Stability of DNA Triplexes on Shuttle Vector Plasmids in the Replication Pool in Mammalian Cells*

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Triple helix-forming oligonucleotides may be useful as gene-targeting reagents in vivo, for applications such as gene knockout. One important property of these complexes is their often remarkable stability, as demonstrated in solution and in cells following transfection. Although encouraging, these measurements do not necessarily report triplex stability in cellular compartments that support DNA functions such as replication and mutagenesis. We have devised a shuttle vector plasmid assay that reports the stability of triplexes on DNA that undergoes replication and mutagenesis. The assay is based on plasmids with novel variant supF tRNA genes containing embedded sequences for triplex formation and psoralen cross-linking. Triplex helix-forming oligonucleotides were linked to psoralen and used to form triplexes on the plasmids. At various times after introduction into cells, the psoralen was activated by exposure to long wave ultraviolet light (UVA). After time for replication and mutagenesis, progeny plasmids were recovered and the frequency of plasmids with mutations in the supF gene determined. Site-specific mutagenesis by psoralen cross-links was dependent on precise placement of the psoralen by the triple helix-forming oligonucleotide at the time of UVA treatment. The results indicated that both pyrimidine and purine motif triplexes were much less stable on replicated DNA than on DNA in vitro or in total transfected DNA. Incubation of cells with amidoanthraquinone-based triplex stabilizing compounds enhanced the stability of the pyrimidine triplex.

Triple helix-forming oligonucleotides (TFOs), or third strands, have received considerable attention because of their potential for intracellular gene targeting (1–11). Many of the biochemical and biophysical properties of triplexes appear to support this application. Triplex formation following the encounter between an appropriate third strand and duplex target has long been recognized as a fundamental structural option of nucleic acids and is not dependent on enzymes or proteins (12). The most stable triplexes are formed on polypurine/polypyrime sequences. However, there is considerable stringency with respect to the specific sequence such that single interruptions in a polypurine run can be destabilizing (13–15). Depending on the actual sequence of the purine/pyrimidine run, triplexes can be formed by third strands composed of either purines or pyrimidines (16), which offers some flexibility in TFO design.

Once formed, triplexes can be quite stable under appropriate conditions. This was demonstrated some time ago with a pyrimidine triplex with a dissociation half-life of many hours (17). Some purine TFOs form even more stable complexes, with melting temperatures equivalent to or greater than the target duplex (18), and with half-lives of days under optimal conditions (19, 20). The persistence of purine motif triplexes formed on DNA fragments and then introduced into mammalian cells has also been measured. A footprinting assay to measure triplexes on transfected fragments as a function of time after transfection reported that the triplexes were stable over a 24-h period (21, 22). Another assay, based on radiofootprinting, came to a similar conclusion (23). These data implied that triplexes were quite stable in cells, similar to the situation under optimal laboratory conditions.

On the other hand, there are limitations to the activity of TFOs and triplex stability. Triplexes formed by pyrimidine third strands containing cytosines are typically unstable under physiological conditions. This is because of the requirement for N3 protonation of cytosines which does not occur at physiological pH. However, the incorporation of 5-methylcytosine (5-MeC) (24, 25) and the 2′-O-methyl (2′-OMe) substitution of the sugar (26) into TFOs enhance the stability of pyrimidine triplexes in “physiological” buffers. The activity of purine TFOs is compromised by the tendency of guanine-rich oligonucleotides to aggregate or form tetraplex structures in physiological levels of K+, which inhibits triplex formation (27–29). Triplex formation in both motifs is dependent on Mg2+, with dramatic differences in affinity apparent over a relatively narrow concentration range (30, 31). The requirement for divalent cation is of concern as triplexes are typically formed in vitro in 5–10 mM Mg2+, while the concentration of Mg2+ inside cells is probably less than 1 mM.

