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*THE BINDING OF AMINOACYL-sRNA'S TO RIBOSOMES STIMULATED BY BLOCK OLIGONUCLEOTIDES**

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The ability of oligonucleotides to function as messenger RNA analogues in protein synthesis has been demonstrated by several groups.[1-3] In particular, Leder and Nirenberg[3] have shown that oligonucleotides as small as the trimer are capable of stimulating the binding of specific aminoacyl-sRNA's to ribosomes. This reaction has proved very useful in determining the sequences of bases within the amino acid coding triplets,[4-6] especially since there are a number of methods by which trinucleotides of known base sequence can be prepared.[6, 7] While trinucleotide messengers are ideally suited for studies of coding specificity, much longer oligonucleotides of unique sequence are desirable for studies of the order of events in messenger-RNA translation.

Recently, we have described a method for the synthesis of block oligonucleotides of unique size and sequence.[8] In the present communication, we have studied the ability of block oligonucleotides to stimulate the specific binding of aminoacyl-sRNA's. Our major purpose in this work was to determine whether there was any triplet "reading frame" within the oligonucleotide chain which constituted a mandatory binding site for aminoacyl-sRNA. If such a site existed, it might well correspond to the initiation point of protein synthesis. Both the 5′ terminal[3] and the 3′ terminal[9] triplet reading frames have been suggested as possible initiation sites.

Materials and Methods.—Synthesis of block oligonucleotides: Polynucleotide phosphorylase from *Micrococcus lysodeikticus* was purified 90-fold following the method of Singer and O'Brien.[10] Oligonucleotides to be used as primers for this enzyme were purified from an alkaline digest of poly U or poly A which had been treated with alkaline phosphatase for the removal of the terminal phosphate. The mixtures of oligonucleotides were fractionated according to chain length by DEAE-cellulose column chromatography. For the synthesis of block oligonucleotides, the following conditions were employed: 0.035 *M* NDP, 1 mM $Mg(OAc)_2$, 0.1 mM $CuSO_4$, 0.2 *M* Tris buffer, pH 8.1, 0.6 mM oligonucleotide primer, 50 μg enzyme, and 0.4–1.5 *M* NaCl. Incubations were conducted at 33°. A detailed description of the polymerization reaction is presented elsewhere[8]. The block oligonucleotide products of the above reaction were purified and fractionated

by paper chromatography as follows. Reaction mixtures were applied to Whatman 3 MM chromatography paper and developed in a solvent composed of 75 vol 95% ethanol:30 vol 1 *M* ammonium acetate. Oligonucleotides, which remained at or near the origin, were eluted in 10 mM NH_4HCO_3 solution, lyophilized, and reapplied to the origin of a second chromatogram. This was then developed in a solvent composed of 50 vol 95% ethanol:50 vol 1 *M* ammonium acetate in which the various components of the oligonucleotide mixtures migrated in discrete bands according to chain length. The chain length of each band was determined by comparing its mobility to that of the unincorporated primer. For example, in the chromatographic purification of the series U_7A_n,[11] the UV-absorbing band which migrated immediately behind the unincorporated U_7 band was identified as U_7A_1, the next band as U_7A_2, and so forth. The reliability of this method for identification was tested by using ADP-H^3 as a reactant and U_8 as a primer. The chain lengths of the products of this reaction mixture (that is, U_8, U_8A_1, U_8A_2, etc.) were then determined independently by UV absorption and radioactivity measurements. The chain lengths determined by this method were identical to those determined by chromatographic mobility.

Ribosome preparation: *E. coli* B cells were grown in minimal medium with glucose as the sole carbon source[12] and harvested in late log phase. Ribosomes were prepared according to the method of Smith[13] which involves two washings in buffer containing a high KCl concentration (3 *M* KCl, 0.04 *M* $Mg(OAc)_2$, 0.01 *M* Tris buffer, pH 7.5). Pellets from the second wash were resuspended in Tris-Mg buffer (0.01 *M* Tris, pH 7.5; 0.01 *M* $Mg(OAc)_2$) and dialyzed against the same buffer overnight.

