Translational Properties of mHNA, a Messenger RNA Containing Anhydrohexitol Nucleotides†

Inna N. Lavrik,‡,§ Olga N. Avdeeva,‡,§ Olga A. Dontsova,‡,§ Matheus Froeyen,|| and Piet A. Herdewijn*,||

Department of Chemistry, Moscow State University, Moscow, Russia, and Laboratory of Medicinal Chemistry, Katholieke Universiteit Leuven, Leuven, Belgium

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ABSTRACT: Short messenger RNAs (mRNAs) with hexitol residues in two codons were constructed and their properties were studied in an *Escherichia coli* in vitro translation system. The replacement of the natural ribonucleotides of mRNA in the AUG start codon and the UUC second codon by hexitol nucleotides did not influence the main steps of translation, as indicated by the same level of binding of mRNA with or without hexitol residues under P-site conditions, and the same yield of tRNA binding to the P- and A-sites. Moreover, both peptide formation and translocation took place on mRNAs with hexitol residues. The presence of an A-type messenger hexitol nucleic acid (mHNA)−transfer RNA (tRNA) duplex is important for efficient translation and the 2′-OH function in mRNA is not necessary for binding and movement through the ribosome. Groove shape recognition of the codon−anticodon complex, more than hydrogen-bond interactions of ribose residues in mRNA, is an important factor for correct translation.

The protein synthesis machinery is composed of a relatively stable decoding and synthesizing system that is programmed by an unstable template (*1*). The replacement of the enzymatically and chemically labile mRNA[1] by a stable RNA mimic may be beneficial for the use of in vitro translation experiments. Here, we report our initial results dealing with the use of artificial mRNA in the translational system (Figure 1). Our findings may also contribute to understanding the molecular basis of the main stages of protein synthesis. The X-ray structure of ribosomal subunits was recently solved with a resolution up to 2.4 Å, which for the first time made it possible to understand the details of the spatial organization of the ribosome (*2a*−*f*). Although details of the mechanism of ribosomal functioning are still lacking, knowledge of the exact structure allows us to design more precise biochemical experiments, which could lead to a better understanding of protein biosynthesis. One of the central problems in understanding translation is how mRNA is recognized and moved by the ribosome. The sugar phosphate backbone plays an important role in this process. Because the coding sequence of mRNAs is highly variable, the sugar phosphate backbone might serve as a universal recognition element, providing a sequence-independent mRNA−ribosome interaction (*3*). The mRNA sugar phosphate backbone inside the ribosome complex is at least partly protected as defined by accessibility to various chemical probes, suggesting that the sugar phosphate backbone is involved in a number of weak interactions with the ribosome (*3, 4*). Phosphorothioate protection studies (*4*) revealed relatively weak interactions of the mRNA phosphate groups with the ribosome, leaving the main component of mRNA binding to be ribose-mediated. It was shown previously that the substitution of ribose residues in mRNA by deoxyribose leads to a loss of translational activity, despite the fact that these DNA analogues were bound to the ribosomes (*5*−*7*). This fact indicates that the sugar structure is recognized by the decoding center of the ribosome. In this study, we selected an oligonucleotide with a conformationally restricted sugar phosphate backbone as a potential mRNA mimic (*8*).

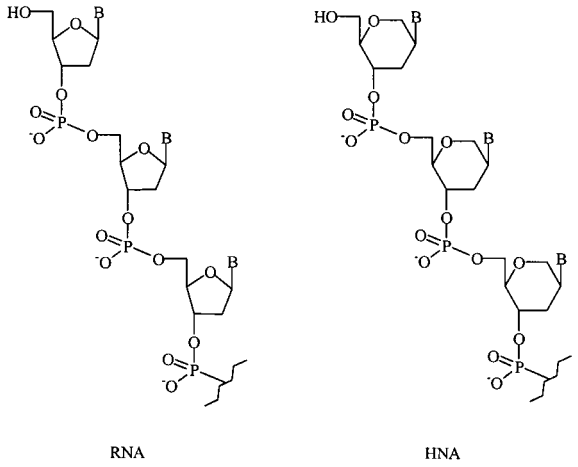

FIGURE 1: Structures of hexitol nucleic acids.

The recognition of mRNA and the 30S ribosomal subunit is based on codon−anticodon interaction between fMet-

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* To whom correspondence should be addressed: Phone +32-16-337387; fax +32-16-337340; e-mail Piet.Herdewijn@rega.kuleuven.ac.be.

‡ Department of Chemistry, Moscow State University, Moscow, Russia.

§ Present address: Department of Immunogenetics, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.

|| Laboratory of Medicinal Chemistry, Rega Institute, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.

[1] Abbreviations: mRNA, messenger RNA; tRNA, transfer RNA; EF-G, elongation factor G; $tRNA^{fMet}$, formylmethionine transfer RNA; fMet-$tRNA^{fMet}$, formylmethionyl-(formylmethionine transfer RNA); $tRNA^{Phe}$, phenylalanine tRNA; Phe-$tRNA^{Phe}$, phenylalanyl-(phenylalanine transfer RNA); HNA, hexitol nucleic acids.

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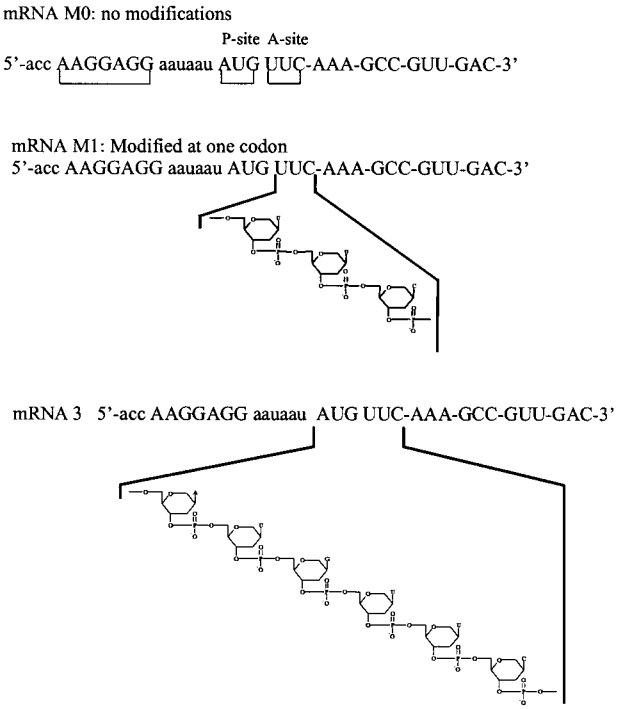

FIGURE 2: Hexitol-containing mRNAs. mRNA M0, control mRNA carrying no hexitol residues; mRNA M1, mRNA carrying three hexitol residues in the UUC codon; mRNA M2, mRNA having six hexitol residues in the AUG and UUC codons.