We are interested in TFOs as reagents for targeting genes in vivo in gene knockout applications (9). Consequently, we are concerned with the stability of triplexes in nuclear compartments, in living cells, in which replication and mutagenesis occur. Given the sensitivity of triplexes to ionic conditions and pH, it is important to note that the measurements of long triplex half-lives in vitro, cited above, were conducted under conditions that were optimal for complex formation and maintenance. These conditions may not accurately reflect those in vivo. Furthermore, although the analysis of triplex stability on
the transfected DNA population is informative, it does not necessarily describe the stability of the complexes on biologically active DNA. In the experiments described here, we have used a plasmid-based mutation assay to report the persistence, in vivo, of triplexes formed prior to transfection. We find that preformed triplexes on DNA that replicated following transfection are less stable than would be predicted by analyses of triplexes in vitro, or on total transfected DNA. However, the stability of triplexes can be extended by treatment of cells with certain compounds that stabilize triplexes in vitro. These compounds may have potential for enhancing the activity of TFOs in vivo, particularly if issues of long-term toxicity can be resolved.

MATERIALS AND METHODS

Cells—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin and streptomycin.

Plasmids—Shuttle vector plasmids were constructed as described previously (32) with the early region and origin of replication from SV40 virus, and the β-lactamase gene and replication origin from pBR322. The plasmids also contained variant supF genes with triplex target and psoralen cross-link sites as described under “Results.” Restriction enzyme sites at the position of the psoralen cross-link in the plasmids (XhoI for the supF5/TC30; BstBI for the supFG1/AG30) were used in restriction protection assays.

Oligonucleotides—We prepared a 5′-psoralen (C6) linked-TC30 TFO (5′-Pso-TCTTTTCTTTTCTTTTCTTCTTTTTTCTTT) with 5-MeC and 2′-OMe modifications on all bases. The AG30 TFO (5′-Pso-AGGAAG-GGGGGGCGTTGGGGGGAGGGGGAG), described previously (33) was also linked (C6) to psoralen at the 5′ end. The three 3′ terminal residues were added as phosphorothioates to provide nuclease protection (34). Affinity constants were determined by duplex band shift as described (33). In 10 mM MgCl2, 20 mM Tris acetate, pH 7.4, the Kd of the TC30 TFO was 29 nM, while the Kd of the AG30 TFO was 5 nM.

Triplex Formation—Triplexes were formed by incubation of 3.5 pmol of the appropriate plasmid with 2 μM TFO in 20 mM Hepes, pH 7.2, 100 mM NaCl, 10 mM MgCl2, 0.2 mM spermine for 24 h at 37 °C. Triplexes were separated from free oligonucleotide by adjustment of the mixture to 2.5 M ammonium acetate, 10 mM MgCl2, and precipitated by the addition of two volumes of alcohol. Control experiments showed that the triplexes were stable during this manipulation, which had the added advantage of sterilizing the samples. In experiments in which preformed triplexes on DNA that replicated following transfection, we find that certain compounds that stabilize triplexes in vitro, or on total transfected DNA. However, the stability of triplexes can be extended by treatment of cells with certain compounds that stabilize triplexes in vitro. These compounds may have potential for enhancing the activity of TFOs in vivo, particularly if issues of long-term toxicity can be resolved.

RESULTS

Restriction Protection and Mutation Assays for Bound TFO—The assays for TFO binding are based on a shuttle vector plasmid described in previous publications (36, 37). In addition to elements that support replication in bacterial and primate cells, the plasmid carries a mutation marker gene, the suppressor tRNA gene, supF. The structure of a transfer RNA is a major determinant of correct processing of the pre tRNA transcript and the activity of the mature tRNA molecule. Consequently, it is possible to make substantial changes in tRNA sequence, while retaining function, as long as the structure of the mature molecule is maintained (32, 38, 39). We have taken advantage of this principle to construct variant, but still functional, supF genes containing sequence elements that served as targets for triplex formation and psoralen cross-linking. These functional variants were used as mutation reporter genes.

