. These results indicate that the triple helix stabilization brought about by rU does not depend on the presence of rC or dC, as the values are similar for all rC families in the same fraction.
Another conclusion is that the presence of rU is not a general guarantee for high stability, as fraction M45 contains an average of only 4.1 rU of 6 possible.

**Partial Alkaline Digestion (PAD)—**At basic pH (pH > 9), ribooligonucleotides undergo strand cleavage by 2',3'-cyclophosphate formation. Deoxyribonucleotides are stable under these conditions. Statistical control of this degradation process allows the reaction to take place at a maximum of one nucleotide per chain, and the 5' end 32P-labeled fragments can then be detected and analyzed by PAGE. The presence of ribonucleotides at each position along the TFO can thus be evaluated. Applied to the library M and its fractions, an average ribonucleotide distribution is obtained at each position in the chain. An example of this experiment is shown in Fig. 4. For the analysis of these data, the signal intensities were corrected to a normalized total reactivity for each fraction and related to those of the whole library M. This method is complementary to analysis by MS since it allows detection of the positions at which ribonucleotides can be found, whereas MS reveals the total number of ribonucleotides in the TFOs of the fraction.

In the stronger binding AC fraction M45, we find a requirement for ribonucleotides in the central part (positions 4–9) and a preference for dT over rU in positions 1 and 2. In positions 10 and 11, similar reactivities were found for all fractions, indicating a lack of preference for these positions. This explains the fact that the average rU content found for M45 by ESI-MS is only 4.1, and that many individuals with one or two dC are present in this fraction. It emerges as a general rule that sequence-dependent differences in triplex stability are more encountered for dT versus rU than for dC versus rC replacements. In the low binding fraction M05, the ribonucleotides are evenly scattered over all positions, and an anomalous migration is found for the fragments containing the nucleotides 8 and 9, which is not reflected in the migration of shorter and longer fragments, as well as in the mass spectra. A possible explanation for this and the poor triple-helix formation propensity by the members of this fraction is that a 2' to 3' phosphate migration of a part of the library had occurred during synthesis or deprotection.

**UV-visible Absorbance—**The UV-visible spectrum (210–350 nm) of RNA and DNA of the same sequence and length differ in the position of the absorption peak, because uridine has a near-UV maximum at 260 nm, rather than the 268 nm for deoxythymidine (33). Therefore, we used the A260/A280 ratio in order to estimate the rU/dT contents of the fractions. As shown in Table I, only little differences were found, with the higher rU/dT ratio occurring in the first and last fractions. These results indicate importantly that a continuously increasing rU/dT ratio in the TFO goes not in parallel with increased stability of the triple helix.

**HPLC—**In IE-HPLC, the whole TFO library M eluted with intermediate retention times compared with the pure TFOs r11d and d (Table I). As expected, M45 and M35 have shorter retention times, indicating a higher ribonucleotide content. During IP-RP-HPLC, average retention times followed the se-
The following conclusions could be drawn from the results obtained. (i) An “RNA core” is necessary for high triple helix stability, (ii) ribonucleotides at the 3′ end of the TFO have a negative effect on stability, and (iii) TFOs with higher rC content show higher triple helix stability in a sequence-independent manner. Taking this into account, we designed and synthesized the sublibrary P corresponding to the all-rC family of M. This library contains only 64 individuals, with all C in the 3′ terminal unit, and dT and rU as variables. With this focused library, a second round of selection was performed.

The TFO sublibrary P (Fig. 5) was prepared much in the same way as described for M. The sublibrary was again assayed for triplex formation by affinity chromatography on the immobilized dh, as before. The resulting elution profile (Fig. 6), as expected, showed the higher populated fractions eluting at higher temperatures (P35 and P45). The small peak arising at 90-ml elution volume is an artifact produced by the change of eluent. The thermal denaturation UV profiles of the collected fractions (Fig. 7) again verify the success of the selection process. UV-visible absorption spectra were measured for all fractions. The highest A260/A280 ratios were found for P45 and P35, indicating a higher content of rU (Fig. 6). The fractions were further characterized as before.

