FORMATION OF A PROTEIN INITIATION COMPLEX ON E. COLI RIBOSOMES DIRECTED BY A EUKARYOTIC VIRUS RNA

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1. Introduction

The mechanism which governs the initiation of protein synthesis in prokaryotic systems is now reasonably well understood. Several translation initiation factors both catalyze the positioning of fMet-tRNA on the messenger–ribosome complex and account for the ribosomal recognition of the messenger initiation region including the AUG triplet. The situation in eukaryotic cells seems to involve the same general principles (for a review, see [1]). A recent advance in our understanding of the specificity underlying ribosome–messenger interaction has been provided by Shine and Dalgarno's observation [2] that the 3′ terminal sequence of 16 S ribosomal RNA exhibits some complementarity to the ribosome-binding sequences of different coliphage RNAs. This interaction may dictate the relative affinity of the ribosome for the cistron concerned [3]. Protein S 1 and initiation factor IF_3 could play a role in reinforcing the pairing between the purine-rich cluster and the 16 S terminus.

Results by Shine and Dalgarno [4] indicate that a similar kind of pairing mechanism, albeit between regions of different base sequences from those involved in prokaryotic systems, is involved during the initiation step in eukaryotes.

The existence of different matching RNA sequences within prokaryotic and eukaryotic initiation complexes probably accounts for the fact that eukaryotic and prokaryotic ribosomes cannot generally be interchanged for the correct synthesis of polypeptides [5].

In particular, phage RNA is poorly and probably not accurately translated by 80 S reticulocyte or ascites cell ribosomes [6]. Conversely, eukaryotic messengers such as 9 S RNA, although they can complex with 70 S *E. coli* ribosomes, cannot direct synthesis of a complete protein product [7]. However, Siegert et al. [8] have reported that RNA extracted from Avian Myeloblastosis virus (AMV RNA) could serve as template for the synthesis of complete virus proteins in an *E. coli* ribosomal system.

In the present work we have reinvestigated the characteristics of the initiation step in such a crossed system, by using washed *E. coli* ribosomes and initiation factors. Our data indicate that AMV RNA, a eukaryotic messenger, permits the correct positioning of *E. coli* fMet-tRNA at the order of 3 initiation complexes can form per RNA chain.

2. Materials and methods

2.1. *Preparation of ribosomes, supernatant and initiation factors*

Frozen bacteria (*E. coli* MRE 600) were sonicated in two vols of TMNSH buffer (Tris–HCl, pH 7.4 (10 mM), NH_4Cl (60 mM), β-mercaptoethanol (7 mM)). The 30 000 *g* supernatant fraction constitutes the source of ribosomes.

Ribosomes were obtained by centrifuging the S 30 fraction for 8 h at 75 000 *g*, the upper 2/3 supernatant S 100 being kept as a source of tRNA synthetase.

The ribosomal pellet was resuspended in TMNSH and centrifuged first for 30 min at 30 000 *g* to discard possible aggregates, then at 75 000 *g*. NH_4Cl-washed ribosomes and initiation factors were obtained from

the pellet. TMNSH made one molar with NH_4Cl was used to resuspend ribosomes and the suspension was layered on 1/3 of its volume of a 20% glycerol cushion made in NH_4Cl containing TMNSH buffer.

After centrifugation for 12 h at 100 000 *g*, the supernatant on top of the glycerol cushion contained crude initiation factor (IF), while the pellet corresponded to NH_4Cl-washed ribosomes.

Ribosomes were resuspended in TMNSH, heated for 20 min to 40°C and stored as small aliquots in liquid nitrogen.

IF was precipitated by addition of ammonium sulfate (35–70% saturation) dialysed extensively against Tris–HCl pH 7.4 (10 mM), NH_4Cl (100 mM), α-mercaptoethanol (7 mM).

S 150 was dialysed 4 h after 3 changes of Tris–HCl pH 7.4 (10 mM), β-mercaptoethanol (7 mM).