Binding assay: The binding of specific aminoacyl-sRNA's to ribosomes in the presence of oligonucleotide messengers has been described by Leder and Nirenberg.[3] We have employed essentially the same techniques, except that NH_4OAc was substituted for KCl, as suggested to us by Dr. G. Spyrides. The binding assay solutions contained 300–400 μg ribosomes, 0.05 *M* Tris buffer, p.H. 7.2, 0.1 *M* NH_4OAc, 0.02 *M* $Mg(OAc)_2$, 20–60 μg sRNA (General Biochemicals) charged with radioactive amino acids following the procedure of von Ehrenstein and Lipmann[14] (C^{14}-lys at 166 μc/μm; H^3-phe at 2.5 mc/μm; H^3-pro at 3 mc/μm; C^{14}-leu at 131 μc/μm; C^{14}-ileu at 131 μc/μm), and varying amounts of oligonucleotide. After incubating 25 min at 25°, the samples were diluted tenfold with buffer solution (0.05 *M* Tris, pH 7.2, 0.1 *M* NH_4OAc, 0.02 *M* $Mg(OAc)_2$) and filtered as described by Leder and Nirenberg.[3] The dried Millipore filters were placed in vials containing 10 ml of a toluene-based scintillator fluid and counted in a Packard Tricarb liquid scintillation counter.

Nuclease assay: In order to determine to what extent oligonucleotides were degraded by nuclease in the above binding assay, incubations were performed in which the oligonucleotide messenger was labeled with C^{14} or H^3. After incubation, the samples were chromatographed as described above. In one experiment, less than 2% of C^{14}-labeled A_6 was degraded to mononucleotides or to smaller oligonucleotides. In another experiment, where the messenger was U_8A_1 with the adenosine residue labeled with H^3, less than 14% of the adenosine terminal residue was released as mono- or oligonucleotide. These levels of nucleolytic damage are so low as to be of no significance to our interpretation of the experimental results presented below.

Results.—Effect of oligo U chain length on the binding of phe-sRNA: The relationship between the chain length of an oligo U messenger and its ability to stimulate binding of phe-sRNA is seen in Figure 1, where the amount bound as a function of the concentration of oligo U is displayed. (Ribosomes are limiting in the experiments; all other components are in excess.) It is apparent that the efficiency with which an oligonucleotide stimulates the binding reaction increases rapidly with the chain length. Thus, in the linear concentration-response range (below 0.025 mM oligo U), U_9 binds more than 12 times as much phenylalanyl-sRNA as does U_3. In contrast to this situation, the levels of phenylalanyl sRNA bound at saturating oligo U concentrations vary less widely. Indeed, repeated experiments have shown that there is no significant difference between the maximum levels attained by U_3, U_4, U_5, U_6, U_7, and U_8.[15] On the other hand, we have repeatedly observed that U_9 binds about 1.7 times as much phe-sRNA at saturation as do shorter oligo U chains.

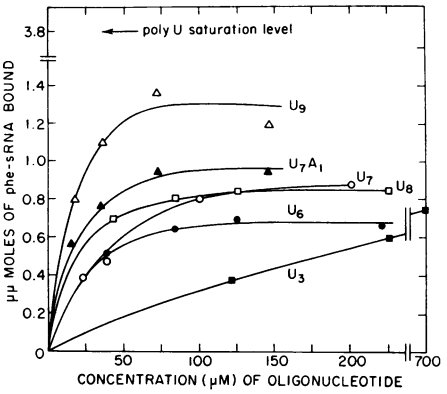

FIG. 1.—Relation between chain length of oligo U and H^3 phe-sRNA binding to ribosome. Each reaction mixture contained 5.0 $\mu\mu$moles of H^3 phe-sRNA, the oligo U's at indicated concentrations and other constituents as described in the text. Symbols represent U_3, (■); U_6, (●); U_7, (○); U_8, (□); U_9, (△); and U_7A_1, (▲).

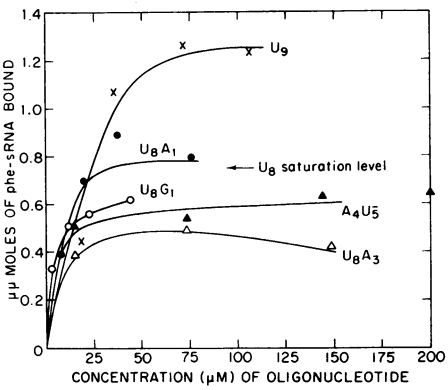

FIG. 2.—Phe-sRNA binding to ribosome directed by some pU-containing block oligonucleotides. Each reaction mixture contained 7.5 $\mu\mu$moles of H^3 phe-sRNA, the block oligonucleotides at indicated concentrations, and other constituents as described in the text. Symbols represent U_8A_1, (●); U_8G_1, (○); U_8A_3, (△); A_4U_5, (▲); and U_9, (×).