In the supF5 construction, we placed the TC30 triplex target sequence in the pre-tRNA region of the gene, immediately adjacent to the start of the 5′ end of the mature gene (Fig. 1). Just inside the mature gene sequence, at the 5′ start of the acceptor stem, we placed a 5′ TA step, the optimal sequence for cross-linking by psoralen. This was at position 99/100 (the numbering was preserved from the original supF scheme) (41). The sequence of the 3′ acceptor stem sequence was adjusted to

\[
3′=\text{ttttctt ttctt ctctt ttttt ttttt (-tcg(-trp)}-\text{psol-5′)}
\]

\[
5′=\text{TTTTCT TTTTT TTTTT TTTTT TTTTT TTTTT}
\]

\[
3′=\text{AAAGAAA AAAAAA AAAAAA AAAAAA AAAAAAA}
\]

FIG. 1. The structure of the variant supF5 gene. The triplex target sequence is immediately adjacent to the mature gene, shown as a DNA sequence in cloverleaf form. The sequence of the psoralen-TC30 TFO is shown above the duplex target. The XhoI site at the psoralen cross-link site is in bold. The sites of the psoralen cross-link are boxed and numbered (residues 99 and 100).

48 h, the plasmids were harvested and then treated with DpnI to remove non-replicated plasmid (35). The plasmids were then introduced into the Escherichia coli indicator strain MBM 7070, which carries an amber mutation in the β-galactosidase gene (36). The bacteria were plated on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside, and the frequency of white or light blue colonies with mutations in the supF gene was determined. In order to compare the relative stabilities of the two triplexes, the data were normalized to the mutation frequency of the appropriate plasmids in which the psoralen cross-link was fixed immediately after electroporation (0 time point). This value was the same as that recovered from plasmids that had been cross-linked in vitro. The frequency of mutations in the supF5 plasmid obtained after replication of the plasmid, cross-linked in vitro, was 11%, while that of the supFG1 plasmid was 6%. The results from the complete time-course measurements were confirmed in independent experiments at a subset of time points.

RESULTS

Restriction Protection and Mutation Assays for Bound TFO—The assays for TFO binding are based on a shuttle vector plasmid described in previous publications (36, 37). In addition to elements that support replication in bacterial and primate cells, the plasmid carries a mutation marker gene, the suppressor tRNA gene, supF. The structure of a transfer RNA is a major determinant of correct processing of the pre tRNA transcript and the activity of the mature tRNA molecule. Consequently, it is possible to make substantial changes in tRNA sequence, while retaining function, as long as the structure of the mature molecule is maintained (32, 38, 39). We have taken advantage of this principle to construct variant, but still functional, supF genes containing sequence elements that served as targets for triplex formation and psoralen cross-linking. These functional variants were used as mutation reporter genes.
Several recent publications have measured triplex stability inside cells by following the level of triplexes on transfected DNA as a function of time after transfection (21–23). The triplexes described in these publications were stable over many hours in transfected DNA. We performed similar experiments with the TC30 and AG30 triplexes. The complexes were formed in vitro, and the triplex-plasmids were electroporated into cells that were incubated at 37 °C. After 0.5, 4, or 8 h, cells were UV-treated, then washed extensively, and total plasmid harvested, without the DpnI treatment used in subsequent experiments to eliminate nonreplicated plasmid. The extent of psoralen cross-linking was then determined by the restriction protection assay. The plot of the data from the supFG1 (AG30) and supF5 (TC30) plasmids is shown in Fig. 3. The t½ of TC30 triplex was 288 min, while that of AG30 triplex was 150 min. These results indicated that both triplexes on total transfected DNA were less stable in cells than in a physiological buffer, and, as before, the TC30 triplex was the more stable of the two.

**Triplex Stability on Replicated Plasmid DNA**—We then measured the stability of triplexes on plasmids that replicated following transfection. Following triplex formation, free TFO was removed (see “Materials and Methods”) and the complexes suspended in medium prewarmed to 37 °C, mixed with cells, and electroporated in prewarmed cuvettes. The cells were incubated at 37 °C for the indicated times and then exposed to UVA to activate the psoralen. The transfected cells were incubated for an additional 48 h. During this time, the plasmids replicated and the psoralen cross-links were removed with or without mutational consequences. Then, the plasmids were harvested and treated with DpnI to remove unreplicated plasmid. The plasmids were introduced into MM7070 and the frequency of colonies with mutations in the supF gene determined. The results showed that, relative to the 0 time point,
the TC30 triplex had a $t_{1/2}$ of 59 min, while the $t_{1/2}$ of the AG30 triplex was 10 min (Fig. 4A). The results of this experiment were in sharp contrast to the data in the preceding figures. However we repeated complete, and abbreviated (1-, 2-, and 4-h time points), versions of the experiment with both triplexes, with different preparations of TFOs and plasmids. As shown in the figure, there was good agreement between data sets (see legend to Fig. 4A).

