Inhibition of Protein Synthesis by Anti-5.8 S rRNA Oligodeoxyribonucleotides*

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To examine the role of the 5.8 S rRNA in ribosome function, oligodeoxyribonucleotides, complementary to chemically accessible sequences, were incubated with rabbit reticulocyte or wheat germ extracts undergoing protein synthesis in vitro. Significant and reproducible inhibitions were observed with several different oligonucleotides, the most inhibitory being specific for the universally conserved GAAC sequence. Mutant or heterologous sequences were substantially less inhibitory, results which clearly implicate the 5.8 S rRNA in the inhibitory process and are consistent with the possibility that the 5.8 S rRNA plays an important role in the binding of tRNA.

Although for two decades the 5.8 S rRNA has been recognized as a separate but integral part of the eukaryotic ribosome (1-3), little is clear about its role in ribosomal function. Numerous studies on the structure of this RNA from divergent organisms have revealed an extensive conservation of structural features (see Ref. 4) and give working models for both the secondary structure and the stable RNA-RNA complex which forms with the 25-28 S rRNA of the large subunit (see Ref. 5).

Several lines of evidence strongly suggest that the 5.8 S rRNA may, at least in part, be localized in the ribosomal interface and play an important role in protein synthesis. For example, comparisons of 5.8 S rRNA reactivity in the free 60 S subunit and whole ribosomes indicate that parts of the molecule become inaccessible when the 40 S subunit is present (6, 7). In addition, studies utilizing affinity chromatography suggest that the molecule may interact with 40 S subunit proteins (8, 9) and ternary (10) or quaternary (11) complexes of the 5 and 5.8 S RNAs and ribosomal proteins or tRNAs which have been formed in vitro. More recently, additional support has come from chemical cross-linking studies (12) which indicate that some of the 5.8 S rRNA binding proteins are proximal to the A-site and further reactivity comparisons (13) provide in situ evidence for the various 5 S rRNA, 5.8 S rRNA, and tRNA containing ribonucleoprotein complexes. Antisense RNAs are being widely exploited as a means to inhibit gene expression at the DNA or mRNA levels (e.g. Ref. 15). Because this approach may be equally effective for the study of ribosome function (e.g. Ref. 5), the effects of anti-5.8 S RNA oligonucleotides were examined in search of further information about this molecule's role in protein synthesis, particularly with respect to the binding of aminoacyl-tRNAs. Complementary oligodeoxyribonucleotides were synthesized using the Biosearch model 8600 DNA synthesizer and incubated with prepared (16) or commercially available micrococcal nuclease-treated rabbit reticulocyte or wheat germ extracts undergoing protein synthesis in vitro.

In our previous studies on the accessibility of the ribosome-associated 5.8 S rRNA (6, 7, 13), very similar results were obtained with intact ribosomes of diverse origins (wheat, yeast, and rat), but striking differences were observed when the results with intact ribosomes were compared with the 60 S subunits or when ribosomes were treated with puromycin. The differences included a universally conserved GAAC sequence region which has been postulated to be involved in tRNA binding (13). Since regions of variable reactivity may reflect a functional role, they were chosen as targets for anti-5.8 S rRNA oligonucleotides were examined in search of further information about this molecule's role in protein synthesis, particularly with respect to the binding of aminoacyl-tRNAs. Complementary oligodeoxyribonucleotides were synthesized using the Biosearch model 8600 DNA synthesizer and incubated with prepared (16) or commercially available micrococcal nuclease-treated rabbit reticulocyte or wheat germ extracts undergoing protein synthesis in vitro.