$tRNA^{fMet}$ and the AUG initiation codon and on base-pairing between the Shine−Dalgarno sequence and the anti-Shine−Dalgarno sequence at the 3′-end of the 16S rRNA (*9*, *10*). For the translation assay we selected an mRNA of 33 nucleotides containing a Shine−Dalgarno sequence, a spacer region, the AUG start codon, and the UUC ($tRNA^{Phe}$) second codon, followed by four other codons (Figure 2). Three messenger RNAs were synthesized and tested: an mRNA with the nonmodified ribonucleic acid sequence (M0); an mRNA with one modified codon, i.e., UUC (M1); and an RNA with two modified codons, i.e., start codon AUG and UUC (M2). Modification consists of replacement of the ribose sugar of the nucleotide tRNAs by a hexitol sugar (Figures 1 and 2). In this way, the correct recognition of the mRNA by tRNAs at both the P- and A-site can be evaluated together with the efficiency of the translocation reaction.

MATERIALS AND METHODS

Materials. T4 Polynucleotide kinase was purchased from Boehringer Mannheim, Germany. [γ-^{32}P]ATP and L-[^{14}C]-phenylalanine (476 mCi/mmol) were purchased from Amersham, U.K. Ultrapure 5′-ATP and 5′-GTP were purchased from Pharmacia, Sweden. Nitrocellulose filters type HA, pore size 0.45 μm, were from Millipore Corp. $tRNA^{Phe}$ and $tRNA^{fMet}$ were from Boehringer Mannheim, Germany.

Synthesis of mRNA Carrying Hexitol Residues. The phosphoramidite HNA building blocks were synthesized as described before (*11*). Eurogentec synthesized the RNAs according to previously described procedures (*12*) and the correct mass was obtained for all three RNAs as determined by matrix-assisted laser desorption ionization mass spectrometry.

5′-End-Labeling of $tRNA^{fMet}$ and mRNA. The 5′-ends of $tRNA^{fMet}$ and mRNA were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase under standard conditions (*13*). The products were purified on 8% polyacrylamide gels.

Aminoacylation of $tRNA^{Phe}$. [^{14}C]Phe-$tRNA^{Phe}$ was prepared by incubating *Escherichia coli* $tRNA^{Phe}$ (4 μM) for 30 min at 37 °C in a buffer containing 30 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 30 mM KCl, 70 mM NH_4Cl, 1 mM DTT, 3 mM ATP, 20 μM L-[^{14}C]phenylalanine, and 0.5 A_{280} unit/mL DEAE-cellulose-purified S100 extract. The reaction was stopped by the addition of 0.1 volume of 3 M NaOAc, pH 5.5, and an equal volume of phenol, followed by vigorous shaking and phase separation. The upper (water) phase was subjected to a second phenol deproteination and was then precipated by the addition of 2.5 volumes of ethanol. The tRNA concentration was determined by use of a Pharmacia UltrospecIII spectrophotometer. The specific activity of [^{14}C]Phe-$tRNA^{Phe}$ was 846 dpm/pmol. The counting efficiency was 55%.

Aminoacylation of $tRNA^{fMet}$. fMet-$tRNA^{fMet}$ was prepared by incubating *E. coli* $tRNA^{fMet}$ (4 μM) for 30 min at 37 °C in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 30 mM KCl, 70 mM NH_4Cl, 1 mM DTT, 3 mM ATP, 40 μM L-methionine, 0.1 mg of citrovorum factor, and 0.5 A_{280} unit/mL DEAE-cellulose- purified S100 extract. The reaction was stopped by the addition of 0.1 volume of 3 M NaAc, pH 5.5, and an equal volume of phenol, followed by vigorous shaking and phase separation. The upper (water) phase was subjected to a second phenol deproteination and was then precipated by the addition of 2.5 volumes of ethanol. The tRNA concentration was determined by use of a Pharmacia UltrospecIII spectrophotometer.

Formation of Ribosomal Complexes. The binary complex mRNA−ribosome was formed in P-site buffer, containing 80 mM potassium cacodylate (pH 7.5), 10 mM $MgCl_2$, 100 mM NH_4Cl, and 1 mM DTT. Ribosomes were first activated in P-site buffer for 5 min at 42 °C and then cooled on ice. mRNA was added in the same buffer and the next incubation lasted for 15 min at 37 °C. The final concentration of ribosomes was 1 μM. The ratio between ribosome and mRNA concentrations ranged from 1:0.5 to 1:10 for analysis by the filter binding assay. In the case of the gradient binding assay this ratio was 1:1.

P-Site Binding. Ribosomes were first activated in P-site buffer for 5 min at 42 °C and then cooled on ice. Ribosomes were incubated with a 3-fold excess of mRNA in P-site buffer for 15 min at 37 °C. tRNA was added in the P-site buffer and the incubation continued for 30 min. The final concentration of ribosomes was 1 μM. The ratio between ribosome and tRNA concentrations ranged from 1:0.5 to 1:10 for the analysis by the filter binding assay. In the case of the gradient binding assay for P-site formation with 5′-labeled mRNA and unlabeled $tRNA^{fMet}$, the incubations were performed in the same way with the ratio of ribosomes:mRNA:tRNA being 1:1:3.

A-Site Binding and Translocation. Prior to the A-site binding, $tRNA^{fMet}$ was bound under P-site conditions in the presence of mRNAs M0, M1, and M2 as decribed above. The ratio between the concentrations of ribosomes and $tRNA^{fMet}$ was 1:5. Then the concentration of $MgCl_2$ was increased to 12 mM, the concentration of NH_4Cl was lowered to 50 mM, and [^{14}C]Phe-$tRNA^{Phe}$ was added at a ratio of 0.5−10 relative to the ribosome concentration. Incubation under these conditions continued for 15 min at 37 °C.

Binding to the A-site was measured by a nitrocellulose binding assay as described in ref *14*. As background the value of [^{14}C]Phe-tRNA^{Phe} absorbed to the nitrocellulose filters was taken. As a control [^{14}C]Phe-tRNA^{Phe} was bound in the absence of mRNA with the P-site filled by tRNA^{fMet}. For the translocation experiments the P- and A-sites were filled with tRNAs first as described above, except that in these experiments fMet-tRNA^{fMet} instead of nonaminoacylated tRNA^{fMet} was used. EF-G at a final concentration of 0.2 μM and GTP (5 mM) were added. The incubation lasted 10 min at 37 °C. The puromycin reaction was performed as decribed in ref *14* except that, after the addition of the second tRNA, every complex was divided into eight aliquots. Two aliquots were subjected to the filter binding assay for A-site binding. Four aliquots were subjected to puromycin reaction. From these four, two aliquots were used as control, without the addition of EF-G as described in ref *14*, and to the other two aliquots were added EF-G and GTP as described above. Two aliquots were used as a background without the addition of puromycin and EF-G. The incubation with puromycin lasted for 60 min at 0 °C, followed by the extraction with ethyl acetate. The yield of the puromycin reaction was calculated in the following way: the values of the puromycin reaction in the presence of EF-G (originally P-site-bound material plus translocated material) are divided by the value of the puromycin reaction in the absence of EF-G (originally P-site-bound material). The puromycin solution was prepared just before use, and the pH of the solution was adjusted to 7.5.