Moreover, above the 9-mer, the amount of phe-sRNA bound increases with increasing chain length, the maximum level reached thus far (with poly U, $n > 500$) being some sevenfold higher than that obtained with U_3. There is no obvious explanation as to why long chains should be able to bind more aminoacyl-sRNA than short ones. Therefore, it is important to bear in mind that chain length may be a critical factor contributing to the binding properties of a given oligonucleotide.

Binding of phe-sRNA stimulated by U-containing block oligonucleotides: Having obtained a general idea regarding the importance of messenger size, in the binding reaction, we turned to examine the effects of base composition at the 3′ and 5′ ends of the oligonucleotide chain. In particular, we were seeking some evidence for a preferred binding site located at either the 5′ (ref. 3) or the 3′ (ref. 9) end of the chain. In the first experiment, a single A residue was added on to the 3′ end of a U_7 chain. The effect of this addition on the binding of phe-sRNA is seen to be (Fig. 1) a slight but reproducible increase in both efficiency and saturation level relative to U_7 (cf. Fig. 3*b*). Indeed, the behavior of U_7A_1 is very similar to that of U_8, the homopolymer of the same chain length. This experiment strongly suggests that the 3′ terminal triplet is not a mandatory initial binding site, since there is no known phenylalanine codon containing an A residue.[16]

Further evidence for this interpretation is obtained from binding studies with a variety of U-containing block oligonucleotides, the results of which are shown in Figure 2. Once again we see that the addition of a heterologous nucleotide residue, in this case A or G, to the 3′ end of an oligo U chain does not prevent the U region from binding phe-sRNA. However, in contrast to the results with U_7A_1, it is interesting to note that U_8A_1 and U_8G_1 bind considerably less phe-sRNA at saturation than does U_9, the homopolymer of the same chain length. This suggests that the feature responsible for the unique behavior of U_9, as compared to shorter oligo U chains (Fig. 1), is not over-all chain length *per se* but rather the number of consecutive U residues in the chain.

These experiments, in conjunction with those performed with U_7A_1, demonstrate clearly that the 3′ end of the messenger chain is not a mandatory binding site for aminoacyl-sRNA, at least under the conditions employed here. That the same statement applies equally well to the 5′ end is shown in binding studies with the block copolymer A_4U_5. In this case the U region, now located at the 3′ end, is quite active in stimulating the binding of phe-sRNA, as shown in Figure 2. However, the maximum amount of phe-sRNA bound is only about half as much as with U_9. In the present case, the interpretation of this observation is complicated by the fact that the chain now contains the triplet A_3 which is well known to be active in stimulating the binding of an aminoacyl-sRNA other than phe-sRNA.[3] This reduction in binding, proportionate to the U content, could result from a competition between the A and U regions for the coding site, that is, the site on the ribosome where messenger and sRNA interact.[17] If this competition were about equally balanced in this case, the observed decrease in the amount of phe-sRNA bound at saturation would be accounted for.

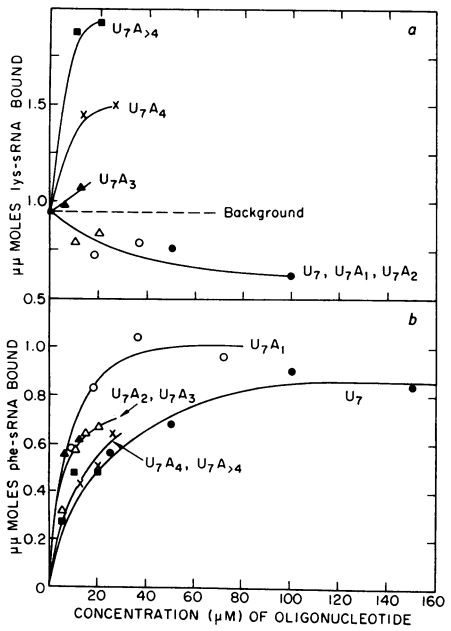

FIG. 3.—Coding efficiency of 3′ and 5′ components of UA block copolymer. (*a*) Binding of lys-sRNA to ribosome directed by U_7, (●); U_7A_1, (○); U_7A_2, (Δ); U_7A_3, (▲); U_7A_4, (×); and $U_7A_{>4}$, (■). Each reaction mixture contained 11.1 μμmoles of C^{14} lys-sRNA, the block oligonucleotide at indicated concentrations, and other constituents as described in the text. (*b*) Binding of H^3 phe-sRNA to ribosome. Explanation of symbols same as for (*a*). The reaction mixture was similar to that described above, except that H^3 phe-sRNA (5.0 μμmoles per reaction mixture) replaced the C^{14} lys-sRNA.

