Hybridization arrest of the cell-free translation of the malarial dihydrofolate reductase/thymidylate synthase mRNA by anti-sense oligodeoxyribonucleotides

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ABSTRACT

In order to inhibit the *in vitro* translation of *Plasmodium falciparum* mRNA coding for the bifunctional enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS), oligodeoxynucleotides (ODNs) were directed against the translation initiation site or a site in the TS-coding region. In both cases considerable hybridization arrest, *i.e.* >50% inhibition, was only achieved if the lengths of the ODNs to the two regions were 30 and 39 nucleotides, respectively, or longer. The ODN with the highest efficiency was a 49-mer directed against the TS-coding region (OTS49); 45 μM was sufficient to inhibit the expression of DHFR-TS by almost 90%. In this case the synthesis of DHFR-TS was interrupted at the binding site of OTS49 by a RNase H-independent mechanism. The resulting polypeptide was smaller (55 kDa) than one subunit of the native protein (71 kDa) and lacked TS activity.

INTRODUCTION

In *Plasmodium falciparum*, like in other protozoa, the dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) and thymidylate synthase (TS; 5,10-methylenetetrahydrofolate: dUMP C-methyltransferase, EC 2.1.1.45) activities reside on a bifunctional polypeptide (DHFR-TS) (1). DHFR-TS provides the sole pathway for the biosynthesis of dTMP and is essential for DNA synthesis and for the maintainance of tetrahydrofolate levels in *P.falciparum* (2). Unfortunately, the treatment of *P.falciparum* malaria with antifolates like pyrimethamine (3) is now rendered ineffective by the appearance of resistant strains.

Rather than selective blockage of enzyme activity, alternate approaches to the inhibition of cellular processes involve interactions between oligodeoxynucleotides (ODNs) and nucleic acids as diverse as messenger, transfer, ribosomal, and small nuclear RNA, as well as double- and single-stranded DNA (4). Selective inhibition of protein synthesis by double-strand formation between mRNA and complementary (anti-sense) DNA (5, 6) and ODN molecules (7) was first demonstrated more than a decade ago. Since then, numerous studies have been devoted to problems like the optimization of critical parameters such as target sequence (8, 9) and oligonucleotide length (10), the role of RNase H (11), and the modifications of ODNs in order to facilitate uptake by a cell, improve nuclease resistance, and/or increase the stability of duplexes formed with the target mRNA (4).

Trypanosomatids are the only parasites to which ODN-mediated translation inhibition in a cell-free system (12, 13), as well as in axenic culture (14), has been studied so far. Our own work on translation inhibition of malaria deals with a special problem. The content of the nucleotides adenine and uridine within *P.falciparum* RNA reaches 70 percent in the coding and 90 percent in the noncoding region (15). Since the base pairing between A und U is considerably weaker than that between G and C, we synthesized ODNs up to 49 nucleotides in length directed against both the translation initiation site and a sequence in the TS-coding region for *in vitro* translation inhibition. We demonstrate that, surprisingly, the 49-mer directed against the coding region is the most efficient ODN and RNase H-like activity is not crucial for its efficiency.

MATERIALS AND METHODS

Parasites

Plasmodium falciparum strain Ro58 (resistant to chloroquine and pyrimethamine and adapted to horse serum) was kindly provided by H. Matile (F.Hoffmann-La Roche, Basel). The parasites were cultured in RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES, 0.37 mM hypoxanthine, 100 mg/l neomycin base, 0.2% $NaHCO_3$, and 10% heat-inactivated horse serum (Amimed). The cultures (10 ml in volume) were kept at 5% hematocrit and 37°C in an atmosphere of 4% CO_2, 3% O_2, 93% N_2, and 94% rel. humidity (16). Human erythrocytes were type A, Rh^+. Parasite cultures were synchronised by two treatments in 5% sorbitol (17) about 40 hours apart. Parasitemia and staging were determined on Giemsa-stained thin-smears. Upon reaching 10 to 15% parasitemia, parasites were harvested and isolated by lysis of the infected erythrocytes with 0.015% saponin in SSC (15 mM sodium citrate and 150 mM NaCl) (18) followed by two washings in SSC.