Gradient Binding Assay. The ribosomal complexes were centrifuged on a 5−20% sucrose gradient, in P-site buffer (SW41 rotor, 40 °C, 18 h). The fractions corresponding to the 70S subunits were taken according to radioactivity, controlled by UV adsorption on an UltraSpecIII spectrophotometer.

Nitrocellulose Binding Assay. The nitrocellulose binding assay was performed as described in ref *14*.

Molecular Modeling. Initially we started from the 3.1 Å 30S structure, including mRNA and tRNA, Pdb entry 1IBM, described by Ogle et al. (*2f*). The mRNA−tRNA duplex in the A-site (chain Z, residues 1−3, and chain Y, residues 34−36) was replaced by an HNA−RNA duplex extracted from the NMR-defined structure (*12*) by use of a quaternion method to fit the bases onto each other (*15*). The nucleobases were replaced to correspond with the A-site sequence 5′-UUC-3′ (mHNA) and 5′-GAA-3′ (tRNA).

RESULTS AND DISCUSSION

Structure of mRNAs. Hexitol residues were incorporated into the A- and P-site codons of short mRNAs (Figure 2). The primary structure of these mRNAs was analogous to the short mRNAs we described before (*16*−*18*). The mRNAs had a length of 33 nucleotides and included the essential signals for translation initiation in prokaryotes, e.g., the Shine−Dalgarno sequence and the initiation AUG codon, resulting in the proper orientation of these mRNAs in the *E. coli* ribosome. Three mRNAs were used in our experiments (Figure 2): a control mRNA carrying no hexitol residues [M0 (for nonmodified)], a mRNA carrying three hexitol residues in UUC codon [M1 (for modified at one site)], and a mRNA having six hexitol residues in the AUG and UUC codons [M2 (for modified at two sites)].

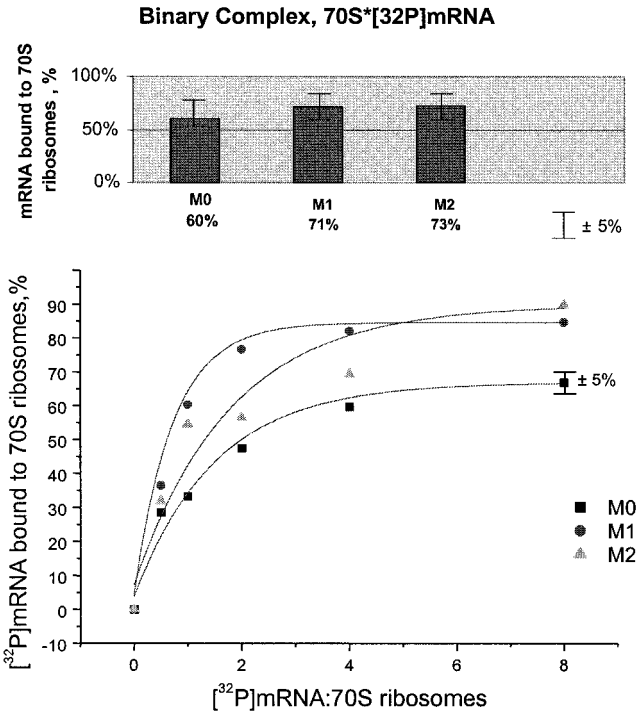

FIGURE 3: (A, upper panel) Results of the gradient binding assay of the binary complex [^{32}P]mRNA−70S ribosomes. The ratio between mRNA and 70S ribosomes was 1:1 for mRNAs M0, M1, and M2. (B, lower panel) Results of the filter binding assay of the binary complex [^{32}P]mRNA−70S. The final concentration of ribosomes was 1 μM. The ratio between ribosome and mRNA concentrations ranged from 1:0.5 to 1:10. (■) mRNA M0, without hexitol residues. (b) mRNA M1, with three hexitol residues at the UUC codon. (▲) mRNA M2, with six hexitol residues at the AUG and UUC codons.

Formation of mRNA−Ribosome Binary Complexes. In the set of first experiments we investigated how the incorporation of hexitol residues into the mRNA structure would influence mRNA−ribosome interactions and if mRNA with hexitol residues would bind to 70S ribosomes at all. To determine the formation of the binary complex mRNA−70S, ^{32}P was introduced at the 5′-end of all mRNAs and a binary complex with 70S ribosomes was formed under conditions described in Materials and Methods. The yield of the mRNA binding was evaluated by a gradient binding assay based on the level of radioactivity in the fractions of the sucrose gradient containing 70S ribosomes. The results of the fractionation in sucrose gradients (Figure 3A) clearly demonstrated that the formation of the binary complex 70S−mRNA took place for mRNAs M1 and M2, carrying three and six hexitol residues, respectively. The yield of the binary complex formation for mRNAs M1 and M2 was about 70%, while for the control nonmodifical mRNA M0 the yield was only 60%. The formation of the binary complex mRNA−70S was also studied with a filter binding assay (Figure 3B). The binding of M1 and M2 was 10% higher than the binding of the control mRNA M0. Thus, two independent techniques demonstrate that the incorporation of up to six hexitol residues into 33-nucleotide-long mRNA did not influence its binding to the ribosome in the absence of tRNA and also indicated a small increase in the level of mRNA binding to the ribosome for hexitol-containing mRNAs.

P-Site Binding. At the next step we studied the initiation complex 70S−mRNA−tRNA formed with hexitol-contain-