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### TABLE I

| Oligonucleotide sequence | Target sequence | Complementarity | Acid-precipitable incorporation |
|-------------------------|----------------|----------------|-------------------------------|
| AATGTTG                    | ACACAU         | Complete | 79.2 ± 6.8 (4) |
| GGCTGAT                    |                | None     | 97.6 ± 1.4 (4) |
| AGCCACAGG                  | CUCUGCGGGUG    | Complete | 83.3 ± 5.4 (9) |
| AGCCCGAG**                 | CUGCGGCGUG     | Partial  | 91.3 ± 0.5 (4) |
| GTTCGATG                   | CAUGACACC      | Complete | 100.1 ± 5.8 (3) |
| AGCTAGCC**                 | CAGCUAGCU      | Complete | 78.3 ± 7.4 (3) |
| TGCGTTCGA                  | UCGAAACCG     | Complete | 68.5 ± 0.4 (6) |
| TGCGTTCGAG**               | CUGCGAAACCA    | Complete | 55.9 ± 0.5 (3) |
| TGAGTCCAAG**               | CUGCGAAACCA    | Partial  | 92.0 ± 1.8 (3) |

* Each reaction mixture containing 20 μl of micrococcal nuclease-treated rabbit reticulocyte lysate mixture (16), 15 ng of rabbit globin mRNA, and 1.3 μCi (26 pmol) of [3H]leucine was incubated at 30°C in the presence or absence of 0.25 A260 unit of oligonucleotides. Aliquots were withdrawn after 20, 40, and 60 min, treated with 0.1 N NaOH, acid-precipitated, and counted as described previously (16). The initial rate of synthesis which was determined from the acid-precipitable counts is given as a percentage of the rate in the absence of an oligonucleotide; the number of experiments is noted in parentheses.

** Complete and partial incorporations are defined as 100% and 50%, respectively.

*** Underlined residues are not complementary to the 5.8 S rRNA.
5.8 S rRNA oligonucleotides. As indicated in examples shown in Table I, nine different short oligonucleotides chosen to be complementary or noncomplementary to five regions of the mammalian 5.8 S rRNA molecule were observed to have a variety of effects on protein synthesis. In each case, the incorporation was essentially linear during the 60 min incubation period and oligonucleotide degradation could be eliminated as a significant factor in the differences which are observed. The most inhibitory was TGCGTTTCGAAG, specific for the universally conserved GAAC site, and these of the 18 and 28 S rRNAs as well as the globin mRNA. No homologous stretches were found with fewer than three mismatched nucleotides.

| Oligonucleotide sequence | Target 5.8 S rRNA | Source of extract | Acid-precipitable incorporation* |
|--------------------------|------------------|------------------|----------------------------------|
| No oligonucleotide       | Rabbit           | Rabbit reticulocyte | 100%                             |
| TGCGTTTCGAAG             | Rabbit           | Wheat germ        | 55.9 ± 0.5 (3)                   |
| TGCGTTTCGAAG             | Rabbit           | Wheat germ        | 63.0 (1)                         |
| TTGCGTTTCGAAG            | Rabbit           | Rabbit reticulocyte | 75.1 ± 1.9 (3)                   |
| TTGCGTTTCGAAG            | Rabbit           | Wheat germ        | 84.9 (1)                         |
| TGCGTTTCGAAG             | Wheat            | Wheat germ        | 51.7 ± 4.3 (2)                   |
| TGCGTTTCGAAG             | Wheat            | Rabbit reticulocyte | 65.5 (1)                         |
| TTGCGTTTCGAAG            | Wheat            | Rabbit reticulocyte | 71.3 (1)                         |
| TTGCGTTTCGAAG            | Wheat            | Rabbit reticulocyte | 89.0 (1)                         |

* Reaction mixtures were prepared and sampled as described in Table I.