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Isolation of total RNA

Parasites in the schizont stage (36 to 48 hours after erythrocyte invasion) had the highest translation level for DHFR-TS mRNA (data not shown). They were freshly isolated and the cell pellet was rapidly lysed in 4 M guanidinium thiocyanate/0.1 M 2-mercaptoethanol/25 mM sodium citrate/0.5% N-lauroylsarcosine (19). The lysate was sheared by several passages through a 22 gauge needle (20). Centrifugation through a discontinous CsCl gradient yielded the total RNA as a pellet (21). In order to isolate tRNA, total RNA was dissolved in 10 mM HEPES (pH 7.5) and one volume of 4 M LiCl was added. This was left overnight at 4°C and then centrifuged (20,000×g, 30 minutes, 0°C). After ethanol precipitation of the LiCl soluble supernatant (22), analysis on a 2% agarose gel revealed a tRNA content of about 80%.

Preparation of mRNA coding for malarial DHFR-TS

The transcription template was constructed by means of two clones which were kindly provided by Alan Cowman: The 1.4 kb *Sph*I-*Xba*I insert of the first clone contained the 5'end of the transcript, including the 5' end (382 bp) of the coding region of the DHFR-TS gene (Alan Cowman, personal communication), and was used without further change. The 4.8 kb *Xba*I insert of the second clone containing the remaining 3' part of the DHFR-TS coding region at its 5'end (23) was cut by *Asn*I 160 bp downstream of the coding region, as was predicted from the DNA sequence (24). The identity of the resulting fragment was verified by Southern blot analysis (25) with the ODN OTS49 (see below) as a probe and the *Asn*I restriction site was transformed into a *Kpn*I site by means of an adaptor. The *Sph*I-*Xba*I (1.4 kb) and *Xba*I-*Kpn*I (1.7 kb) fragments were cloned into a *Sph*I/*Kpn*I cut pGEM-4 (Promega) vector carrying T7 and SP6 promoters on either side of the polycloning site (26).

After *Kpn*I digest the template was transcribed, by means of T7-RNA polymerase (TransProbe T Kit, Pharmacia) according to the manufacturers instructions. The radiolabeled product was analysed under denaturing conditions on a 1% agarose/formaldehyde gel. The size of the transcript agreed with the size of the insert of the plasmid (3.1 kb). The corresponding unlabeled transcription product was divided into 5 aliquots and one aliquot was used for each *in vitro* translation reaction.

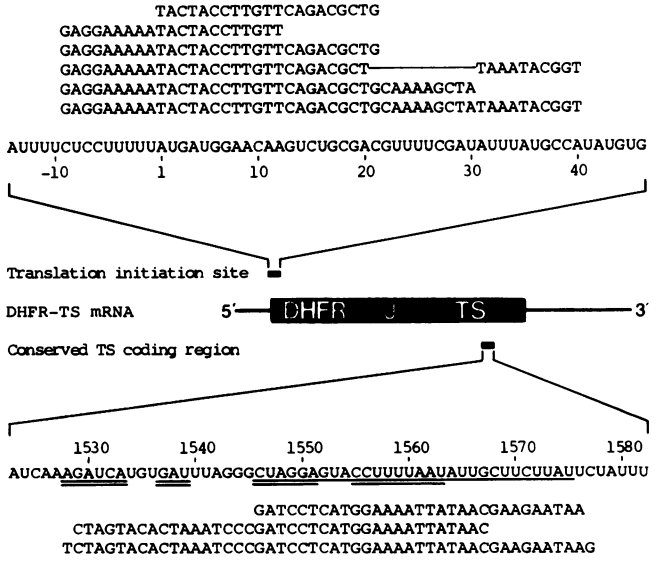

Figure 1. Anti-sense oligonucleotides complementary to *Plasmodium falciparum* DHFR-TS mRNA. J represents the part of the mRNA that codes for the polypeptide strand joining the DHFR and the TS coding moieties. The upper group of oligonucleotides is complementary to the translation initiation site, the lower group is designed to hybridize to a mRNA sequence coding for a conserved part of the TS moiety. The lower most sequence represents the oligonucleotide OTS49. The underlined parts of the mRNA sequence refer to the degree of homology of the corresponding amino acids with those of the TS enzyme of other species. In a comparison of the *P. falciparum* TS moiety with the TS of nine other species, double and simple underlining indicates homology in all, or in 9 out of 10 cases (24).

Preparation of oligonucleotides

ODNs were synthesized on an Applied Biosystems 380B synthesizer using reagents from Milligen. The analysis and purification was made by electrophoresis on urea-polyacrylamide gels.