2.2. *Preparation of AMV RNA*

Plasma from AMV-infected chickens was the gift of Dr J. Beard (Duke University, Durham, N. C., USA). Virus was concentrated from the plasma by sedimentation for 1 h onto a cushion of 50% (w/w) sucrose in 0.05 M Tris–HCl, pH 8, 0.1 M NaCl, 0.001 M EDTA (STE), and partially purified by resuspension and centrifugation through a step gradient of 20% (w/v) sucrose and 50% (w/w) sucrose in STE buffer for 2 h. Centrifugations were at 27 000 rev/min (100 000 *g*) at 4°C in a Spinco SW 27 rotor. The virus was resuspended and pelleted for 1 h at 27 000 rev/min in a SW 27.1 rotor. Pelleted virus was resuspended in STE buffer, incubated with 0.5 mg/ml pronase and 0.5% SDS at 37°C for 30 min, and deproteinised by two extractions with phenol and 1% SDS, at room temperature. The total viral RNA was precipitated by the addition of two vols of ethanol, and 0.4 M NaCl. AMV 70 S RNA was prepared by centrifugation of total RNA on a linear 5–20% sucrose gradient, prepared in STE buffer containing 0.1% SDS. Centrifugation was at 45 000 rev/min (190 000 *g*) for 1 h at 20°C in a Spinco SW 50.1 or SW 56 rotor.

35 S RNA was kindly prepared by Dr Jacquet by heat denaturing 70 S in the presence of formamide and purified on a sucrose gradient.

Total cytoplasmic poly-A containing RNA from calf skeletal myoblasts was a gift of Dr Caput and Dr Jacquet. Poly-A containing RNA from AMV-infected chick myoblasts was a gift of Dr B. Koller.

[^{3}H]fMet-tRNA was prepared according to Lelong et al. [9], its specific activity was 1000 cpm/pmole.

E. coli stripped tRNA was from Schwarz Bioresearch. Methionine, ATP and GTP were from Sigma. [^{3}H]methionine was from CEA (Saclay, France). T_4-mRNA prepared according to Salzer was kindly provided by Dr Jeantet.

3. Results

3.1. *AMV-RNA dependent-stimulation of fMet-tRNA binding to* E. coli *ribosomes*

Fig.1 describes the concentration dependence of [^{3}H]fMet-tRNA binding to *E. coli* 70 S ribosomes when using specific mRNA or crude total AMV-RNA as messengers. It is clear that in low ranges of concen-

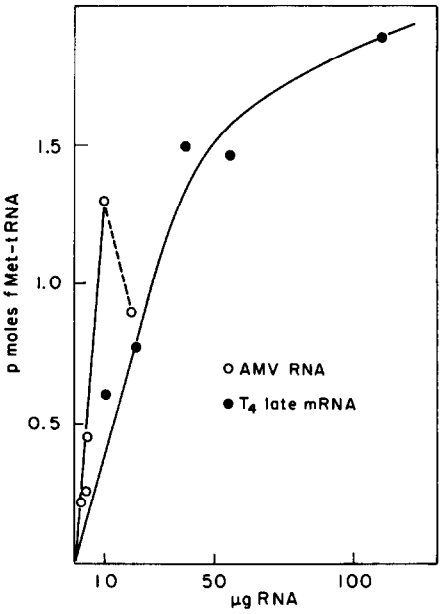

Fig.1. Dependence of fMet-tRNA binding on RNA concentration. The reaction mixture (50 µl) contained: Tris–HCl, pH 7.4 (50 mM), NH_4Cl (80 mM), $MgCl_2$ (8 mM), β-mercaptoethanol (7 mM), ribosomes, 0.5_{OD} unit, 1 mM GTP, IF 33 µg, plus 0.69_{OD} unit tRNA charged with 8.9 pmol of formyl-[^{3}H]methionine; incubation was at 37°C for 30 min. T_4-mRNA (●——●), AMV-RNA total (○——○). After incubation, the mixture was filtered through nitrocellulose, and filters were washed with buffer containing the above concentrations of Tris–HCl, NH_4Cl, $MgCl_2$ and β-mercaptoethanol. Bound activity on the air-dried filters was measured by counting in toluene-based scintillator.

Table 1
Dependence of AMV-RNA-directed binding of fMet-tRNA on GTP, IF and ribosomes

	−RNA	+ AMV −RNA
Complete	0.384	0.754
Complete + minus Rib.	0.025	0.025
Complete + minus IF	0.059	0.108
Complete + minus GTP	0.372	0.412

The conditions were the same as in fig.1 with 4 μg crude AMV-RNA added to the mixture. Values are expressed in pmoles of fMet-tRNA.

tration, AMV-RNA markedly stimulates initiator tRNA binding, its effect being about 2–3 times more pronounced than that of T_4 mRNA on a weight basis. But it is to be recalled that the T_4 is largely contaminated with ribosomal RNA. At high AMV-RNA/ribosome ratios, a rapid decline in the binding reaction appears.