**Design of Sublibrary P and Second Round of Selection**

ESI-MS—Seven different masses are possible for the TFOs of P, ranking between 3589 for the one having six rU (r11d) and 3577 for the one with six dT (r6d; see Fig. 5, 6). This small difference in mass did not allow us to obtain resolved mass peaks by ESI-MS. Therefore, only an average rU/dT ratio could be obtained from the mass spectra of each fraction. The obtained values are shown in Fig. 5. P05 and P15 are around the average value of the library. P25 has the lowest mass of the P fractions, with an average of 2.6 rU per TFO, still not far from the 3 rU average of the library. P45 shows the highest mass, with a corresponding average of 3.8 rU per TFO. This value fits well with the results obtained with M45, the most stable TFO fraction from the library M, showing a preference for rU at positions 3, 4, 6, and 9, and for dT at positions 1 and 2. In summary, a maximum difference of 1.2 average rU per TFO has been found, between P25 and P45, which is in agreement with the results obtained from the previous selection step with the library M.

**Partial Alkaline Digestion**—The results of these experiments clearly show a stronger reactivity of P45 at positions 6 and 9, indicating, as previously shown for the M library, that an RNA core is required in the TFO for stronger triple helix stability (Fig. 8). Another feature of P45 is a low reactivity at positions 1 and 2. This means that dT is preferred over rU at these positions of the TFO. This is the opposite behavior with respect to positions 6 and 9. The P25 and P35 fractions also show interesting PAD profiles. At positions 6 and 9, their reactivities are average, being a little higher in the case of P35 compared with P25. Focusing on positions 1–4, distinctly different reactivities are found. P25 has the highest rU content of all the fractions at positions 1 and 2 and the lowest (together with P35) at position 3. P35 has the lowest signals at positions 3 and 90-ml elution volume is an artifact produced by the change of eluent. The thermal denaturation UV profiles of the collected fractions (Fig. 7) again verify the success of the selection process. UV-visible absorption spectra were measured for all fractions. The highest A260/A280 ratios were found for P45 and P35, indicating a higher content of rU (Fig. 6). The fractions were further characterized as before.

**Design of Sublibrary P and Second Round of Selection**

ESI-MS—Seven different masses are possible for the TFOs of P, ranking between 3589 for the one having six rU (r11d) and 3577 for the one with six dT (r6d; see Fig. 5, 6). This small difference in mass did not allow us to obtain resolved mass peaks by ESI-MS. Therefore, only an average rU/dT ratio could be obtained from the mass spectra of each fraction. The obtained values are shown in Fig. 5. P05 and P15 are around the average value of the library. P25 has the lowest mass of the P fractions, with an average of 2.6 rU per TFO, still not far from the 3 rU average of the library. P45 shows the highest mass, with a corresponding average of 3.8 rU per TFO. This value fits well with the results obtained with M45, the most stable TFO fraction from the library M, showing a preference for rU at positions 3, 4, 6, and 9, and for dT at positions 1 and 2. In summary, a maximum difference of 1.2 average rU per TFO has been found, between P25 and P45, which is in agreement with the results obtained from the previous selection step with the library M.

