Synthesis and functional activity of translation initiation regions in mRNA

20-base polyribonucleotides from the replicase gene of phage MS2 and fr

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Three 20-base polyribonucleotides, AAACAUGAGGAAUACCCAUG (I), AAACAUGAGGAAAACCCAUG (II), AAACAUGAAGAAUACCCAUG (III), corresponding to the minimal initiation region for the replicase gene of phage MS2 and fr or having some differences were synthesized using enzymatic methods The template activity of the synthesized polynucleotides in initiation and their capacity to bind phage coat protein were studied under conditions optimal for native mRNA Polynucleotides I and II exhibit template activity comparable to that of the native phage RNA fragments Polynucleotide III with the destroyed SD sequence dit not manifest any functional activity either as template or in binding to MS2 phage coat protein

Polyribonucleotide *Enzymatic synthesis* *Protein synthesis initiation* *Template activity*

1. INTRODUCTION

A ribosome, on initiation of protein biosynthesis, recognizes selectively the mRNA region at the beginning of the gene and forms with this region an initiation complex [1].

A detailed investigation of template activity of RNA fragments of different length from phage MS2 and fr belonging to the beginning of the phage replicase gene permitted us to determine the approximate boundaries of the given initiation region, and a corresponding structural model was proposed [2,3]. In accordance with this model, the specificity of initiation complex formation is determined by the minimal initiation region preceding the coding part of the gene. To take up a study of the structural determinants of the ribosome binding sites in mRNA, we have carried out enzymatic synthesis of several 20-nucleotide long fragments corresponding, in structure and size, to the minimal initiation region for the replicase gene of phage MS2 and fr or having some differences.

2. MATERIALS AND METHODS

Isolation of the corresponding enzymes and the synthesis of decaribonucleotide fragments (5′-deca and 3′-deca) will be described separately.

5′-Phosphorylation of 36 nmol 3′-deca (VI) (see fig.1) was carried out for 1 h at 37°C in 100 μl buffer (0.15 M Tris-HCl, pH 7 5; 0.015 M $MgCl_2$, 0.015 M β-mercaptoethanol) with the addition of 30 μl T4 polynucleotide kinase (1500 act.units/ml). The separation of the reaction mixture was performed on a 0.3 ml DEAE-Sephadex A-25 column in 0.02–1.0 M $(NH_4)_2CO_3$ gradient (75–75 ml each) followed by evaporation in a vacuum. For the synthesis of 20-base polynucleotide I, a mixture of 18 nmol 5′-deca (IV) and the 5′-phosphorylated 3′-deca (VI), 36 nmol ATP and 20 μl buffer (0.1 M Tris-HCl, pH 8.3; 0.02 M $MgCl_2$, 0.002 M DTT) was evaporated and then incubated for 16 h at 14°C with 18 μl T4 RNA-ligase (50000 act.units/ml) manufactured by the Enzymatic Preparations Fac-

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tory in Vilnius, USSR, in 50% glycerol, 0 01 M Tris-HCl (pH 7.5), 0.0001 M EDTA, 0.05 M KCl and 0.001 M DTT The reaction mixture was maintained with 10 μl formamide dyes for 1 min at 100°C before gel electrophoresis. The separation was in a 25% polyacrylamide gel (0.04 × 20 × 40 cm) containing 7 M urea in 0.1 M Tris-borate (pH 8.3), 0.002 M EDTA for 5 h at 1300 V The band just below xylene cyanol was cut off and the product was isolated as in [4]; after resedimentation 3 nmol product (yield 18%) was obtained The binding of MS2 phage coat protein was conducted according to [5]. The initiation activity of polynucleotides was studied as described earlier [4]. RNase-free *E. coli* ribosomes were a kind gift from Dr S.N. Kirillov (Leningrad Institute of Nuclear Physics, USSR) Initiation factors IF1, IF2, IF3 were purified as in [6] with some modifications.