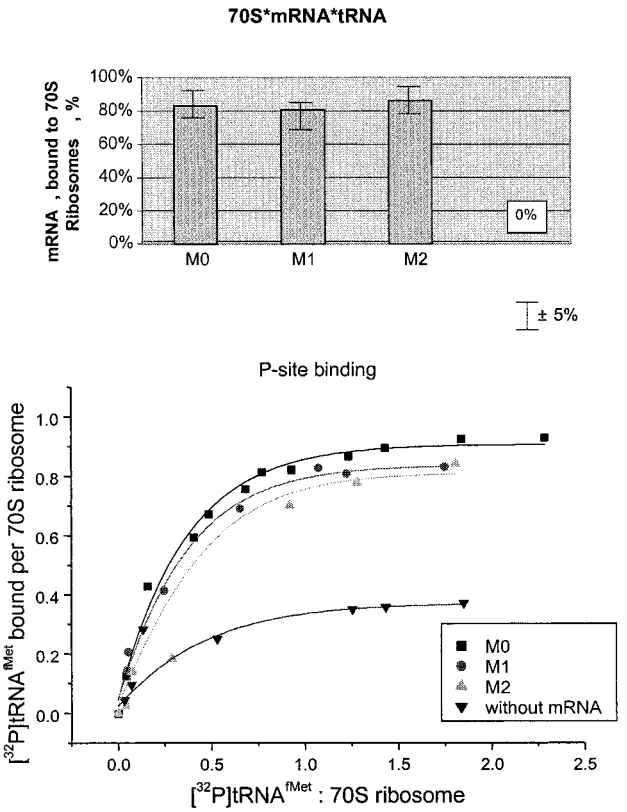

FIGURE 4: (A, upper panel) Results of the gradient binding assay of the complex [^{32}P]mRNA−70S−tRNA^{fMet} ribosomes. The ratio between mRNA, 70S ribosomes, and tRNA was 1:1:3. (B, lower panel) Results of the filter binding assay for the binding of [^{32}P]-tRNA^{fMet} to the P-site. The final concentration of ribosomes was 1 μM. The ratio between ribosome and tRNA^{fMet} concentrations ranged from 1:0.5 to 1:10. (■) Binding in the presence of mRNA M0, without hexitol residues. (b) Binding in the presence of mRNA M1, with three hexitol residues at the UUC codon. (▲) Binding in the presence of mRNA M2, with six hexitol residues at the AUG and UUC codons. (▼) mRNA-independent binding.

ing mRNAs. We first assayed the binding of mRNA and then focused on the details of tRNA binding in the P-site. Both gradient and filter binding assays were used to evaluate the formation of the complexes. The gradient binding assay with the complex 70S−[^{32}P]mRNA−tRNA^{fMet} indicated that the level of mRNA binding to the ribosomes was increased by 20% in comparison to the binary complex (Figure 4A). The yield was the same for nonmodified mRNAs and for those modified at one and two codons. The observed increase in the mRNA binding in the presence of tRNA was described earlier (*17*) and can be used as an indication of the formation of the ternary complex. The P-site binding of tRNA was also studied by filter binding assays measuring the incorporation of radioactively labeled tRNA into the complex. Two independent series of experiments were performed: addressing binding of deacylated [^{32}P]tRNA^{fMet} to the AUG codon and binding of Ac-[^{14}C]Phe-tRNA^{Phe} to the UUC codon. In the first series of experiments, the deacetylated tRNA^{fMet} was 5′-labeled with [γ-^{32}P]ATP and then four types of complexes were studied by the filter binding assay. Three 70S−[^{32}P]-tRNA^{fMet}−mRNA complexes were formed with nonmodified and two types of modified mRNA (M1 and M2), and a fourth one, 70S−[^{32}P]tRNA^{fMet}, was formed without mRNA (Figure 4B). The fourth complex was used as a control, because tRNA could be bound to the ribosome in a mRNA-independent way, with lower yields (*2d, 19*−*21*). The results of the filter binding assay demonstrated that 80% of the ribosomes bound tRNA^{fMet} in the presence of mRNAs M0, M1, and M2 and 40% of the ribosomes bound tRNA^{fMet} without mRNA. The saturation level was reached with the same concentration of tRNA^{fMet} for all three mRNAs. Thus, we could observe no difference in the tRNA binding under P-site conditions for mRNA M2 carrying hexitol modifications in all three positions of the AUG codon as compared to nonmodified mRNAs and M1, having no modification in the AUG codon.

It should be noted that we did not reach a 100% binding level of tRNA to the ribosomes either in the presence of the control mRNA M0 or in the presence of mRNAs M1 and M2, which should be the case for active ribosomes, having only one P-site per molecule. Thus, we can conclude that the activity of the ribosomes we were using was below 100%. However, this should be of minor importance to our results because we were mainly comparing the differences in the P-site binding between different mRNAs.

Similar results were obtained in the second series of experiments with *N*-Ac-[^{14}C]Phe-tRNA^{Phe}, which was bound under P-site conditions (data not shown). The binding of Ac-[^{14}C]Phe-tRNA^{Phe} to the ribosome in the presence of mRNAs M1 and M2 carrying a UUC codon with hexitol residues was the same as the binding in the presence of the control nonmodified mRNA.

Thus, we concluded that binding occurred similarly for nonmodified and modified mRNAs. The presence of hexitol residues did not influence tRNA binding to the P-site and the yield of mRNA binding to the ribosome was the same regardless of the presence of hexitol residues.

A-Site Binding and Translocation. The next step in our experiments was the study of the subsequent stages of the elongation cycle, i.e., the binding to the A-site with the following translocation reaction catalyzed by elongation factor EF-G.

Prior to A-site binding, the P-site was filled by tRNA^{fMet} in the presence of mRNAs M0, M1, and M2. The conditions for the tRNA^{fMet} binding to the P-site were analogous to our previous experiments. An excess of tRNA^{fMet} was used to reach 100% occupation of the P-site. [^{14}C]Phe-tRNA^{Phe} was bound to the A-site under nonenzymatic conditions. The conditions used for the nonenzymatic A-site binding were as reported (*14, 22, 23*). Due to the fact that mRNAs with different codons for the P- and A-sites were used, we could quantitatively evaluate the percentage of binding to the A-site from the amount of the ^{14}C radioactivity absorbed to the nitrocellulose filters. The results of the binding are presented in Figure 5. For 100% binding we took the amount of radioactivity in [^{14}C]Phe-tRNA^{Phe}, which could be bound to the ribosomes at a ratio 70S:[^{14}C]Phe-tRNA^{Phe} of 1:1. We did not observe any drastic differences in the binding of the second [^{14}C]Phe-tRNA^{Phe} to the ribosome for mRNAs M0, M1 and M2 (Figure 5, curves A−C). It might be suggested that the A-site binding in the case of mRNA M2 is up to 5% higher (curve C) than for mRNA M0 (curve A). However, within the nitrocellulose filter approach used for these measurements we do not consider this value as a serious functional difference and rather conclude that the same level of A-site binding is reached for all three mRNAs. Thus, the incorporation of hexitol residues at only the A-site (mRNA