**Partial Alkaline Digestion**—The results of these experiments clearly show a stronger reactivity of P45 at positions 6 and 9, indicating, as previously shown for the M library, that an RNA core is required in the TFO for stronger triple helix stability (Fig. 8). Another feature of P45 is a low reactivity at positions 1 and 2. This means that dT is preferred over rU at these positions of the TFO. This is the opposite behavior with respect to positions 6 and 9. The P25 and P35 fractions also show interesting PAD profiles. At positions 6 and 9, their reactivities are average, being a little higher in the case of P35 compared with P25. Focusing on positions 1–4, distinctly different reactivities are found. P25 has the highest rU content of all the fractions at positions 1 and 2 and the lowest (together with P35) at position 3. P35 has the lowest signals at positions 3 and
4. A logical explanation fitting with these reactivities is that TFOs with two dT at positions 6 and 9 mainly belong to the fraction P25, with also some of them in P15. TFOs with only one dT at position 6 or 9 belong to fraction P35, with some, that carry rU at positions 1 and 2, falling into P25. The TFOs having the RNA core (rU in central positions 6 and 9) are in the more stable TFO fraction P45, except for a few individuals with mainly rU in positions 1 and 2, which go to P35. These results confirm the slightly destabilizing effect of rU at positions 1 and 2, and the opposite effect for positions 3 and 4. Looking at fractions P05 and P15, we can observe a regular reactivity all along the nucleotides, with the same anomalous migrating product between positions 8 and 9 that was present in the fraction M05 of the first TFO library M (Figs. 3 and 6), indicating again partial 3’ → 2’ phosphodiester isomerization during synthesis and deprotection of the library P.

**IP-RP-HPLC**—The reduced number of individuals present in the sublibrary P, as compared with M (2º versus 2¹), facilitates the resolution of the different AC fractions by HPLC. The average retention times of P and its fractions followed the sequence P45 < P35 < (P ~ P05 ~ P15) < P25 (Fig. 9). This is in agreement with the results of ESI-MS showing the same order for the average rU content of the fractions. P35 counts for some 35 individuals of the library, giving rise to ~20 peaks and shoulders. In the case of P45, the ~16 individuals that are contained are almost resolved by LC.

The large range of retention times of the TFOs in P45 allowed us to separate this fraction into five well resolved subfractions (Fig. 10, top). The subfraction 1 corresponds to r11d, the only TFO of this library to have six rU residues, which gives it a unique retention time. The other subfractions were assayed by PAD (Fig. 10, bottom), and the results confirmed our expectations. No differences were found between the subfractions in the reactivity at positions 6 and 9, confirming that all products within P45 have an RNA core. The sum of the reactivities at positions 1–4 point to one missing rU-unit per subfraction when going from 2 to 5, confirming that the “peak families” are mostly separated by ribonucleotide content. 2 contains at least two products of the four possible that carry only one dT at positions 1 to 4, 3 contains individuals with two dT (6 possibilities), with positions 1 and 2 being favored for the presence of dT. The reactivity of 4 is consistent with the presence of three dT, with high probabilities for positions 1 and 2. Finally, the two peaks in the chromatogram corresponding to 5 represent the TFO bearing four dT in positions 1 to 4, plus the one containing three dT in positions 1–3 and one rU in position 4. These conclusions are in agreement with UV-visible, ESI-MS, and PAD, which foresaw an average of three to four rU units in P45.

**Single TFO Synthesis and Validation of the Combinatorial Approach**

In order to validate these results and conclusions, we undertook the synthesis of representative individuals of the library P. The synthesized TFOs are described in Fig. 11. r11d and r6d are the maximum and minimum ribonucleotide-containing TFOs included in P. T12 and T34 and 4T allow for the verification of the sequence dependent effects of the rU → dT substitution at positions 1–4 observed during the selection process. T6 and T9 are expected to be members of P35, and T9 should be a representative of the P25 fraction. dU5 was synthesized in order to assess the effect of the 5-Me group of thymines on the triple helix stability. The TFOs were synthesized, purified, and characterized (Fig. 11) as described previously. PAD was performed in order to verify the reactivity of the mixed sequences. The expected patterns were observed, with digestion signals only present at ribonucleotide positions (data not shown).

Thermal stabilities of the corresponding triple helices were measured. As shown in Fig. 11, the conclusions from the study of the combinatorial libraries are fully confirmed. The Tₘ values of the TFOs fit within the values of the fractions to which they were allocated. In particular, the Tₘ of P25th is similar to that of r6dth. The conclusion that this fraction is mainly composed by TFOs completely lacking the RNA core is therefore verified. The Tₘ of P35 is just 2.7–3.0° lower than the Tₘ of T₆ and T₉, which also fits with our conclusions. The Tₘ of P45 is
similar to that of 4T, lower than that for T12 and r11d, but higher than that for T34. All these TFOs have, as expected for TFOs synthesized separately.