In order to test this view of competition between different regions of the same oligonucleotide for the coding site, the ability of the block U_8A_3 to stimulate the binding of phe-sRNA was studied. In this case (Fig. 2), both the efficiency and the maximum level of phe-sRNA binding is seen to be considerably reduced with respect to U_8 and U_8A_1 even though the U_8 block component is unchanged. As suggested above, this seems most probably due to the ability of the 3′ terminal A_3 triplet, which codes for lysine, to compete with the U_8 region for coding site(s) on the ribosome. That the terminal A_3 is capable of coding in this situation, and hence competing with the U_8 region of the chain, is proved by the fact that U_8A_3 (but not U_8A_2 or U_8A_1) stimulates the binding of lys-sRNA. The binding of lys-sRNA has been carefully studied for the series U_7,U_7A_1, U_7A_2, U_7A_3, U_7A_4, and $U_7A_{>4}$. In Figure 3*a*, it is seen that U_7A_1 and U_7A_2 actually depress the background level of bound lys-sRNA, as does U_7. However, U_7A_3 gives a small but significant stimulation, while U_7A_4 and $U_7A_{>4}$ show an increasing efficiency corresponding to the increase in the size of the A region.

The fact that U_7A_4 is considerably more efficient than U_7A_3 in the binding of lys-

sRNA is consistent with our view that the A and U regions of the block compete for the coding site(s) of the ribosome. Moreover, it implies that any triplet, regardless of its distance from the 3′ or 5′ ends of the chain, is a potential competitor for the coding site(s), or, in other words, that the choice of a reading frame within the messenger is random. Thus, in U_7A_3 only one of the 8 available reading frames can code for lysine, whereas two of the 9 frames in U_7A_4 can. Hence, if the choice of a reading frame is truly random, U_7A_4 should bind nearly twice as much lys-sRNA as U_7A_3. That this may indeed be the case is evident from Figure 3*a*, where U_7A_4 is roughly twice as efficient as U_7A_3 in stimulating the binding of lys-sRNA.

A preliminary study of the binding of phe-sRNA with the U_7A_n series is shown in Figure 3*b*. Although the data are incomplete, it is nevertheless evident that increasing the length of the 3′ A region decreases the binding of phe-sRNA, as expected. That this decrease is not due to hydrogen bond formation between the A and U regions of the blocks has been established from studies of the hypochromicity: U_7A_2 showed no hypochromicity in the range from 7° to 50°, while U_7A_4 had a T_m of about 8°. From these studies we conclude that there is no secondary structure in either of these blocks at 25°, the temperature at which the binding reactions were conducted.

For the most part, these experiments have been conducted using sRNA charged only with a single radioactive amino acid. However, we have also used sRNA charged with both amino acids (lys and phe), one labeled with C^{14} and the other with H^3. In binding experiments conducted with doubly charged sRNA, the results were identical to those obtained when singly charged sRNA was used. This observation confirms the finding of others[3, 18] that stripped sRNA binds as well as charged sRNA.

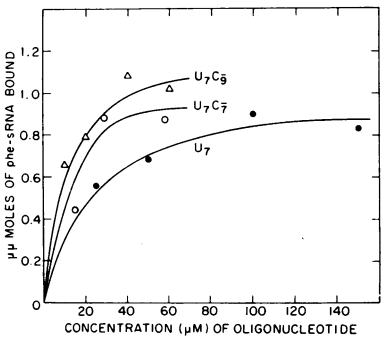

FIG. 4.—Phe-sRNA binding to ribosome directed by oligo U block copolymers. The reaction mixture contained 5.0 μμmoles of H^3 phe-sRNA, oligonucleotide at indicated concentrations, and other constituents as described in the text. Symbols represent U_7, (●); U_7C_7, (○); and $U_7C_9^-$, (△).