3. RESULTS AND DISCUSSION

First, two 20-base ribonucleotides AAACAUGAGGAAUACCCAUG (I) and AAACAUGAGGAAAACCCAUG (II) corresponding to the minimal initiation region of the MS2 and fr phage replicase gene – R(−17⟶3) [2,3] were synthesized. Both polynucleotides contain the native SD sequence UGAGGA and end in the initiator codon; they can form a secondary structure (fig.1) haracteristic of phage RNA fragments [4,7]. For the sake of convenience, two nucleotide substitutions were introduced into polynucleotide I (synthesized by coupling IV and VI), as opposed to the natural structures; the substitution $U_{-6}\longrightarrow A_{-6}$ made it distinguishable from the MS2 RNA region [4], and $C_{-17}\longrightarrow A_{-17}$ from the fr RNA region [7]. Polynucleotide II carrying a $U_{-5}\longrightarrow A_{-5}$ modification in the loop part of the hairpin was synthesized by coupling decamers IV and VII. The third polynucleotide AAACAUGAAGAAUACCCAUG (III) obtained by joining V with VI contained, unlike the first one, a $G_{-9}\longrightarrow A_{-9}$ substitution leading to destruction of the SD sequence by transforming it into UGAAGA. Simultaneously this substitution must have destabilized the secondary structure of the polynucleotide. The structures of all hepta- and decamers were confirmed by sequence determination according to [8]. The struc-

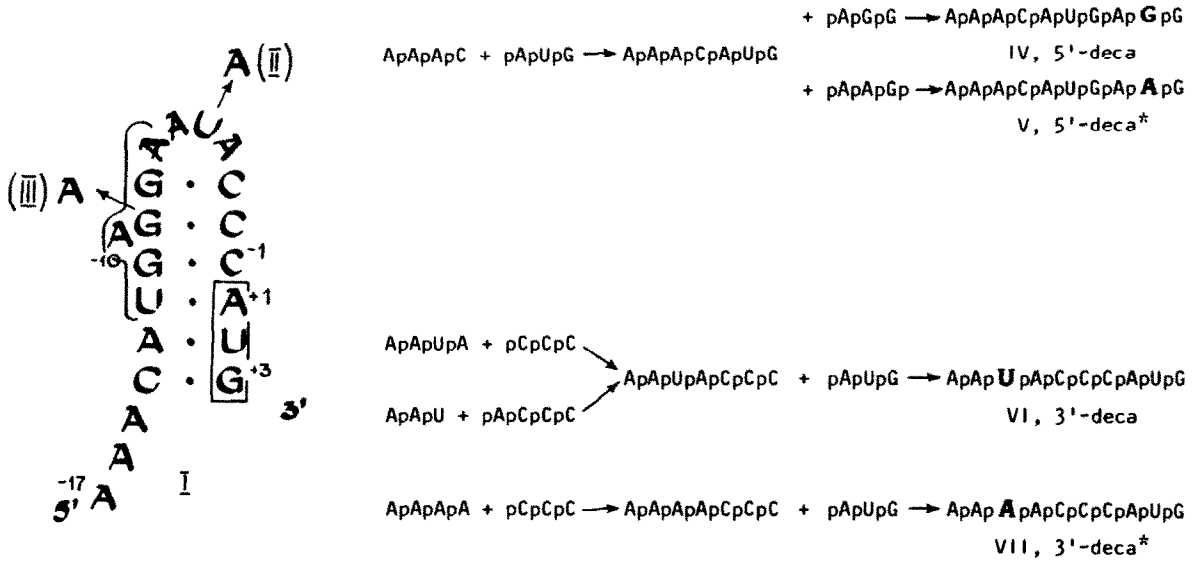

Fig 1 Polynucleotide I with structural variants II and III and the scheme for the synthesis of constituent decamers IV–VII The initiator codon is framed; the SD sequence is underlined. The substituted nucleotides are shown in bold type