The single TFOs were characterized separately by their retention times in IP-RP-HPLC and then were co-injected (Fig. 12). The retention times are, as shown previously, mainly a function of the ribonucleotide content of the oligonucleotide.

Some isomers carrying dT nucleotides in the same number but at different positions within the TFO, as, e.g., for T12, T34, and T69, are also resolved. By comparison to the chromatograms of the P fractions (Fig. 9), the major part of the peaks, or groups of peaks, could thus be assigned with high reliability.

**DISCUSSION**

Oligonucleotide triple helices composed of DNA or RNA third strands dramatically vary in stability, and are structurally heterogeneous in backbone conformation (9–16). No general rules are yet available to predict conformational preferences for any given TFO to its specific DNA duplex target, rendering the design of high affinity TFOs difficult. Thus, a combinatorial method allowing for a conformational screen of TFOs, as the one presented here, is an appropriate method for defining the rules that govern high triple helix stability. When compared with the commonly used methods for selection and amplification (34, 35), the method described here shows some distinct advantages. (i) No enzymes are required at any step of the selection cycles, so that not only chimeric ribo- and deoxyribonucleotides, but a wide variety of sugar or base-modified ribo- and deoxyribonucleotide analogues can in principle be used. (ii) The approach is target-driven. This property is of considerable importance when there is need to design a strong binding TFO to a predefined DNA double helix sequence. (iii) The selection process can be performed using a variety of physical selection criteria, as, e.g., pH or ionic strength, in addition to temperature. This can be important when studying the effect of substituents of protonated cytosines in the TFO (see Ref. 17 and reference therein), or in the context of non-anionic backbone (see Refs. 6, 36, and 37 for review). Applied to the model triple helix investigated here, the following conclusions can be drawn:

**Conformational Diversity and Triple Helix Stability**—Certainly one of the most important conclusions of the present study is that the introduction of conformational variability in the backbone by using concomitantly deoxy- and ribonucleotides in the TFO clearly does not lead to substantially enhanced binding affinity to a DNA target compared with an all RNA TFO. However, there exist subtle sequence effects, which are more pronounced for rU/dT than for rC/dC pairs. Looking at the results in detail we find an increase of 4.3 °C (0.9°/nucleotide) by exchanging five dC to rC units (d → r6d). All ribocytidines seem to contribute equally to stability of the triple helix irrespective of their sequence context. This is true, however, for dT to rU substitutions. Here, considerable sequence-dependent effects on stability were observed. For positions 6 and 9, in which the U(T) residues are flanked by protonated cytosines, differences in Tm rank from +6.4 to +6.7 °C modification, in favor for the ribonucleosides. Surprisingly, this is a higher effect on thermal stability for one single substitution as compared with changing all the (non-terminal) dC to rC units. Both protonated rC and dC of the TFOs are expected to be preferentially in N-type conformation, due to the anomic effect (38, 39), whereas rU and dT are preferentially in N-type and S-type conformation, respectively. Thus, changing dC to rC in the TFO is not expected to significantly alter the overall conformation of the TFO, whereas changing dT to rU introduces conformational inhomogeneity. The fact that C/T alternating sequence tracts in particular are sensitive to dT/rU exchanges shows that introduction of local conformational inhomogeneity in this base sequence context creates a dramatic destabilizing effect. In a T(U)-rich sequence context, the differences between deoxy *versus* ribo-substitutions are much less pronounced to almost inexist. Residues at positions 3 and 4 still exert a non-negligible effect of 2.3°/modification (probably due in large part to positions 4, as it is neighboring a cytosine). In contrast, an exchange of rU by dT at positions 1 and 2 leads to a slight increase in stability by 0.5°/modification. Even given that 5′- and 3′-terminal deoxycytosines as in r11d and T12 may stabilize the helix more efficiently than ribonucleosides due to more efficient stacking and/or more favorable solvation, it becomes clear that within homo(A)-homo(T) tracts of a target duplex, there is no significant destabilization arising from conformational inhomogeneity in the third strand. This is rather surprising but might have its origin in the special structural features of the poly(dA)-poly(dT) helix (44).