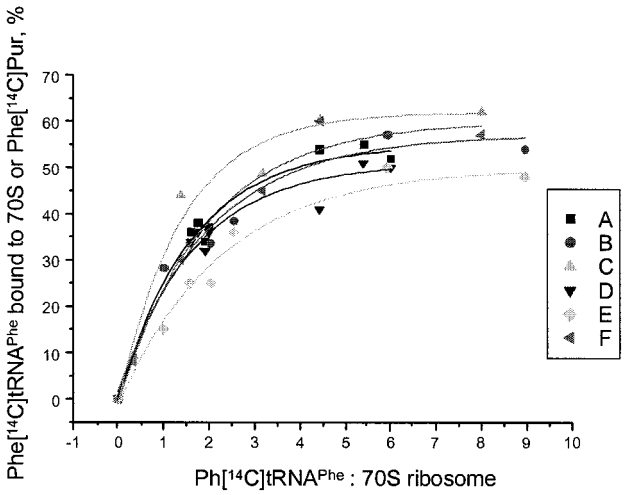

FIGURE 5: A-site binding and puromycin test. The final concentration of ribosomes was 1 μM. The ratio between ribosome and Phe-[^{14}C]tRNAPhe concentrations ranged from 1:0.5 to 1:10. The percent binding to the A-site is shown in the presence of mRNA M0, without hexitol residues (A, ■); in the presence of mRNA M1, with three hexitol residues at the UUC codon (B, ●); and in the presence of mRNA M2, with six hexitol residues at the AUG and UUC codons (C, ▲). The percentage of radioactivity found in the peptidyl−puromycin complex is shown in the presence of mRNA M0, without hexitol residues (D, ▼); in the presence of mRNA M1, with three hexitol residues at the UUC codon (E, ◆); and in the presence of mRNA M2, with six hexitol residues at the AUG and UUC codons (F, left arrowhead). As 100% we considered the amount of Phe[^{14}C] that could be bound to 70S ribosomes in Phe[14C]tRNAPhe at a ratio 70S:Phe[^{14}C]tRNAPhe of 1:1. The A-site binding and puromycin reaction were performed in the same experiment as described under Materials and Methods.

M1) and at both the P- and A-sites (mRNA M2) showed the same level of second tRNA binding as mRNA M0 without hexitol residues. The binding curve reached saturation with an excess of [^{14}C]Phe-tRNA^{Phe} over the ribosome, which corresponded to the data reported in the literature (*14*).

A control experiment run in parallel was [^{14}C]Phe-tRNA^{Phe} binding to the ribosome under the same ionic conditions for nonenzymatic binding to the A-site, with preliminary blocking of the P-site but without mRNA. It is well-established that the binding to the A-site is strictly mRNA-dependent (*14*). No [^{14}C]Phe-tRNA^{Phe} was detected in the ribosome in these control experiments, demonstrating that under the conditions chosen in our work there was no unspecific binding, and measuring ^{14}C radioactivity indeed showed the occupation of the A-site.

To check the next stage of the elongation cycle, the reaction of translocation with elongation factor EF-G was performed. For these experiments, the P-site was blocked with an excess of fMet-tRNA^{fMet} instead of nonaminoacylated tRNA^{fMet}, which was used in all our previous experiments. Then the nonenzymatic binding of [^{14}C]Phe-tRNA^{Phe} to the A-site was performed (*14*). The binding to the A-site was controlled by use of a filter binding assay. Afterward elongation factor G was added to ribosomes in catalytical amounts with bound fMet-tRNA^{fMet} and [^{14}C]Phe-tRNA^{Phe}. The yield of translocation was measured by the puromycin reaction as reported in ref *14*. The antibiotic puromycin is known to occupy the A-site if it is free, which could only be the case if translocation occurs (*13*). Then the peptide from the peptidyl-tRNA in the P-site is transferred to puromycin, and the yield of translocation can be estimated from the amount of radioactivity in the peptidyl−puromycin complex.

Table 1: Results of Functional Assays for mRNAs M0, M1, and M2[a]

	binding of tRNA^{fMet} to P-site (%)	binding of Phe[^{14}C]tRNA^{Phe} to A-site (%)	yield of puromycin| reaction for translocation (%)
mRNA M0	85 ± 5	55 ± 5	90 ± 5
mRNA M1	80 ± 5	55 ± 5	90 ± 5
mRNA M2	80 ± 5	60 ± 5	90 ± 5

[a] The data from the binding assays and the puromycin reaction were highly reproducible; two single values deviated from the average value by less than ±5 %.

To measure the amount of radioactivity in the P-site and the spontaneous translocation before the addition of elongation factor G, puromycin was added to the ribosomes with bound fMet-tRNA^{fMet} and [^{14}C]Phe-tRNA^{Phe}. The amount of radioactivity in this case was less than 10% of the value of radioactivity bound at the A-site. These experiments demonstrate that spontaneous translocation did not take place and serve as an additional control that [^{14}C]Phe-tRNA^{Phe} indeed was bound to the A-site. To escape several rounds of translocation, the EF-G was added in catalytical amounts and the puromycin reaction was performed at 0 °C. These conditions were reported to give reliable information on the extent of translocation (*14*). The yield of the puromycin reaction was estimated as described under Materials and Methods.

Our data show that translocation took place with the same yield on mRNAs M1 and M2 carrying hexitol residues, as well as on mRNA M0 without hexitol residues (Figure 5D−F, Table 1). In Figure 5 (curves D−F) the amount of radioactivity found at the peptidyl−puromycin complex is presented. As 100% we considered the amount of the [^{14}C]-Phe radioactivity that could be bound to 70S ribosomes at the ratio 70S:[^{14}C]Phe-tRNA^{Phe} of 1:1. Almost all [^{14}C]Phe radioactivity was found at the P-site after the addition of the elongation factor EF-G. The amount of peptidyl−puromycin slightly differed between mRNAs M1, M2, and M0, being a little bit higher for mRNA M2 (Figure 5). However, this difference correlates with the slightly different levels of A-site-bound tRNA for mRNAs M1, M2, and M0, which was discussed above. The difference between the amounts of ^{14}C radioactivity bound at the A-site and found at the peptidyl−puromycin complex (compare curves A and D, B and E, and C and F in Figure 5) is about 5−10%, indicating almost quantitative translocation. The yield of translocation (Table 1) calculated for each mRNA was 90%. Thus, it can be concluded that the formation of peptide, as well as mRNA translocation, took place, showing that the incorporation of hexitol residues into two codons allows a ribosome to fulfill its major function.

However, it remains to be investigated if oligopeptide synthesis will take place with a mRNA having hexitol residues instead of ribose in all positions.