Cell-free translation

Total RNA and synthetic mRNA were translated in a micrococcal nuclease-treated rabbit reticulocyte cell-free system (Amersham). The typical reaction mixture (8 μl) contained 80% (v/v) lysate, 8 μg total RNA, 3 mM magnesium acetate, 200 mM potassium acetate, and 0.4 MBq [^{35}S]methionine (~40 TBq/mmol) and was incubated for 1 hour at 30°C. About 5 μg tRNA from *P.falciparum* was included in each reaction using the synthetic mRNA in order to improve the translation efficiency (22). To achieve hybridization arrest of the translation, the ODNs were annealed to the corresponding RNA molecules. RNA and ODNs were dissolved in 8 μl 10 mM HEPES, pH 7.5, kept at 65°C for 5 minutes, allowed to cool down to 30°C over a period of 1 hour, and dried in a Speed-Vac concentrator (Savant).

The translation products were analysed by SDS polyacrylamide gel electrophoresis (7.5% acrylamide) (27). After fixing, the gels were incubated in sodium salicylate (28) and exposed to preflashed film at −70°C. For quantitative analysis of protein

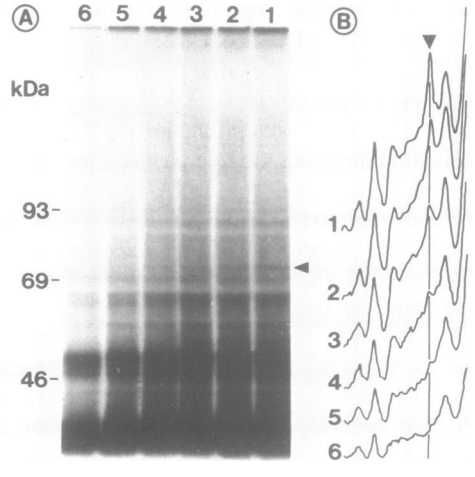

Figure 2. Products of the *in vitro* translation of *Plasmodium falciparum* total RNA in the absence of an anti-sense oligonucleotide (lane 1) or after annealing at 65°C with 1.8 (lane 2), 4 (lane 3), 8.9 (lane 4), 20 (lane 5), and 45 μM (lane 6) of the 49-mer oligonucleotide directed against the TS coding region (OTS49). The densitometric scans (B) are taken from the corresponding lanes of the autoradiograph (A) and cover the region between 65 and 95kDa. The arrows indicate the 71kDa protein corresponding to the DHFR-TS monomer.

synthesis, the autoradiographs were scanned and peak areas were determined densitometrically.

Specific labeling of DHFR-TS and assay of TS activity

DHFR-TS was specifically labeled by formation of a covalent ternary complex between N^5,N^{10}-methylene tetrahydrofolate, [6-^{3}H]-5-fluorodeoxyuridine monophosphate ([6-^{3}H]FdUMP, 740 GBq/mmole, Moravek Biochemicals, Brea, CA), and the TS moiety of the enzyme (29). The enzymatic TS activity was assayed by a modification of a published isotopic method (30). Both reactions were carried out with either the product of cell-free translation or the parasite lysate, both 20μl in volume. The latter was prepared by diluting the packed isolated parasites 4-fold in H_2O containing 2% Triton X-100, 200μg/ml phenylmethylsulfonylfluoride, 50μg/ml soybean trypsin inhibitor, and 1% 2-mercaptoethanol (30).

The measurements were made in triplicate. The error values given correspond to the average deviations from the mean values.

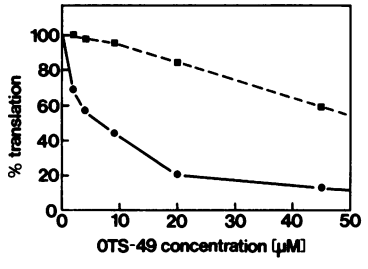

Figure 3. Effect of the concentration of the 49-mer oligonucleotide directed against the TS coding region (OTS49) on the general translation efficiency as determined from the (mean) absolute density of the bands at 69 and 89kDa (---) and on the specific expression of the 71kDa protein as determined from the ratio of the density of the 71kDa band to the combined densities of the 69 and 89kDa bands (——). The quantitative analysis is based on the scans of the autoradiograph shown in Fig. 2.

RESULTS

Effect of oligodeoxynucleotides on synthesis of DHFR-TS

The ODNs and their binding sites on the DHFR-TS mRNA are shown in Figure 1. The oligomers are complementary to two distinct regions on the mRNA: the translation initiation site, including the AUG codon, and a part of the thymidylate synthase coding region. Two effects of the ODNs on the expression of proteins during *in vitro* translation are obvious. At high ODN concentrations there is a general decrease in translation efficiency as judged from a comparison of the density of identical bands (except the 71 kDa band mentioned below) in different lanes (compare Figure 2). This effect is dependent on the length of the ODN. The concentration eliciting a 50% reduction in protein expression ranges from about 220 μM for the 21-mer ODNs to about 70 μM for the 49-mer ODN (Figure 3, dashed line).