Triplex formation by purine motif third strands has been shown to be compromised by competing self-structure formation (G tetraplexes) by the TFO at temperatures below 37 °C (27). We were concerned that this might be an issue during the replication process (G tetraplexes) by the TFO at temperatures below 37 °C (27). We were concerned that this might be an issue during the manipulation of the supFG1-AG30 complex prior to electroporation. Accordingly, we repeated the experiment by preparing and maintaining the complex at 37 °C at all times before electroporation, without a precipitation step to remove unbound TFO. The results were the same as before.

These data demonstrated that both the pyrimidine and purine motif triplexes were much less stable on DNA that entered the replication pool, than in physiological buffer in vitro, or in total transfected DNA.

The data (Fig. 4A) were analyzed by plotting the natural log of mutation frequency versus time (Fig. 4B). The plot of the TC30-supF5 complex was approximately linear, indicating first order kinetics with a single exponential decay. This suggested that decline in mutation frequency was due to the simple dissociation of the triplex. The plot of the AG30 data was multi-component, with at least a two-phase exponential decay, indicating a more complicated decay process (Fig. 4C).

**Mutation Specificity**—The validity of the mutagenesis assay as a measure of triplex stability demanded that the mutation frequency reflected mutagenesis at the site of the cross-link placed by the precise positioning of the psoralen by the TFO. We addressed this issue by analyzing the sequence of mutant supF genes isolated from experiments in which the cells were treated with UVA 3 h after transfection. We found that the pattern of mutations with the supFG1-AG30 triplexes was the same as that reported earlier from experiments with the complexes that had been cross-linked in vitro or in vivo (33, 44). Plasmids with mutant supFG1 genes contained single base substitutions located at the positions of the psoralen cross-link as well as deletions of 5–75 bases of sequence in the triplex target region. We performed the same analysis on mutant supF5 plasmids. Approximately 25% of the plasmids contained single base deletions in the triplex target and supF gene ranging from 2 to 95 bases. Another 45% contained single base substitutions at position 99, while the remainder had single base substitutions at position 100, the sites of the psoralen cross-link (see Fig. 1). T → C and T → A were the principal mutations. Similar results were obtained with supF5 plasmids on which the psoralen cross-link was set in vitro prior to transfection. These mutation data with both the supFG1 and supF5 plasmids demonstrated that the mutagenesis of the plasmid in the stability assays was indeed a measure of the precise positioning of the psoralen cross-link by the TFO.

**Influence of Psoralen Linkage and Cell Type**—The striking differences between our data and the predictions based on the published, and our own (Figs. 2 and 3), in vitro, experiments led us to consider explanations for the results unrelated to triplex stability. Our assay assumed the integrity of the psoralen linkage to the oligonucleotide during the time course. Clearly, if the psoralen were cleaved from the TFO during the experiment, interpretation of the data would not be possible. We repeated the mutagenesis time-course experiment with a version of the Pso-TC30 TFO in which the phosphate linkage of the psoralen was protected from nuclease attack by phosphorothioation (34).

**Fig. 4.** A, stability of the TC30 (upper curve) and AG30 (lower curve) triplexes in replicated DNA. The plasmid-triplex complexes were introduced into cells and at the indicated times the cells were treated with UVA. After an additional 48 h the plasmids were harvested, treated with DpnI, and the frequency of mutations in the replicated plasmid population determined by screening in E. coli MBM7070. The results were normalized to the 0 time (100%) value for each cross-linked plasmid-triplex complex (see “Materials and Methods”). The curves are based on data from two complete experiments, as well as abbreviated versions repeated several times. For example, there were 10 measurements of the TC30 triplex stability at the 4-h time point. The range was 9–20%, with a mean of 15.1%, and a standard deviation of 3.0. B, semilog plot of the data with the TC30-supF5 triplex. C, semilog plot of the data with the AG30-supFG1 triplex.
However, the results were the same as before. Consequently, it seemed unlikely that loss of psoralen was responsible for the loss of mutational signal.