DISCUSSION

It is well-known that modulation of the mRNA secondary structure within the ribosome binding site plays an important role in translation efficiency. Translational efficiency is

influenced by the competition between intramolecular base pairing within the ribosome binding site of the mRNA and intermolecular base pairing between the Shine−Dalgarno sequence and the rRNA (*9, 10*). Hairpin loops are involved at every step in the control of mRNA function (initiation, elongation, termination, and mRNA lifetime) and the presence of higher-order structures of the mRNA may explain partly the difference in efficiency of translation of individual mRNAs (*24*). Hairpins in the mRNA can also affect translation by other mechanisms, such as interacting directly with the ribosomal RNAs and proteins or binding to nonribosomal proteins (*25*). Another determinant of the translational efficiency is the cytoplasmic mRNA local concentrations and thus the rate of enzymatic degradation of mRNA. For both factors, we decided to study hexitol nucleic acids (HNA) as artificial mRNA (i.e., mHNA). It has been demonstrated previously that HNA hybridizes with RNA and that the HNA−RNA hybrid has an A-type geometry, comparable with the geometry of the helix formed during RNA−RNA interactions (*12, 26, 27*). This may be explained by the conformation of the hexitol ring, which can be considered as a mimic of a furanose ring in the C_3-endo sugar puckering. Thus, HNA has similar structural properties as RNA, but lacks the 2′-OH function. Therefore, it allows one to study the shape recognition of the ribosome for mRNA and to investigate the importance of the presence of the 2′-OH group in RNA for mRNA binding and movement through the ribosome. For (tetraloop) hairpin formation in RNA, the middle nucleosides should have conformations predominantly C_2-endo. This puckering in conjunction with changes in the backbone angle allows nucleotides to reverse the direction of the strand and to form a loop. HNA, however, cannot adopt a C_2-endo mimicking state and therefore it is very difficult, if not impossible, for HNA to adopt the hairpin structures (*28*). Therefore, we expect that pausing of translation at specific locations due to the presence of secondary structures will occur less frequently with HNA as template.

Additionally and importantly, HNA is stable against degradation by phosphodiesterases (*29*). This means that the lifetime of HNA in a cell will be much greater than that of RNA and will be primarily defined by excretion of HNA rather than by degradation. As a result, protein synthesis may go on for a longer time. Finally, we have demonstrated recently that HNA can be brought into cells by using cationic lipids as transport system (*30*), so that the uptake of small mHNA fragments may not be an obstacle for the present approach.

In this study we attempted to provide functional characterization of mRNA with hexitol residues during the main stages of translation. We find that in a binary complex (mRNA−70S), reflecting one of the initiation ribosomal complexes, the presence of hexitol residues in the coding part of the mRNA results in an increased mRNA binding. Several physicochemical, biochemical, and structural studies of binary 70S−mRNA complexes suggested that the mRNA occupies the channel formed in the 30S ribosomal subunit, having a strong interaction with the ribosome in the region of the Shine−Dalgarno sequence, and with the other part of the mRNA not really being fixed to the ribosome (*2d, 17, 19*). The coding part of mRNA in this complex most probably interacts with the ribosome by the sugar phosphate backbone. Introduction of hexitol residues in the coding part increases the binding of mRNA in a binary complex.

A more proper fixation of the coding part of the mRNA is achieved during the next stage of translation, when the initiator tRNA is bound to the ribosome and, subsequently, the coding part of the mRNA is being stabilized due to the Watson−Crick interactions between the codons (*17, 19*). However, upon the binding of initiator $tRNA^{fMet}$ we did not observe any difference in binding of mRNAs with or without hexitol residues. This observation can be explained by the fact that Watson−Crick interactions of the codon−anticodon duplex at the P-site are important for the orientation of the coding part of the mRNA and that the hexitol ring can successfully mimic the interactions of the ribose moiety.

Additionally, we did not observe any difference in tRNA binding to the P-site when hexitol residues were present in the P-codon of the mRNA. Also, no difference in tRNA binding to the P-site was observed for mRNA with six hexitol residues in two codons, AUG and UUC, either for the binding of $tRNA^{Phe}$ to the UUC codon or for the binding of $tRNA^{fMet}$ to the AUG codon. In a previous study (*5*) the same level of tRNA binding to the P-site was observed for mRNA with DNA residues at the P-site. It was concluded that the P-site is insensitive to the presence or absence of 2′-OH groups in the codon and that tRNA binding to the P-site is also not affected if the A-site codon is lacking the 2′-OH groups. Our results are in agreement with the observation that, for the formation of the P-site, the structure of the sugar phosphate backbone of the mRNA may not be strictly limited to a ribose phosphate (*5*). It is noteworthy that the incorporation of DNA residues at the A-site codon of the mRNA leads to drastic changes in the presence of the deoxy codon at the A-site. The tRNA binding to the A-site was decreased and no formation of peptide can be observed with deoxynucleotides (*5*). This might suggest that the 2′-OH of mRNA is involved in hydrogen bonding. An alternative explanation, however, is that the 2′-OH is involved in a preorganization rather than in H-bonding. Indeed, DNA is not a good model to study the relative importance of shape recognition and 2′-OH recognition on translation, because DNA is not preorganized. In contrast to this, tRNA binding to the A-site for mRNA with hexitol residues was detected as well as the subsequent formation of a peptide bond and the mRNA translocation. Thus, we can conclude that, in contrast to deoxyribose, the incorporation of hexitol into the mRNA does not block the translation.

This conclusion could be further explained by the structural differences between ribose, deoxyribose, and hexitol. Hexitol has an additional carbon atom as compared to deoxyribose and ribose. Structural studies of 1′,5′-anhydrohexitol nucleosides suggest a good fit with a natural furanose nucleoside in its 3′-endo conformation (*12, 27*). Hexitol nucleotides stabilize A-form duplexes, and this shape recognition of the codon−anticodon complex seems to be more important than the recognition of the 2′-OH group of ribose residues in mRNA. Therefore, the hexitol residues do not create any steric problems for the correct positioning of the mRNA, and hexitol-substituted mRNA possesses all major functions of mRNA.

Previously it was reported that the 2′-OH groups of mRNA are important for translation (*31*−*34*). However, the reason for this was not clear. The acetylation of the 2′-OH group in RNA (*31*) or complete 2′-O-methylation (*32*) renders an

RNA inactive as a template. However, low levels of substitution with 2′-O-methylnucleotides in a RNA polymer do not eliminate its template activity (*32*). Fukui et al. (*35*) demonstrated that polynucleotides with a 2′-halogen substituent in the ribo configuration [in particular poly(2′-fluoro-2′-deoxyadenylic acid)] can be used as enzymatically stable mRNA in protein synthesizing systems in vitro, and these results are, likewise, in agreement with the recognition by the ribosomes of A-type RNAs. However, these conclusions have not been supplemented by functional studies and are not fully consistent with the results published by Aurup et al. (*33*), who reported that 2′-fluoro-2′-deoxyadenosine-modified mRNA is transcribed at a 10-fold lower efficiency than unmodified mRNA, and that no detectable translation was obtained with 2′-fluoro-2′-deoxypyrimidine-modified mRNA, due to premature termination of translation (*33*). A possible explanation given is that the 2′-fluoro modification enhances thermal stability of polynucleotide duplexes and, hence, the secondary structure formation, which may hamper the translation initiation or translocation of mRNA on the ribosome (*33*). The difference between the effect of 2′-fluoro-2′-deoxyadenosine and of 2′-fluoro-2′-deoxypyrimidine was not explained. On one hand, the experiments with 2′-fluoro-2′-deoxynucleotides do not allow one to discriminate between the importance of shape recognition and the potential of H-bond formation of the 2′-substituent. The 2′-fluoro substituent can still act as a proton acceptor in molecular interactions. On the other hand, it was reported that substitution of the 2′-OH by 2′-H (deoxynucleotides) in the A-site codon decreases the binding affinity of tRNA but does not affect P-site tRNA binding (*5*).