In addition to the general decrease in translation efficiency, the ODNs used in this investigation cause a more or less pronounced reduction of the expression of a 71kDa protein identified as DHFR-TS by comigration of specifically labeled DHFR-TS from lysed parasites. This specific effect is quantitized according to the ratio $[p71/(p69+p89)]_{oli}/[p71/(p69+p89)]_0$ where p71, p69, and p89 are the intensities of the corresponding bands in the presence (oli) or the absence (0) of oligonucleotide.

The specific hybridization arrest of DHFR-TS expression by ODNs directed to the translation initiation site ranges from 30% for a 21-mer to 60% for the 30-mer and the 49-mer (Table 1). The 39-mer is much less efficient than both the 30- and the 49-mer, but replacement of nucleotides 21 to 30 by the subsequent ten bases (31 to 40) renders the resulting 39-mer almost equally efficient as the 30- and 49-mer. Nevertheless, in no case does the maximum level of translation arrest exceed 60% and this is only achieved at high ODN concentrations (≥200μM).

Among the ODNs directed against the TS coding region, the 30-mer only inhibits the expression of DHFR-TS by 15%. Its efficiency is, thus, even poorer than that of the 21-mers of the

Table 1. Effect of oligonucleotides (ODNs) directed against the translation initiation site or a part of the thymidylate synthase coding region on the specific expression of the 71kDa polypeptide band during *in vitro* translation. The results are corrected for unspecific effects. Specific and unspecific effects are defined in Figure 3 and in the text

Target sequence[a]	Length of ODN	Maximum level of translation arrest (Highest ODN conc.)	ODN concentration for 50% arrest (estimated)
Translation initiation site			
1 to 21	21	30% (190μM)	–
−9 to 12	21	35% (285μM)	–
−9 to 21	30	60% (200μM)	160μM
−9 to 30	39	35% (270μM)	–
−9 to 20 and 31 to 40	39	55% (200μM)	170μM
−9 to 40	49	60% (190μM)	150μM
21 to −9[b]	30	0%	–
TS conserved region			
1546 to 1575	30	15% (230μM)	–
1529 to 1567	39	55% (280μM)	260μM
1528 to 1576[c]	49	90% (45μM)	6μM
1567 to 1529[b]	39	0%	–

[a] The nucleotide numbers of the target sequence refer to the DHFR-TS cDNA sequence published by Bzik *et al.*(24).
[b] The base composition agrees with the corresponding anti-sense oligonucleotide but the synthesis was made in the opposite direction (syn-sense).
[c] This ODN is called OTS49 throughout the text.

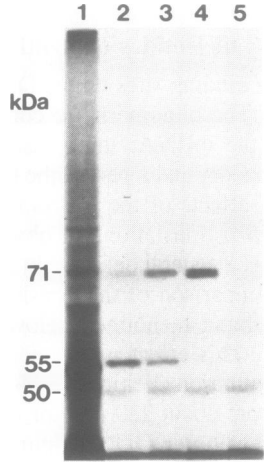

Figure 4. Expression of DHFR-TS after *in vitro* translation of the corresponding synthetic mRNA in the absense of (lane 4) or after annealing with 5 (lane 3) or 25 μM (lane 2) of the 49-mer TS-directed oligonucleotide (OTS49). The *in vitro* translation of *Plasmodium falciparum* total RNA (lane 1) and the DHFR-TS coding mRNA (lanes 4 and, to a lower extent, lanes 2 and 3) exhibit a 71kDa protein at identical positions. Upon annealing with OTS49 (lanes 2 and 3) an arrested 55 kDa polypeptide appears. The *in vitro* translation without RNA (lane 5) serves as control.