COS-7 cells express the SV40 T antigen constitutively (45). T antigen is a helicase that can unwind triplexes (46). Although T antigen is also encoded by the plasmids used in this report, the gene must be transcribed and translated and the protein accumulated before reaching the levels found in the COS-7 cells. We reasoned that, if the T antigen were unwinding the triplexes, there would be a lag before this would occur in cells other than COS-7. Consequently, we repeated the experiment with the TCG30\sup F5 complex in CV1 cells and Ad293 cells, which do not express T antigen. However, we again found similar decay kinetics for the mutagenesis signal. These data suggested that T antigen was not the reason for the loss of signal in the COS-7 cell experiments.

Influence of Temperature on Triplex Stability in Vivo—The relative stability of the triplexes in vitro under the conditions described in Fig. 2 suggested that the sharp decline in triplex level in the replication compartment was due to factors other than physiological temperature, or K\(^+\), and Mg\(^{2+}\) concentrations. If the triplex loss followed from the action of the cellular functions (enzymes, proteins, etc.), then a reduction in cellular temperature during the incubation prior to psoralen activation would be expected to preserve higher levels of complex, and yield higher mutation frequencies. Accordingly, we performed the experiment by electroporating the complexes into cells maintained at 20 °C prior to the UVA treatment. The results (Fig. 5A) showed that the TC30 triplex was indeed more stable at the lower temperature, such that more than 70% of the starting signal was recovered at 8 h after electroporation. The AG30 triplex was also more stable, with a \(t_{1/2}\) of about 300 min. As expected the semilog plot of the \sup F5/TC30 data reflected the greater stability of the complex (Fig. 5B). Interestingly, the semilog plot of the \sup F5/AG30 data was linear, suggesting that, at the lower temperature, the AG30 triplex dissociation followed first order kinetics (Fig. 5C).

Ligand Stabilization of the TC30 Triplex—The greater stability of the triplex formed by the TC30 TFO led us to focus on this TFO and consider ways to improve the residence time in vivo. One approach was suggested by reports that certain intercalators stabilize pyrimidine motif triplexes in vitro (2, 47–49). Recently, several amidanthraquinone (AQ) derivatives were shown to effectively stabilize pyrimidine triplexes at concentrations that were compatible with cellular viability (50–52). We asked if incubation of cells with some of these compounds (Fig. 6) following introduction of the \sup F5/TC30 triplex would stabilize the complex. In initial experiments, we incubated the cells with the compounds, introduced the TC30-supF5 complex, and continued the incubation with the compounds for 4 h, at which time the cells were treated with UVA (Table I). The cells were then placed in fresh medium without compound and processed as in the previous experiments. A concentration of 1 \(\mu\) M was chosen for the AQ derivatives because each of the compounds was active at this concentration (51), and the cells would tolerate this condition. As shown in the table, \sup F5 was not mutagenized by passage through cells treated with the compounds, consistent with earlier studies showing that they were not mutagenic (53). There was also no mutagenesis in experiments with the triplex complexes if the psoralen was not photoactivated. In addition to the AQ derivatives, we tested the effect of 8 \(\mu\) M coralyne which is also a triplex stabilizer (2, 47, 54). The results indicated that, although coralyne was not effective, incubation of the cells with the AQ derivatives during the time prior to UVA treatment did enhance triplex stability. This stimulation in mutation fre-
quency required UVA treatment, indicating that it was psoralen-dependent.

Based on these results, we performed a more detailed analysis of the stability of the TC30-supF5 complex in cells incubated with the 2,7-pyrrolidine AQ, which was the most active derivative. The $t_{1/2}$ of the complex in the presence of this derivative was extended to 150 min, a 2.5-fold increase in stability (Fig. 7).