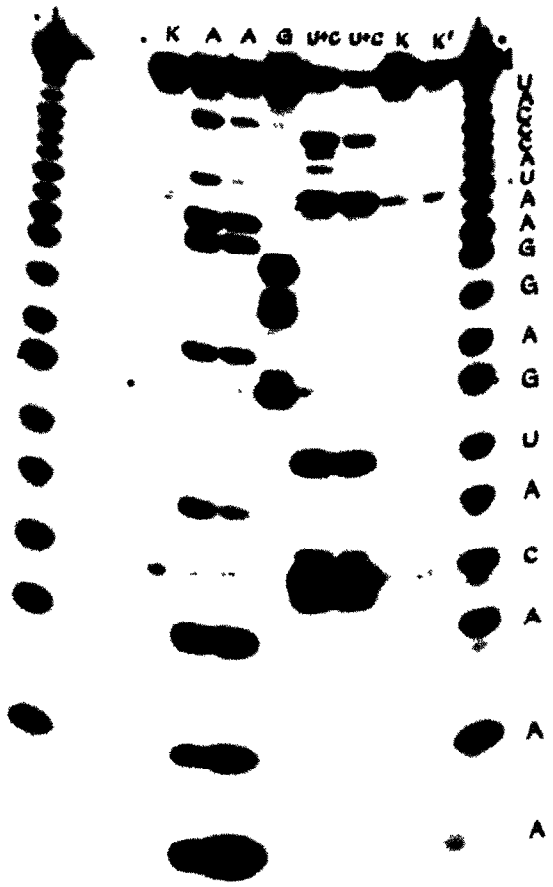

Fig 2. Gel sequencing of polyribonucleotide I (5′-^{32}P)-labelled polynucleotide I was partially hydrolyzed with U_2 RNase (tracks A), T_1 RNase (track G), pancreatic RNase (tracks U+C) K, starting product; K′, starting product incubated at 100°C for 1 min, L, ladder (hydrolysis of the starting product with 90% formamide for 30 min at 100°C) Resolution was in 25% polyacrylamide gel with 7 M urea, pH 8 3 (●) Xylene cyanol, (○) bromophenol blue

tures of the isolated 20-base products were confirmed by gel sequencing (fig.2).

The activity of synthesized polynucleotides in the formation of initiation complexes in vitro was studied under conditions optimal for the native mRNA [4]. Two methods were employed – template-dependent binding of f[^{3}H]Met-tRNA by the ribosomes (fig.3a) and gradient centrifugation of the 70 S initiation complex formed by (5′-^{32}P)-labelled polynucleotides (fig.3b–d). The trinucleotide ApUpG and MS2 RNA fragments MS2 R(−53⟶3) and MS2 R(−53⟶6) carrying the full-length initiation region of the MS2 phage replicase gene were used for comparison. The initiatory activity of MS2 R(−53⟶6) is close to that of the native phage RNA but its shortened analogue MS2 R(−53⟶3) ending like I–III in the initiator codon does not differ functionally from MS2 R(−53⟶6) [4]. As can be seen in fig.3a, polynucleotides I and II are equally active in stimulating the binding of f[^{3}H]Met-tRNA to ribosomes. The template activity of I and II as compared with ApUpG, and the decanucleotides AAACAUGAGG (IV) and AAUACCCAUG (VI) constituting I, is relatively high, although not so high as that of MS2 R(−53⟶3).

For quantitative estimation of the 70 S initiation complex formation we used ^{32}P-labelled polynucleotides at concentrations close to saturation for fMet-tRNA binding (cf fig.3a). Under the experimental conditions, out of 30 pmol templates used only 10.4 pmol MS2 R(−53⟶6) and 5.8 pmol I were incorporated into the 70 S complex (fig 3b,c). Product III has a very low activity in initiation (0.6 pmol bound; fig.3d), obviously due to the destruction of the SD sequence.

Polynucleotide I is also active in binding MS2 phage coat protein (table 1). The substitution U_{-5}⟶A_{-5} has a negative effect on activity and hence polynucleotide II is less active. A considerable decrease in coat protein binding activity is reached by the G_{-9}⟶A_{-9} substitution in polynucleotide III. Our findings are in agreement with the results of detailed coat protein binding investigations by Uhlenbeck et al. [9] who used similar experimental systems.

The experience gained permits synthesis of other variants of minimal initiation regions of mRNA, namely by changing the distance between the SD sequence and the initiation codon, by varying the structure of these intermediate nucleotides and the structure of the polypurine track, and by changing the secondary structure of polynucleotides. The study of the functional activity of these polynucleotides will contribute to a better understanding of the template specificity of translational initiation in prokaryotes.