Studies with C-containing blocks: We have seen above that block copolymers containing A and U regions are capable of stimulating the binding of both lys- and phe-sRNA, and that the two block components appear to compete for the coding site(s) of the ribosome. In this connection it is interesting to note that oligo U and oligo A are each by themselves quite effective in stimulating the binding of their respective aminoacyl-sRNA's. But not all homo-oligonucleotides are equally effective in stimulating specific aminoacyl-sRNA binding; in particular, oligo C is very poor in stimulating the binding of pro-sRNA under the conditions employed here. In view of the mediocre binding qualities of oligo C by itself, it was of interest to see whether a C region in a block oligonucleotide could effectively compete with an adjacent U region.

In order to investigate the possibility of competition between U and C regions, the binding of phe-sRNA in the presence of $U_7C_7^-$ and $U_7C_9^-$ was studied. The results of these experiments are shown in Figure 4, where it is clearly seen that at saturation both these blocks bind phe-sRNA as well as the U_7 control. Thus, we conclude that the C region is not competing for the coding site. Consistent with this

TABLE 1

AMINOACYL S-RNA BINDING SPECIFICITY FOR INTERNAL CODEWORDS

Oligonucleotide	Concentration (μM)	Stimulation above Background ($\mu\mu$moles sRNA bound) Phe	Lys	Ileu
U_7A_4	13	0.43	0.48	0.03
	26	0.64	0.52	0.10
A_4U_5	72	0.75	0.23	—
	144	1.21	0.32	0.54
	288	1.24	0.32	0.50
A_1U_2	180			0.09
	180			0.12
	360			0.10
U_6	80			0.07
A_6	150			0.07
(Background)		(0.45)	(0.70)	(0.50)

The reaction mixture contained in a final volume of 50 μl, the oligonucleotides at indicated concentrations, aminoacyl-sRNA as follows: H^3 phe-sRNA, 5.0 $\mu\mu$moles; C^{14} lys-sRNA, 22.0 $\mu\mu$moles; and C^{14} ileu-sRNA, 16 $\mu\mu$moles. Other constituents and conditions of incubation are as described in the text.

conclusion is the finding that neither of these blocks stimulates the binding of pro-sRNA to a significant extent, although poly C ($n > 500$) alone is quite capable of stimulating binding under these conditions.

A second interesting feature of Figure 4 is the fact that both $U_7C_7^-$ and $U_7C_9^-$ at low concentrations are considerably more efficient than U_7 in binding phe-sRNA. This fact implies that the C region of the block, although incapable of competing with the U region for the coding site, nevertheless actively participates in the binding reaction. This result is consistent with the idea that nucleotide residues in the messenger which are not specifically involved in coding can nevertheless bind to the ribosome, thus increasing the stability (or the rate of formation) of the aminoacyl-sRNA-messenger-ribosome complex as a whole. This model would explain in part the results shown in Figure 1, where the binding efficiency of the series U_3 to U_8 increases sharply while the amount of phe-sRNA bound at saturation remains virtually constant.

Recently, we have also studied the ability of blocks containing X regions (such as $U_6X_6^-$, etc.) to bind aminoacyl-sRNA's specific for the second block component.[19] Results of these experiments support the view that noncoding oligonucleotide residues may play an important role in increasing the rate of formation or the stability of specific complexes.

Binding of internally coded aminoacyl-sRNA's: The preceding experiments demonstrate clearly that both the 3′ and 5′ components of a block oligonucleotide are capable of binding specific aminoacyl-sRNA's in the *in vitro* system. Moreover, the results of the competition experiments (Fig. 3) suggest that internal triplets may also bind. If this is indeed the case, one would expect block copolymers to stimulate the binding of aminoacyl-sRNA's other than the two coded by the homogeneous triplets at either end. In order to test this hypothesis, we have studied the binding of a variety of aminoacyl-sRNA's with $A_4U_5^-$ and U_7A_4. The results in Table 1 show that isoleu-sRNA is bound by $A_4U_5^-$ but not by U_7A_4, A_6, or U_6. Since the isoleucine coding triplet is known to contain both A and U residues,[16] it seems fairly certain that one of the internal triplets in $A_4U_5^-$, either AAU or AUU, is specifically coding for isoleucine in this case.

To determine which of these two possible triplets actually codes for isoleucine, the following trinucleotides[20] have been tested for the binding of ileu-sRNA: AUU,

TABLE 2

TYROSYL AND ISOLEUCYL-sRNA BINDING TO RIBOSOME DIRECTED BY AU CODONS

Expt.	Oligomer	Concentration (μM)	Tyr-sRNA bound ($\mu\mu$moles)	Ileu-sRNA bound ($\mu\mu$moles)
1.	UAU	190	1.55	—
	UAA	180	0.57	—
	AUU	180	0.42	—
	UAC*	190	0.82	—
	UAC*	160	1.09	—
	—	—	0.64	—
2.	UAU_7^-	40	1.26	—
	UAU_7^-	80	1.40	—
	UAU_7^-	120	1.40	0.77
	—	—	0.84	0.63

* These results obtained with two different preparations of UAC.