The simplest explanation of these data was that the AQ derivative had stabilized the TC30 triplex in the cells. However, there was a formal possibility that residual compound present in the cells after the UVA treatment could increase the probability of mutagenesis of the cross-links. If this were true, then a higher mutation frequency could be derived from the same number of triplexes and cross-links as compared with the experiments without compound. We tested this possibility by incubating cells in 1 $\mu$M 2,7-AQ for 2 h prior and 24 h following introduction of the supF5-TC30 complex previously cross-linked in vitro. The mutation frequency of the sample from the cells incubated with the AQ derivative was 14%, while the control (cells without AQ) was 11%. Thus, although residual compound might have provided a slight increase in the mutation frequency, it cannot have accounted for the 2.5-fold increase in $t_{1/2}$ in the experiment of Fig. 7. Consequently, we concluded that the increase was due to the stabilization of the triplex by the AQ derivative.

**DISCUSSION**

The long standing interest in the DNA triple helix is based, in part, on the notion that triplex-forming oligonucleotides might be used as gene-targeting reagents in living cells (3, 5, 6, 9). Triplexes have been shown to be quite stable in vitro, with half-lives that would appear to be appropriate for biological utility (17, 18, 20, 22, 55). However, the conditions under which these measurements were made may or may not reflect conditions in vivo. One of the important features of our shuttle vector plasmids is that they are transcribed and replicated as minichromosomes in the nucleus. The design of the mutagenesis assay limits the analysis to those plasmids that actually replicated (35). This is an important distinction because much of the DNA that enters a cell via transfection, including electroporation, does not enter the nucleus (56, 57) and does not become functional. Furthermore, even nuclear localization, as indicated by physical measurement, is no guarantee of biological activity. It has become clear in recent years that the nucleus is not a “randomly arranged bag of molecules” but instead is functionally compartmentalized (58), and a molecule may or may not be in the compartment of interest. Thus, it is important to employ functional rather than physical assays when considering the fate of triplexes following introduction into cells.

In the experiments described here, we found that triplexes on plasmids that replicated were much less stable than triplexes in total transfected DNA (Figs. 3 and 4). One implication of this observation is that complexes in the “total” plasmid population did not bleed steadily into the replication compartment after the initial distribution of the triplexes following electroporation. If they had, the half-life of triplexes on the replicated DNA would reflect the longer life of the triplexes in the total transfected DNA. Thus our data support the simple conclusion that both the purine and pyrimidine triplexes, in the replication compartment, were much less persistent than predicted by measurements of stability in physiological buffers in vitro, or in total transfected DNA.

There are two explanations for this. Triplex stability is sensitive to the pH and ionic environment, and the actual conditions in vivo could be different, and less supportive, than anticipated by our laboratory formulations. Another possibility is that cellular enzymes or other proteins might disrupt the triplexes.

**Purine Triplex Instability**—The decay kinetics of the transfected AG30-supFG1 complex suggest that both explanations may apply to this purine triplex. The fast initial decay of this triplex seen in Fig. 4 is consistent with an immediate change in
the effective ionic environment. One possibility is that upon entry into the replication compartment a significant fraction of the AG30 triplexes dissociate rapidly due to the formation of self-structures by the AG30 TFO. These structures, such as G tetraplexes, are incompatible with triplex formation (27, 29, 59, 60). An initial decay was also seen in the experiments with total plasmid DNA (Fig. 3), although not as dramatic as in Fig. 4. This probably also reflects a change in the environment, although not as pronounced as for the complexes that enter the replication compartment.

An alternate explanation might be that the divalent cation concentration in the active compartment is inappropriate for triplex stability. Other studies have shown a rapid loss of purine triplexes following reduction in Mg²⁺ concentration (31). However this would also have been true for the TC30 complex, which was much more stable than the AG30 triplex.

In other experiments, we have used the mutation assay to measure the stability of a 16-base purine triplex (5'-GGAAAGGAGGGGGG), which, according to our gel analyses, is less inclined to form self-structures. This triplex, which also requires Mg²⁺, shows the same t₅₀ as the TC30 triplex. Consequently, we favor the self-structure scenario as an explanation for the rapid loss of the AG30 triplex following electroporation. If this is correct, then, as emphasized above, the use of physiological K⁺ concentrations (which stabilize self-structure formation by guanine rich sequences) in the experiment in Fig. 2 did not effectively model the actual cellular environment insofar as the stability of the AG30 triplex was concerned.