Recently, the crystal structure of functional complexes of the complete *Thermus thermophilus* 70S ribosome, containing mRNA, up to 7.8 Å resolution has been reported, and this work provided a more detailed insight into the structure of the codon−anticodon complex and more specifically to the function of the universally conserved bases A1492 and A1493. In the original model (*19*) it was established that both adenines are positioned far away from the codon−anticodon helix. Yoshizawa et al. (*36*) published studies on the molecular basis for ribosomal recognition of the codon−anticodon pair. On the basis of their work it was hypothesized that the 2′-OH groups within the mRNA codon interact by hydrogen bonding with the N1 positions of the bases A1492 and A1493 of rRNA and that the mRNA forms an A-form duplex with a cognate tRNA anticodon (*36*). A flipped-out position of A1492 and A1493 is required for these interactions as well as for the readout of the minor groove and the fidelity of translation. Our results allow us to hypothesize that groove shape recognition and steric hindrance, more than hydrogen-bond interactions (*34*, *37*), may be important factors for correct translation. In case of correct base-pairing between mRNA and tRNA, both adenine bases may recognize the groove of the duplex. Our data confirm that the A-form helical structure formed between codon mRNA and anticodon cognate tRNA is indeed important for ribosome recognition. In case of mismatches, bases in the duplex are either pulled out from the Watson−Crick regions into one of the grooves or grooves may change their widths, resulting in disruption of an efficient readout process by the two adenine bases. In case of incorrect base-pairing, the adenine bases might flip back to the position 15 Å away of the mRNA−tRNA complex (*19*) and disrupt the translation process.

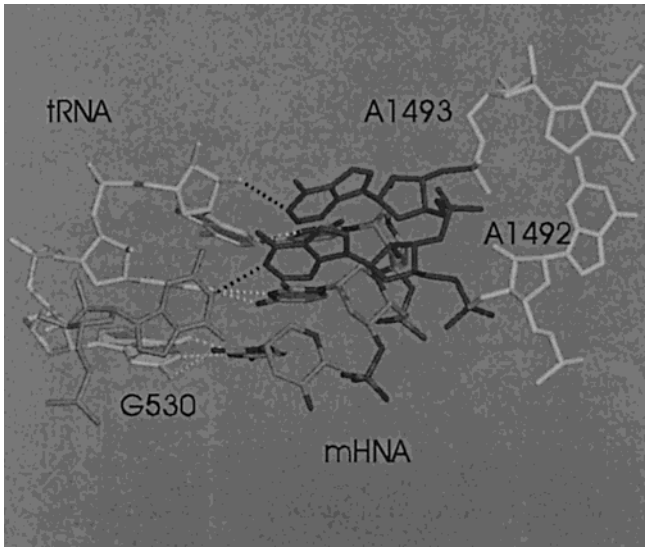

FIGURE 6: Side view of the modeled complex between the mHNA−tRNA duplex and the flipped-out adenosine residues A1492 and A1493. The adenine bases enter from the top of the minor groove. A hydrogen bond (dotted black) is formed between the N1 of A1493 and the 2′-OH group of residue 36 of tRNA (chain C, 1IBM numbering). It is not possible to have additional hydrogen bonds between A1492 and the 2′-OH group of residue 35 without alteration of the geometry of the loop. Instead the A1492 base nitrogen N1 forms a hydrogen bond to N1 of G530.

With the crystal structure of the 30S subunit from *T. thermophilus*, refined to 3 Å resolution (*2b*) and superpositioned onto the 70S tRNAs and mRNA, more detailed functional studies were carried out by Carter et al. (*37*). The flipped-out bases, A1492 and A1493, point into the A-site and are positioned to interact with the minor groove of the codon−anticodon helix and form a portion of the decoding surface. The energy needed for the flip-out of the adenine bases must be compensated by the formation of favorable interactions with the codon−anticodon helix. It was proposed that the A1492 and A1493 adenine bases hydrogen-bond simultaneously to 2′-OH groups on both sides of the codon−anticodon helix. Two adenines can monitor a large portion of the minor groove of three consecutive base pairs (*37*). The building of the interaction surface is further completed by recruiting other RNA residues. It is not clear, however, if the hydrogen bonding of the adenine residues with the 2′-OH groups on both strands involves adenines N1, N6, and N3 or adenines N1, N6, and N7. However, recently the crystal structure of the 30S ribosomal subunit in complex with mRNA and tRNA in the A-site has been solved at 3.1 Å (*2f*). From this structure it is clear that the two flipped-out adenines A1492 and A1493 form hydrogen bonds via their N1 atoms to the 2′-OH of residue 36 of tRNA and to the N1 of G530. This observation fits with our model where hydrogen bonding between the adenine base of 16S RNA and the 2′-OH of the codon is not needed for a stable interaction, as demonstrated in Figure 6. The flipped-out adenine residues can be accommodated in the minor groove of the mHNA−tRNA duplex. The mHNA−tRNA duplex has a minor-groove shape, which is typical for an A-type double-stranded RNA helix. Hydrogen bonding is possible between the N1 group of A1493 and the 2′-OH group of

residue 36 of tRNA. This adenine residue nicely fills the minor groove of the duplex. The hexitol residues lack a free hydroxyl group, and no hydrogen-bond interactions with A1492 and A1493 are possible.

CONCLUSION

Replacement of mRNA by a nuclease-stable artificial nucleic acid opens the way to many new applications, such as the production of peptides and proteins via in vitro translation systems and bypassing nuclear pathways of mRNA synthesis. We demonstrated that mRNAs of 33 nucleotides containing three and six modified hexitol nucleotides function as template in the translation process. It remains to be demonstrated if homogeneous mHNA still function in the same way. Structurally, our data are in contrast with the observation that tRNA binding to the A-site (as well as the elongation process) depends on the presence of 2′-OH groups in the corresponding codon. Our results are in agreement with the hypothesis that the conformation of the codon−anticodon duplex is more crucial than its constitution and that the ribosomal RNA uses an analog readout system rather than a digital readout system for recognition of the codon−anticodon duplex. This implies not only a correct positioning of the sugars and phosphate groups but also the pseudoaxial positioning of the base moieties with respect to the sugars (*12*). Our data allow us to hypothesize about the function of A1492 and A1493 in the translation process.