Table 2. Effect of the anti-sense 49-mer oligonucleotide directed against part of the TS coding region (OTS49) on the *in vitro* translation of synthetic DHFR-TS mRNA. The translation products were analysed by SDS-polyacrylamide gel electrophoresis and subsequent densitometric determination of the 71 and 55kDa bands on the autoradiograph or by measuring the TS activity in the lysate after translation. In both cases, experiments carried out in the absence of RNA served as controls

OTS49 concentration [μM]	relative density of band [kDa] 71	55	55_{corr}[a]	$71+55_{corr}$[a]	relative TS activity
0	1	0	0	1	1 ($\pm$0.16)[b]
5	0.48	0.39	0.47	0.95	0.63 ($\pm$0.21)
25	0.11	0.46	0.55	0.66	0.12 ($\pm$0.13)

[a] The density of the 55kDa band is corrected for the lower number incorporated methionine residues (14) as compared to the full-length protein (17).
[b] Values in ( ) are the average deviation from the mean value of the results of three independent experiments.

former group. But with increasing length of the anti-sense molecules, the maximum level of translation arrest increases dramatically, reaching 90% at only 45 μM for the 49-mer (OTS49). The concentration necessary for 50% arrest is more than one order of magnitude lower than for the 49-mer of the other group.

In order to ensure that the specific interaction between the above mentioned ODNs and their target mRNA sequences correlates with the specific hybridization arrest of the DHFR-TS mRNA translation, ODNs were tested which are identical in sequence to the 30-mer of the former and the 39-mer of the latter group but are synthesized in the opposite orientation. Both synsense molecules inhibit translation nonspecifically to the same extent as the anti-sense molecules but do not elicit a specific inhibition of DHFR-TS synthesis.

Translation of synthetic DHFR-TS mRNA and its arrest

In order to investigate the efficient inhibition of expression of the 71kDa DHFR-TS by OTS49, we have prepared synthetic

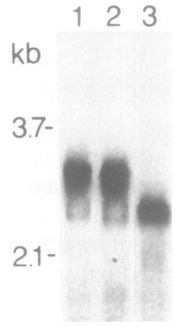

Figure 5. Effect of rabbit reticulocyte lysate on the size of the *in vitro* translated DHFR-TS mRNA after annealing with oligonucleotide OTS49. The size (*ca.* 3.1 kb) of the synthetic mRNA (lane 1) is not affected by the reticulocyte lysate after annealing with 30 μM OTS49 (lane 2). Incubation with 1 unit RNase H after annealing leads to a reduction of the mRNA length by *ca.* 0.5 kb (lane 3).

DHFR-TS mRNA which was then translated *in vitro* in the absence of OTS49 or after annealing to this ODN. In both cases the autoradiograph (Figure 4) shows a band at the same position where the 71kDa DHFR-TS band appears after *in vitro* translation of *P.falciparum* total RNA. With increasing concentrations of OTS49, this 71kDa band becomes weaker and a new band at about 55kDa increases in intensity. OTS49 hybridizes with bases 1528 to 1576 of the DHFR-TS coding region. In case of an interruption of translation at that site, the resulting polypeptide should be about 510 amino acid residues, or about 56kDa. The latter value agrees well with the size determined for the smaller band appearing after hybridization arrest of *in vitro* translation of synthetic DHFR-TS mRNA (Figure 4). To determine the degree of hybridization arrest by OTS49, the autoradiograph in Figure 4 was evaluated densitometrically. The results are given in Table 2. If the 39-mer syn-sense ODN (see Table 1) was used as a control, the decreased density of the 71kDa band corresponded to the unspecific effects described above. A new band at 55kDa does not appear.

Since the arrested translation product contains only 14 methionine residues instead of the 17 in the full-length protein, the density of the 55kDa band, which depends on the amount of incorporated [^{35}S]methionine, had to be corrected to calculate the molar ratio between the 55 and 71kDa translation products. The sum of the densities of the 71kDa and the corrected 55kDa band decreases with increasing OTS49 concentration in the same way as the product of *in vitro* translation of total RNA does (Figure 3, dashed line). Both effects reflect the non-specific inhibition of translation. The ratio between the densities of the 71kDa band and the sum of the 71kDa and the corrected 55kDa band reflects the specific hybridization arrest. Its dependence on the OTS49 concentration also agrees with the results obtained using total RNA (Figure 3; solid line). We tried to verify the above-mentioned results by means of TS activity assays. The use of synthetic mRNA instead of total RNA led to a ratio between exogenous and endogenous TS activity which was sufficiently high to ensure statistical significance of the former. Although there is a large statistical variation in the the relative values for exogenous TS activity (Table 2), the assays clearly show the same tendency as the densitometric measurements do.

In order to find out if the truncated 55kDa DHFR-TS is the result of RNase H-mediated mRNA degradation after hybrid

formation with an ODN, ^{32}P-labeled DHFR-TS mRNA was translated *in vitro* with or without annealing with 30 μM OTS49. The size of both mRNAs remained identical after incubation (Figure 5). In a control experiment, incubation of the mRNA:OTS49 hybrid with RNase H leads to a decrease of the length of the RNA molecule by *ca.* 0.5 kb, which corresponds to a cut of the DHFR-TS mRNA at the OTS49 binding site.