**Contribution of the 5-Me Group of Thymine to Triple Helix Stability**—The introduction of a 5-Me group on deoxytidine or deoxyuridine has been shown to stabilize the double and triple helices, probably via an entropic effect (40–43), but other studies carried out by isothermal titration calorimetry (20) or with cytosine analogues (21) failed to show any effect. In order to verify the extent of this contribution in the system investigated, we synthesized the TFO dU6, which can directly be compared with r11d. The stability of dU6,th was found to be half-way between those of T6th and r11dth, indicating that the 5-Me group of thymine at position 6 has a nearly similar negative effect on the thermal stability of the triple helix as has the absence of the 2′-OH group when compared with r11d. Such a negative effect, which is opposite to what is found for cytidine and 5-methylcytidine, has never been described before. It cannot be excluded that the 5-Me group of dT within an overall A-like conformation of such a chimeric DNA/RNA leads to unfavorable steric interactions.

In this model study, we exploited differences in chemical and physical properties (chemical reactivity, mass, hydrophobicity,
absorption spectra) in order to differentiate between ribo- and deoxyribonucleotides in TFO libraries and fractions. In particular, IP-RP-HPLC coupled with mass spectrometry and PAD have revealed as versatile analytical tools that enable separation, constitutional identification, and positional assignment of modifications within a set of TFOs. The results obtained here highlight the power of combinatorial methods for understanding and improving molecular recognition in interacting systems, where rational prediction (yet) falls short due to the complexity of the system.

Acknowledgments—We thank Drs. J. Schaller and S. Schurch for help with the M3 measurements.