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REFERENCES

1. Jacob, F., and Monod, J. (1961) *J. Mol. Biol. 3*, 318−356.
2. (a) Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000) *Nature 289*, 905−920. (b) Wimberly, B. T., Brodersen, D. E., Clemons, W. M., Jr., Morgan-Warren, R. J., Carter, A. P., Vonrhein, C., Hartsch, T., and Ramakrishnan, V. (2000) *Nature 407*, 327−339. (c) Clemons, W. M., May, J. L. C., Wimberly, B. T., McCutcheon, J. P., Capel, M. S., and Ramakrishnan, V. (1999) *Nature 400*, 833−840. (d) Schluenzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F., and Yonath, A. (2000) *Cell 102*, 615−623. (e) Yusupov, M. M., Yusupova, G. Z. R., Baucom, A., Lieberman, K., Earnest, T. N., Cate, J. H. D., and Noller, H. F. (2001) *Science 292*, 883−896. (f) Ogle, J. M., Brodersen, D. E., Clemons, W. M., Tarry, M. J., Carter, A. P., and Ramakrishnan, V. (2001) *Science 292*, 897−902.
3. Huttenhofer, A., and Noller, H. (1994) *EMBO J. 15*, 3892−3901.
4. Alexeeva, E. V., Shpanchenko, O. V., Dontsova, O. A., Bogdanov, A. A., and Nierhaus, K. H. (1996) *Nucleic Acids Res. 24*, 2228−2235.
5. Potapov, A., Triana-Alonso, F., and Nierhaus, K. (1995) *J Biol. Chem. 270*, 17680−17684.
6. Soldatkin, K., Koval'chuke, O., Potapov, A., and El'skaya, A. (1992) *Biochim. Biophys. Acta 1130*, 38−40.
7. Potapov, A., Soldatkin, K., Soldatkin, A., and El'skaya, A. (1988) *J. Mol Biol. 203*, 885−893.
8. (a) Herdewijn, P., De Winter, H., Doboszewski, B., Verheggen, I., Augustyns, K., Hendrix, C., Saison-Behmoaras, T., De Ranter, C., and Van Aerschot, A. (1994). Hexopyranosyl-like oligonucleotides, in *Carbohydrate Modification in Antisense Research* (Sanghvi, Y. S., and Cook, P. D., Eds.) ACS Symposium Series, pp 80−99, American Chemical Society, Washington, DC. (b) Van Aerschot, A., Verheggen, I., Hendrix, C., and Herdewijn, P. (1995) *Angew. Chem., Int. Ed. Engl. 34*, 1338−1339.
9. de Smit, M. H., and van Duin, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7668−7672.
10. de Smit, M. H., and van Duin, J. (1994) *J. Mol. Biol. 244*, 144−150.
11. De Bouvere, B., Kerremans, L., Rozenski, J., Janssen, G., Van Aerschot, A., Claes, P., Busson, R., and Herdewijn, P. (1997) *Liebigs Ann./Rec.*, 1453−1461.
12. Lescrinier, E., Esnouf, R., Schraml, J., Busson, R., Heus, H. A., Hilbers, C. W., and Herdewijn, P. (2000) *Chem. Biol. 7*, 719−731.
13. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Rheinberger, H., Geigenmuller, U., Wedde, M., and Nierhaus, K. (1988) *Methods Enzymol. 164*, 658−570.
15. Quatfit program from the computational chemistry list.
16. Dontsova, O., Dokudovskaya, S., Kopylov, A., Bogdanov, A., Rinke-Appel, J., Junke, N., and Brimacombe, R. (1992) *EMBO J. 11*, 3105−3116.
17. Rinke-Appel, J., Junke, N., Brimacombe, R., Lavrik, I., Dokudovskaya, S., Dontsova, O., and Bogdanov, A. (1994) *Nucleic Acids Res. 22*, 153018−153025.
18. Sergiev, P. V., Lavrik, I. N., Wlasoff, V. A., Dokudovskaya, S. S., Dontsova, O. A., Bogdanov, A. A., and Brimacombe, R. (1997) *RNA 3*, 464−475.
19. Cate, J. H., Yusupov, M. M., Yusupova, G. Z., Earnest, T. N., and Noller, N. F. (1999) *Science 285*, 2095−2104.
20. Moazed, D., and Noller, H. (1986) *Cell 47*, 985−994.
21. von Ahsen, U., and Noller, H. (1995) *Science 267*, 234−237.
22. Garret, R. A., Douthwaite, S. R., Liljas, A., Matheson, A. T., Moore, P. B., and Noller, H. F., Eds. (2000) *The Ribosome: Structure, Function, Antibiotics and Cellular Interactions*, pp 129−154, American Society for Microbiology, Washington, DC.
23. Stark, H., Rodnina, M., Wieden, H.-J., van Heel, M., and Wintermeyer, W. (2000) *Cell 100*, 301−309.
24. Wolin, S. L., and Walter, P. (1988) *EMBO J. 7*, 3559−3569.
25. Wyatt, J. R., and Tinoco, I., Jr. (1993) in *the RNA World* (Gesteland, R. T., and Atkins, J. F., Eds.) pp 465−496, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. Hendrix, C., Rosemeyer, H., De Bouvere, B., Van Aerschot, A., Seela, F., and Herdewijn, P. (1997) *Chem. Eur. J. 3*, 1513−1520.
27. De Winter, H., Lescrinier, E., Van Aerschot, A., and Herdewijn, P. (1998) *J. Am. Chem. Soc. 120*, 5381−5384.
28. Lescrinier, E., Esnouf, R. M., Schraml, J., Busson, R., and Herdewijn, P. (2000) *Helv. Chim. Acta 83*, 1291−1310.
29. Hendrix, C., Rosemeyer, H., Verheggen, I., Seela, F., Van Aerschot, A., and Herdewijn, P. (1997) *Chem. Eur. J. 3*, 110−120.
30. Atkins, D., Miller, M., De Bouvere, B., Van Aerschot, A., and Herdewijn, P. (2000) *Die Pharmazie 55*, 615−618.
31. Knorre, D. G., Sirotuk, V. I., and Stephanovich, L. E. (1967) *Mol. Biol. 1*, 837−841.
32. Dunlap, B. E., Friderici, K. H., and Rottman, F. (1971) *Biochemistry 10*, 2581−2587.
33. Aurup, H., Siebert, A., Benseler, F., Williams, D., and Eckstein, F. (1994) *Nucleic Acids Res. 22*, 4963−4968.
34. VanLoock, M. S., Easterwood, T. R., and Harvey, S. C. (1999) *J. Mol. Biol. 285*, 2069−2078.
35. Fukui, T., Kakiuchi, N., and Ikehara, M. (1982) *Biochim. Biophys. Acta 697*, 174−177.
36. Yoshizawa, S., Fourmy, D., and Puglisi, J. D. (1999) *Science 285*, 1722−1725.
37. Carter, A. P., Clemons, W. M., Brodersen, D. E., Morgan-Warren, R. J., Wimberly, B. T., and Ramakrishnan, V. (2000) *Nature 407*, 340−348.

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