DISCUSSION

Hybridization arrest of translation by anti-sense ODNs is generally possible during translation initiation, *i.e.*, before complete assembly of the ribosome. The mRNA cap region and the sequences around the AUG codon are better targets than the sequences in between (8). Since the sequence of the 5'end of DHFR-TS mRNA is not known, our investigation has been restricted to the translation initiation site. Although ODNs up to 20 bases in length inhibit expression of the gene product almost completely in some cases (31), our ODNs directed against the translation initiation site were not very efficient (Table 1). Even with a 30-mer the arrest did not cause more than 60% inhibition. The reasons could be the relatively high content (>60%) of adenine and uridine in the mRNA, secondary structures of the mRNA in the region immediately upstream of the AUG codon, or intermolecular or intramolecular duplex formation of the relatively long ODNs which would all result in a weakening of the DNA:mRNA interactions.

After complete assembly the ribosome induces a helix-destabilizing activity (32). The coding region of the mRNA was, therefore, considered to be an inefficient target sequence for hybridization arrest of translation (31, 33). This is still consistent with our findings that a 30-mer ODN directed against an internal part of the coding region inhibits translation considerably less than the one blocking the initiation site. The inhibitory effects of the 39- and 49-mer ODNs targeted to the coding region, however, were respectively equal to and greater than those of ODNs of the same length targeted to the initiation site. Another mechanism for translation inhibition by ODNs directed against the coding region was therefore investigated, namely that which involves the RNase H activity found in freshly prepared rabbit reticulocyte lysates (34). This does not seem to operate in our case since, although hybrid formation between the oligonucleotide OTS49 and the mRNA occured, the mRNA was not degraded by RNase H (Figure 5). The appearance of the truncated 55kDa protein in the absence of RNase H seems to contradict the fact that ribosome-associated unwinding should take place during elongation (32). In similar studies of Minshull and Hunt (11), on the other hand, one out of four cDNA clones directed against the mRNA downstream of the AUG codon was capable of hybridization arrest in the absence of RNase H activity. Since this particular cDNA was not even the longest one, prediction of efficient translation arrest seems to require the inclusion of more parameters than are known at present.

In all cases of ODN or cDNA molecules directed against the coding region at least partial hybridization occurs since translation is always inhibited in the presence of RNase H activity. Additional requirements have to be fulfilled to arrest the translation without degradation of the mRNA. Certain sequences could promote the formation of hybrids which are particularly stable if exposed to the helix-destabilizing activity of the ribosome. Also certain ODN or cDNA molecules could form more complete and, therefore, more resistent complexes with mRNA than others. In that sense, a crucial factor could be competition between the formation of intramolecular double-strands within one RNA molecule and intermolecular RNA:DNA hybrids during the rehybridization. Although the ODN is short as compared to the mRNA, the extensive base-pairing, including numerous mismatches, within the latter molecule is counterbalanced by the exact match between the highly concentrated ODN and its target site in the RNA. The situation becomes even more favorable for the RNA:DNA hybrid if the extent of potential secondary structure formation within the mRNA at or around the ODN binding site is reduced. After minimum Gibbs energy calculations, including long range interactions, (35) we found a stretch of 11 ribonucleotides within the OTS49 target site which are not involved in base-pairing since they form a hairpin loop.

For the use of ODNs as potential anti-parasitic drugs the mechanism underlying its efficiency is not important since RNase H is present in all dividing cells. Nevertheless the use of the coding region for translation inhibition is advantageous if it contains sequences which code for conserved amino acid sequences in the translation product. The use of ODNs as a drug will put the target organism under considerable pressure. One response could be one or several mutations within the drug binding site. When mRNA is the target, substitution of bases within the coding region will often change the amino acid sequence of the coded protein. Although substitution of similar amino acid residues does not lead to a substantial change of the protein in most cases, this is not so if the target site on the mRNA codes for a conserved amino acid sequence. It should be mentioned that long ODNs like OTS49 are more likely to show unspecific effects in intact cells than shorter ones. Additionally, some useful ODN modifications like methylphosphonates or poly(L-lysine) conjugates cannot be used for long sequences (36). Finally, it is the annealing step necessary for efficient hybridization in our experiments which is the major obstacle to the development of these ODNs to anti-malarial drugs.

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