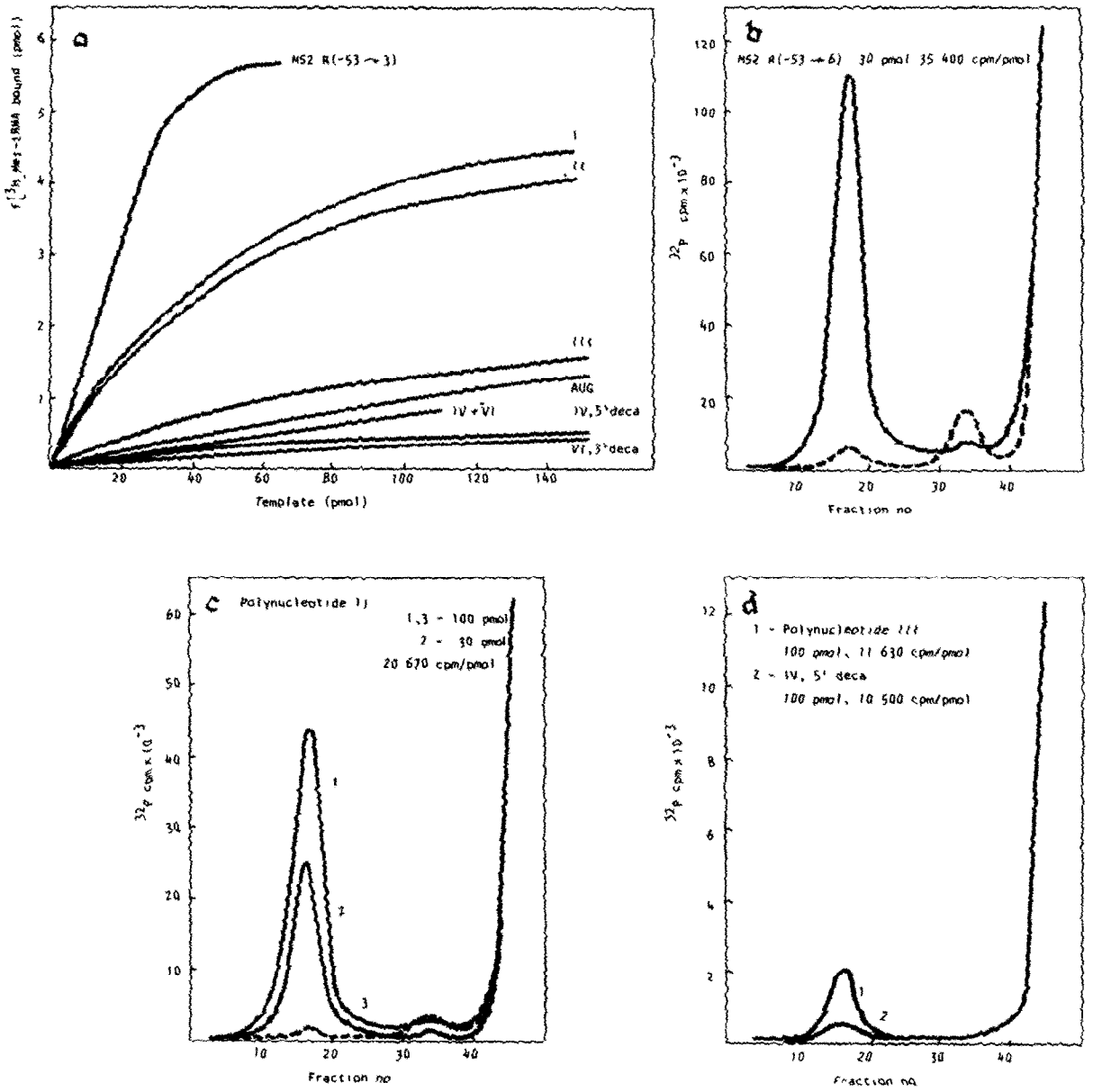

Fig.2. Gel sequencing of polyribonucleotide I. (5'-^{32}P)-labelled polynucleotide I was *partially* hydrolyzed with U_2 *RNase (tracks A),* T_1 *RNase* (track *G*), pancreatic RNase (tracks U + C) *K*, *starting product; K', starting product incubated at 100°C for 1 min; L, ladder (hydrolysis of the* starting product with 90% *formamide* for 30 min at 100°C). *Resolution* was in 25% *polyacrylamide gel with* 7 M urea, pH 8.3 (●) *Xylene cyanol,* (○) bromophenol blue

Table 1

MS2 coat protein binding to 20-base polynucleotides I–III

Polynucleotide	Initial ^{32}P-labelled polynucleotide (cpm)	Bound ^{32}P-labelled polynucleotide (cpm)	(%)
MS2 R(−53→6)	93600	56200	60 0
(I)	57510	23140	40 2
	52700	23195	45 4
(II)	101360	32500	27 2
	79440	22200	27 9
(III)	61160	6305	10 3
	55240	7255	13.1

10 pmol (5′-^{32}P)-labelled polynucleotide and 200 pmol coat protein were incubated for 10 min at 2°C The complex was trapped on nitrocellulose filters Background radioactivity (without addition of coat protein) was subtracted

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