The reaction mixture contained 17 $\mu\mu$moles of C^{14} tyr-sRNA (or C^{14} ileu-sRNA), indicated amounts of the oligonucleotides, and other constituents as described in the text. Incubation at room temperature for 20 min.

AAU, AUA, UAA, and UAU. Of these, AUU has given a marginal stimulation above background, while all the others depress the background.

The extremely low efficiency at which the trinucleotide AUU binds ileu-sRNA is in marked contrast to the relatively good stimulation obtained with $A_4U_5^-$. This result is similar to that observed in the case of U_7C_n blocks, and implies that the over-all chain length of the oligonucleotide may play a vital role in stabilizing the formation of a given aminoacyl-sRNA-triplet-ribosome complex.

Further studies on the binding of ileu-sRNA have been conducted using as a messenger the oligonucleotide $U_1A_1U_7^-$. As in the case of A_4U_5, we again observe reasonably good binding of ileu-sRNA (Table 2). This result re-emphasizes the fact that the ileu-sRNA-AUU-ribosome complex either cannot form or is unstable unless the coding triplet is part of a longer oligonucleotide chain. Moreover, it provides further evidence that internal triplets in block oligomers can code, since the 5′ terminal triplet, UAU, is specific for tyrosine, as will be demonstrated below.

The peculiar inactivity of the AUU trinucleotide contrasts sharply with the ability of the homotrinucleotides, AAA and UUU, to stimulate the binding of lys-sRNA and phe-sRNA, respectively. To test whether heterotrinucleotides might be inherently less active than homotrinucleotides, we examined the ability of UAU and UAC to stimulate the binding of their corresponding aminoacyl-sRNA. That both these trinucleotides are efficient in stimulating the binding of tyrosyl-sRNA may be seen in Table 2. This result clearly demonstrates that the inactivity of AUU is not due to a base composition effect, but is specific for the sequence AUU. Thus it seems likely that the peculiar instability of the ileu-sRNA-AUU ribosome complex may reflect some property of the ileu-sRNA molecule itself, as, for example, its affinity for the sRNA binding site on the 50s ribosomal subunit.[21]

This phenomenon is not peculiar to ileu-sRNA. Bernfield and Nirenberg[6] were unable to demonstrate specific binding of leu-sRNA in the presence of trinucleotides containing U and C, although a random copolymer containing the same bases stimulated binding reasonably well. We have confirmed these results.

Conclusion.—The results presented above clearly demonstrate that any trinucleotide sequence within a short messenger chain is potentially capable of coding for the specific binding of an aminoacyl-sRNA. The question now arises as to what significance our findings have for the problem of the initiation of mRNA translation and protein synthesis *in vivo*, where it seems highly likely that some mechanism which

ensures initiation only at a specific point(s) on the mRNA chain must exist. The lack of specificity found in the *in vitro* binding studies implies that one or more additional components are required for specific initiation. For example, it is possible that an initiator enzyme which normally enables the selection of only one end of the messenger chain is absent in our *in vitro* binding system. A second possibility is that *in vivo* the polarity of mRNA synthesis or its release from a DNA-protein complex determines the initiation point for protein synthesis. Finally, it is possible that there is some chemically distinct feature of the natural mRNA chain which codes for initiation, such as an initiator coding triplet, a sequence of methylated bases, or a terminal phosphate group.[3]

Note added in proof: We have recently studied the binding of phe- and lys-sRNA stimulated by UA block copolymers carrying a phosphate residue at the 5′ end of the chain. The presence of a 5′ phosphate did not alter the random binding pattern observed with the block oligonucleotides studied above.

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[11] Oligonucleotide nomenclature: $A(pA)_5$ is abbreviated as A_6; $U(pU)_6(pA)_3$ is abbreviated as U_7A_3. The 5′ end is on the left, the 3′ end on the right. If a block copolymer is heterogeneous with regard to the chain length of one component, then a number average chain length is given, this being indicated by a bar over the numerical subscript, e.g., $A_4U_{\bar{5}}$. Nucleotide residues are designated by a capital letter, e.g., (pA) is abbreviated as A.

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