REFERENCES

1. Felsenfeld, G., and Rich, A. (1957) Biochim. Biophys. Acta 26, 457–468
2. Felsenfeld, G., Davis, D. R., and Rich, A. (1957) J. Am. Chem. Soc. 79, 2923–2924
3. LeDoan, T., Perrousault, L., Praseuth, D., Habhoub, N., Decout, J. L., Thuong, N. T., Hiomme, J., and Hélène, C. (1997) Nucleic Acids Res. 15, 7749–7761
4. Maser, H. E., and Dervan, P. B. (1987) Science 236, 645–650
5. Radhakrishnan, I., and Patel, D. J. (1994) Biochemistry 33, 11405–11416
6. Sun, J. S., and Hélène, C. (1993) Curr. Opin. Struct. Biol. 3, 345–356
7. Vasquez, K. M., and Wilson, J. H. (1998) Trends Biochem. Sci. 23, 4–9
8. Roberts, R. W., and Crothers, D. M. (1992) Science 256, 1463–1465
9. Liquier, J., Cofinier, P., Firon, M., and Taillander, E. J. (1991) Biochem. J. 302, 437–445
10. Macaya, R. F., Schultz, P., and Feigon, J. (1992) J. Am. Chem. Soc. 114, 781–783
11. Liquier, J., Taillander, E., Klinck, E., Guittet, E., Gouyette, C., and Huyah-Dinh, T. (1995) Nucleic Acids Res. 23, 1722–1728
12. Bartley, J. P., Brown, T., and Lane, A. N. (1997) Science 278, 14502–14505
13. Tarko, Y., Mauz, A., Faucon, B., Giovannangeli, T., Garestier, T., and Hélène, C. (1992) Biochemistry 31, 9269–9278
14. Gotfredsen, C. H., Schultz, P., and Feigon, J. (1998) J. Am. Chem. Soc. 120, 4281–4289
15. Assenio, J. L., Carr, R., Brown, T., and Lane, A. N. (1999) J. Am. Chem. Soc. 121, 10963–10970
16. Shimizu, M., Konishi, A., Shimada, Y., Inoue, H., and Ohtsuka, E. (1992) FEBS Lett. 302, 155–158
17. Escudé, C., François, J. C., Sun, J. S., Ott, G., Sprinzl, M., Garestier, T., and Hélène, C. (1993) Nucleic Acids Res. 21, 5547–5553
18. Noronha, A., and Damha, M. (1998) Nucleic Acids Res. 26, 2665–2671
19. Han, H., and Dervan, P. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3806–3810
20. Toragoe, H., Shimizune, R., Sarai, A., and Shindo, H. (1999) Biochemistry 38, 14655–14659
21. Hildbrand, S., Blaser, A., Parel, S. P., and Leumann, C. J. (1997) J. Am. Chem. Soc. 119, 5499–5511
22. Inanishi, T., and Obika, S. J. (1999) Synth. Org. Chem. Jpn. 57, 77–88
23. Prakash, T. P., Manoharan, M., Fraser, A. S., Kawasaki, A. M., Lesnik, E. A., and Owens, S. R. (2000) Tetrahedron Let. 41, 4855–4859
24. Amersham Pharmacia Biotech. Users Manual 56-1111-56: Gene Assembler Special/4 Primers, Amersham Pharmacia Biotech, Piscataway, NJ
25. Usman, N., Ogilvie, K. K., Jiang, M. Y., and Cedergren, R. J. (1987) J. Am. Chem. Soc. 109, 7845–7854
26. Scharinge, S. A., Francklyn, C., Usman, N. (1990) Nucleic Acids Res. 18, 5433–5441
27. Beier, H., and Gross, H. J. (1991) in Essential Molecular Biology: A Practical Approach (Brown, T. A., ed) pp. 221–236, IRL-Oxford University Press, New York
28. Puglisi, J. D., and Tinoco, J. Jr. (1999) Methods Enzymol. 281, 104–125
29. Maher, L. J., III, Dervan, P. B., and Wahl, B. J. (1990) Biochemistry 29, 8820–8826
30. Rougé, M., Faucon, B., Barcelo, F., Giovannangeli, T., Garestier, T., and Hélène, C. (1991) Biochemistry 31, 9269–9278
31. Shindo, H., Toragoe, H., and Sarai, A. (1993) Biochemistry 32, 8963–8969
32. Xodo, J. D., and Tinoco, I., Jr. (1989) Biochemistry 28, 918–926
33. Cantor, C. R., and Schimmel, P. R. (1980) Biophys. Chem. 2, 382–383
34. Pei, D., Ulrich, H. D., and Schultz, P. G. (1991) Science 253, 1408–1411
35. Handeler, P., and van Dyke, M. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 93, 2811–2816
36. Nielsen, P. E. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 167–183
37. Sun, J. S., Garestier, T., and Hélène, C. (1996) Curr. Opin. Struct. Biol. 6, 327–333
38. Plavec, J., Tong, W. M., and Chattopadhyaya, J. (1993) J. Am. Chem. Soc. 115, 9734–9746
39. Thibaudon, C., Plavec, J., and Chattopadhyaya, J. (1996) J. Org. Chem. 61, 266–268
40. Porse, T. J., and Dervan, P. B. (1989) J. Am. Chem. Soc. 111, 3059–3061
41. Plum, G. E., Park, Y. W., Singleton, S. F., Dervan, P. B., and Breslauer, K. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9436–9440
42. Xodo, J. E., Manzini, G., Quadri, P., van der Marel, G. A., and van Boom, J. H. (1991) Nucleic Acids Res. 19, 5625–5631
43. Leitner, D., Schroder, W., and Weisz, K. (2000) Biochemistry 39, 5886–5892
44. Sanger, W. (1984) Principles of Nucleic Acid Structure, p. 271, Springer Verlag, New York