Table 1 indicates that AMV dependent initiator tRNA binding exhibits a normal dependence upon initiation factors and GTP.

The time course of the binding reaction illustrated in fig.2 shows that although different plateau levels are reached, the overall kinetics of fMet-tRNA binding are the same in the presence of AMV-RNA as with T_4-mRNA, the reaction being complete after 30 min in the experimental conditions; of interest is the fact that, when messenger is limiting, more IF is required to reach maximum binding with the eukaryotic messenger than with T_4-mRNA (fig.3). This may possibly reflect a lower affinity of at least one factor component for AMV-RNA initiation sequences or for the AMV-RNA–ribosome complex.

3.2. *fMet-puromycin formation*

The possibility did exist that, although it can direct initiator tRNA binding to *E. coli* ribosomes, AMV-RNA does not permit formation of a correct initiation complex. In order to ascertain that fMet-tRNA when bound to an AMV–ribosome complex

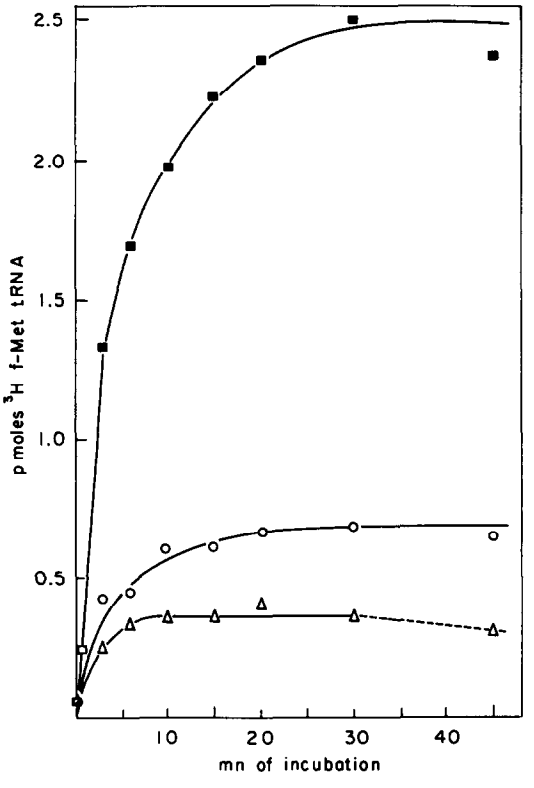

Fig.2. Time course of AMV-RNA and T_4-mRNA directed fMet-tRNA binding to *E. coli* ribosomes. The conditions were the same as in fig.1. (□—□) With 110 μg of T_4-mRNA. (○—○) With 4 μg of AMV-RNA. (△—△) Without messenger.

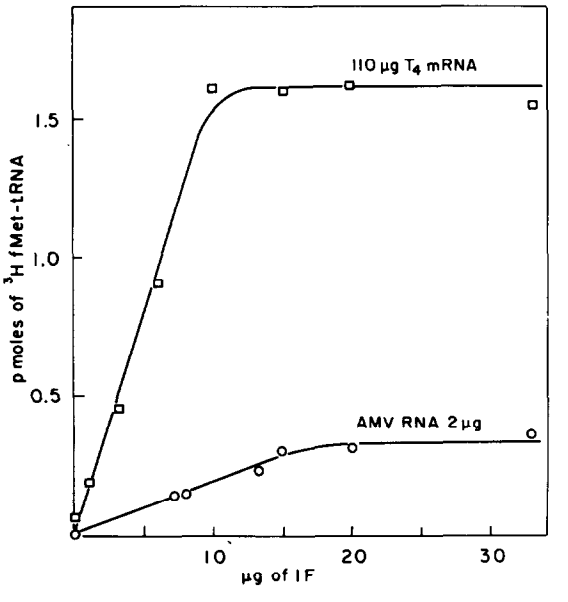

Fig.3. AMV-RNA or T_4-mRNA-dependent binding or [^{3}H]-fMet-tRNA as a function of *E. coli*. (□—□) With 110 μg of T_4-mRNA. (○—○) With 2 μg of AMV-RNA. Conditions as in fig.1.