Effect of oligonucleotide size on the inhibition of in vitro protein synthesis

| Oligonucleotide sequence | Size | Source of extract | Target sequence | Acid-precipitable incorporation* |
|--------------------------|------|------------------|----------------|----------------------------------|
| No oligonucleotide       |      |                  |                | 100%                             |
| TGCGTTTCGA               | 9-mer| Rabbit           | CGACACUUGAAGCAGACUUCG | 68.6 ± 2.1 (6) |
| TGCGTTTCGA               | 10-mer| Rabbit          | CGACACUUGAAGCAGACUUCG | 53.6 ± 5.1 (4) |
| TGCGTTTCGA               | 11-mer| Rabbit          | CGACACUUGAAGCAGACUUCG | 55.9 ± 0.5 (3) |
| TTGCGTTTCGAAG            | 12-mer| Rabbit          | CGACACUUGAAGCAGACUUCG | 75.1 ± 1.9 (3) |
| CAGAGGCGGTTTCGAAG        | 17-mer| Rabbit          | CGACACUUGAAGCAGACUUCG | 71.5 ± 1.4 (3) |
| GCAAGGCGGTTTCGAAGTCTGC   | 21-mer| Rabbit          | CGACACUUGAAGCAGACUUCG | 72.5 ± 3.1 (3) |
| TGAGTCACCA               | 10-mer| Rabbit          | CGACACUUGAAGCAGACUUCG | 71.6 ± 0.4 (2) |
| TGCCGTTCAAA              | 10-mer| Wheat           | CUUGAAGCGA       | 51.7 ± 4.3 (2) |
| TGCCGTTCAAG              | 11-mer| Wheat           | CUUGAAGCGA       | 55.5 (1)                         |
| TTGCGTTCAAG              | 12-mer| Wheat           | CUUGAAGCGA       | 71.3 (1)                         |
| TTGCGTTCTTG*             | 11-mer| Wheat           | CUUGAAGCGA       | 92.5 (1)                         |

* Reaction mixtures were prepared and sampled as described in Table I.

As a final confirmation of a specificity for the 5.8 S rRNA, the inhibitory effect was also examined in heterologous systems. While the GAAC sequence is common to all 5.8 S rRNAs (4), the region surrounding it is often species-specific. As indicated in Table II, when rabbit-specific oligomers of differing length were incubated with an active wheat germ extract, substantially less inhibition was observed in each instance. Similarly, when a wheat germ-specific sequence was examined, it was found to be substantially more inhibitory with wheat germ extract than in the reticulocyte system. Again, the 5.8 S RNA-specific nature of the inhibitory process is clear. While globin mRNA was used in all the experiments shown in Table II, comparable results were also obtained with Brome mosaic virus RNA (results not shown).

Since sequences were initially synthesized to specifically complement chemically accessible regions, oligonucleotides of different lengths were used (e.g. Table I). However, different degrees of inhibition were also observed as the length of a specific nucleotide was varied (e.g. Table II) with longer as well as shorter oligomers. To determine potential steric restrictions on the inhibitory oligomers, the influence of chain length was further examined with the universal GAAC site. As indicated in Table III, the size of the oligonucleotide was more critical than anticipated. With rabbit reticulocyte extract, maximum inhibition was observed with a 10-11-nucleotide oligomer and longer chain lengths resulted in a reduced inhibition. A similar reduction was observed with wheat germ extract; the addition of one nucleotide reduced the inhibition by about 20%. With each type of extract, mutated sequences
were again observed to significantly reduce the level of inhibition, consistent with a 5.8 S rRNA-specific interaction. Using the data in Fig. 1, the molar concentration was found to be a significant factor with the longest oligomers, but the large change with the addition of a single nucleotide could not be accounted for in this way. Instead, steric hindrance appears to offer the best explanation. Localized ribosomal binding sites such as a site binding the T4CG arm of a tRNA molecule could be expected to be sterically restricted as compared to free longer oligonucleotides which are not folded into a tRNA structure.

In 1974, Nishikawa and Takemura (14) noted a GAAC sequence in the eukaryotic 5.8 S rRNA and suggested that this new sequence may have evolved to replace the putative general tRNA binding activity of the prokaryotic 5 S rRNA. It had earlier been suggested by Erdmann and co-workers (17) that the GAAC sequence in the prokaryotic 5 S rRNA interacted with the highly conserved T4CG arm of tRNA molecules. While these ideas were entirely speculative, the indirect evidence which was cited earlier, and the more direct evidence in this study, are fully consistent with the eukaryotic 5.8 S rRNA playing an important role in the ribosomal binding of tRNA.

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