In addition to the self-structure possibility, it is likely that there were additional effectors of the stability of the AG30 triplex since the semilog plot showed at least two exponentials. The situation is clearly complex since the triplexes remaining after the initial phase decayed more slowly. This implies a cellular component that stabilized the complex. At the same time, we suggest that there were cellular factors whose activity contributed to the loss of the triplex. This is based on the observation that the AG30 triplex was more persistent at 20 °C than it had been in the 37 °C experiment (see below).

Pyrimidine Triplex Instability—In the case of the pyrimidine triplex formed by supF5 and TC30, we suggest that the decay in vitro is the consequence of dissociation mediated by temperature dependent functions (perhaps enzymatic) as well as normal kinetic dissociation. This conclusion is based on the results of the experiment in which the cells were incubated at a lower temperature resulting in a dramatic increase in triplex stability. It could be argued that the reduction in temperature simply increased the physical stability of the TC30 triplex since it has been shown that pyrimidine triplex dissociation rates decrease as the temperature is lowered (61). Although this would make a contribution, we think it unlikely to be a complete explanation. This is because the TC30 triplex was quite stable in vitro at 37 °C in physiological buffer (Fig. 2), while it was clearly much less stable at the same temperature in the replication compartment (Fig. 4). Thus, the 37 °C incubation temperature of the cells in the in vitro experiments was not destabilizing per se. Consequently, the increased stability at the lower temperature must be due, at least in part, to a reduction in the activity of a temperature-dependent destabilizing activity, possibly protein-based. Triplexes have been shown to be unwound by helicases (46), and there are reports of triplex-binding proteins in mammalian cells (62–64), some of which could be destabilizing under appropriate conditions. The plasmids are templates for transcription and replication, and it is also likely that these processes are inimical to triplex stability (65). (However, our observation of identical decay kinetics in cells without an endogenous T antigen suggests that the disruption of the complex occurs too rapidly to be due to replication.) The identification of cellular activities that destabilize triplexes, and the improvement of inhibitors of those activities, could be important for improved TFO targeting protocols.

The data presented here raise two issues that are central to the improvement of TFO as gene-targeting reagents. The first is the question of which properties of a TFO, determined in biochemical analyses in vitro, are predictive for biological activity in vivo. The greater stability of the TC30 triplex, formed by a TFO with a weaker affinity than AG30, suggests that K₅ₐ determinations under optimal conditions in vitro may not be reliable predictors. Of course, an answer to this question will require more accurate approximations of the conditions in vivo. For example, several authors have called attention to “molecular crowding,” which occurs in the intracellular environment but not in conditions typically employed in laboratory experiments (66–68). As additional data are derived from in vivo experiments, with a broader range of TFOs, it is likely that we will begin to identify which in vitro measurements are indicators of in vivo behavior.

The second concern is how to enhance the stability of triplexes formed by the TFOs. It was possible to increase the stability of the pyrimidine triplex by incubation of the cells with compounds shown to stabilize triplexes. These results may offer some insight as to the affinity of the compounds necessary to show activity. Thus, coralyne has been shown by several groups to stabilize pyrimidine triplexes in vitro at micromolar concentrations (47, 48, 54, 69). It was also used to enhance triplex formation on a genomic target in permeabilized cells (2). However, it was inactive in the TC30-supF5 stability assay in vivo. On the other hand, the amidoanthraquinone derivatives, with submicromolar dissociation constants (70), were effective stabilizers in vivo. These compounds appear to offer promise as adjuvants for gene targeting by TFOs, especially if derivatives with lower cellular toxicity can be developed.

Direct chemical modification of the oligonucleotides may also prove efficacious for enhancing triplex stability in vivo. Sugar modifications that enhance the stability in vitro of pyrimidine motif triplexes have been characterized recently (71). In addition, oligonucleotides with novel backbones can form quite stable triplexes (72, 73). Purine TFOs with base and backbone modifications that increase triplex stability have also been described (40, 74). It will be of interest to examine the stability of triplexes formed by TFOs with these modifications in the assay discussed here.

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