Table 2
AMV-RNA and T_4-mRNA-directed fMet-puromycin formation

Reactions	−RNA	+ AMV-RNA	+ T_4-mRNA
fMet-tRNA bound	0.304	0.564	1.780
fMet-puromycin	0.378	0.753	2.523

fMet-tRNA binding was measured as in fig.1 after 30 min. At the end of this incubation period fMet-puromycin formation was measured in parallel samples by making the reaction mixture 1 mM with respect to puromycin and by incubating 10 min more at 37°C. [^{3}H] fMet-puromycin was extracted at pH 5 in ethylacetate according to Leder and Bursztyn [8], and radioactivity was measured using Bray's scintillator. Values are expressed in pmoles of fMet-tRNA. The background without puromycin (0.020 pmol) was neglected.

is correctly positioned, its ability to react with puromycin to form a dipeptide bound has been examined.

As shown in table 2, when the initiation complex is just allowed to form under the conditions previously described and the reaction mixture is further incubated with puromycin for a subsequent 10 min, very efficient transfer to puromycin is observed both with AMV- and T_4-RNA. In both cases the amount of fMet-puromycin formed at the end of the reaction suggests that fMet readily recycles from the initiation complex.

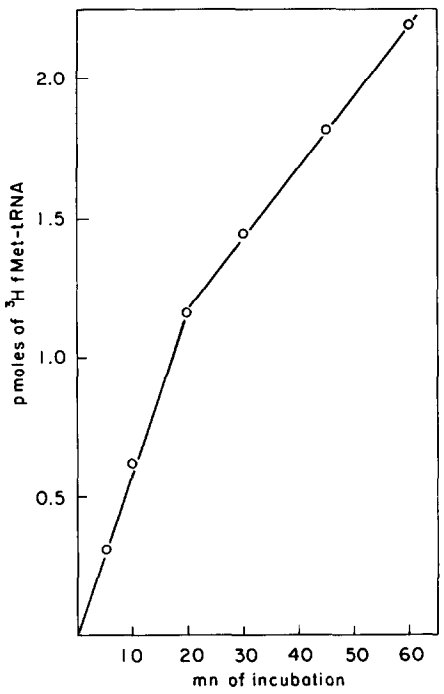

Fig.4. AMV-RNA dependent fMet-puromycin formation. The conditions were the same as in table 2 except that only 2 μg of AMV-RNA were added and puromycin was introduced at time zero.

From the experiment of fig.4, in which puromycin was added at the onset of the binding reaction and the amount of fMet-puromycin was measured as a function of time, the rate of fMet-puromycin formation can be estimated to be of the order of 0.06 pmol/min, a value which represents the initial rate of the binding reaction. Since fMet transfer to puromycin occurs at a rate which is immediately maximum, even after all AMV initiation sites have been saturated with fMet-tRNA, fig.5 it is unlikely that there is heterogeneity with respect to fMet-tRNA positioning on the AMV–ribosome complexes.

3.3. *Comparative activities of AMV-RNA subcomponents*

All the experiments described above were performed with total crude AMV-RNA. Since total RNA extracted from the virion could possibly be contaminated with host-specific components, it was important to examine the effect on fMet-tRNA binding of purified AMV-RNA chains. Binding experiments as well as puromycin transfer studies were repeated using native 70 S AMV-RNA or the formamide denatured 35 S RNA species.

As can be seen in Table 3, both species possess a clear capacity to stimulate IF-dependent fMet-tRNA binding to *E. coli* ribosomes as well as fMet-puro-

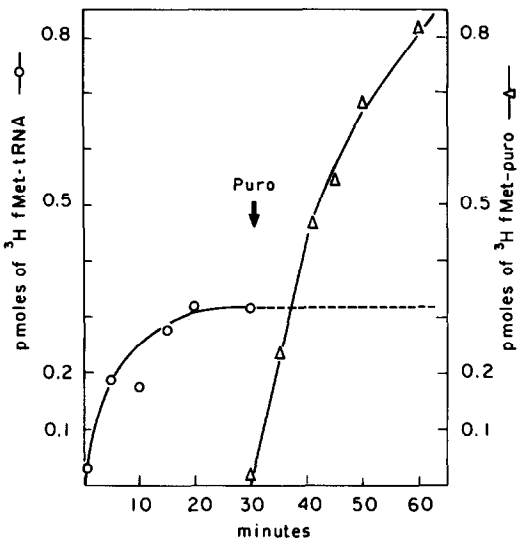

Fig.5. Kinetics of AMV-RNA directed fMet-puromycin formation. The conditions were the same as in table 2 except that the volume of the mixture was 650 μl and the amount of AMV-RNA added was 26 μg. 50 μl were sampled at intervals, and the amount of fMet-puromycin formed was determined after extraction with ethylacetate. For binding and transfer to puromycin, background without messenger was deducted.

mycin formation. On a weight basis, purified 70 S AMV-RNA is somewhat more efficient in promoting initiator tRNA binding than is crude AMV-RNA, while the 35 S form is 3–4 times more stimulatory than the 70 S form.

Table 3
Comparison of fMet-tRNA binding and the fMet-puromycin formation directed by crude AMV RNA, purified 70 S AMV RNA or 35 S AMV RNA

	Crude	70 S	35 S
Binding	0.110	0.170	0.620
fMet-puro.	0.063	0.068	not tested

The conditions were the same as in table 2. For binding experiments, data are calculated from kinetic experiments at the plateau with limiting amounts of RNA and expressed in pmole of fMet bound to the initiator complex directed by μg of RNA. For fMet-puromycin, results are expressed in pmole of fMet-puromycin formed per minute in the presence of 2 μg of messenger (initial rate).

A possible explanation for this fact could be that the combined effects of formamide and heat treatment would expose initiation triplets and regions that are masked within the 70 S secondary structure.

3.4. *Binding experiments with other eukaryotic messengers*

The capacity of directing fMet-tRNA binding to *E. coli* ribosomes by a eukaryotic messenger does not appear to be restricted to AMV-RNA. Cytoplasmic poly A-containing RNA from AMV-infected chicken myeloblasts or from foetal calf muscles also strongly stimulate binding as evidenced by the nitrocellulose filtration assay described in fig.6.

4. Discussion

The present study indicates that a messenger of eukaryotic origin, AMV-RNA, is correctly recognized by the elements of the *E. coli* initiation machinery.

The arguments supporting this view are:

(i) That AMV-RNA strongly stimulates fMet-tRNA binding to *E. coli* 70 S ribosomes in a reaction which

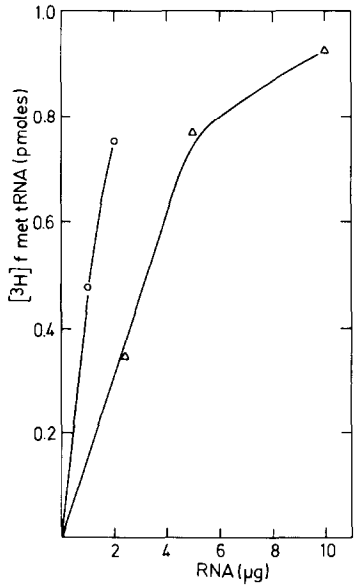

Fig.6. Binding of fMet-tRNA to the ribosomes in the presence of poly A-containing RNA from chick myeloblasts and from calf muscle. (○—○) Calf myoblast RNA. (△—△) Chick myeloblast RNA.

is fully dependent upon *E. coli* initiation factors plus GTP.

(ii) fMet-tRNA thus bound can react with puromycin, suggesting that tRNA is positioned at the 'P' site. Moreover, the amount of fMet-puromycin thus formed indicates that the initiator tRNA can readily recycle from the complex.

That more IF is required to reach maximum binding at limiting messenger concentration with AMV- than with T_4-RNA may however suggest a weaker affinity of the bacterial factors for the eukaryotic template. This observation deserves further investigation with purified initiation factors in order to specify which factors would be limiting in the recognition of the heterologous tRNA. Both purified 70 S and 35 S chains are active in catalyzing initiation complex formation. From the experiment of fig.1 in which a strict proportionality is obtained between the amount of fMet-tRNA bound and the amount of AMV-RNA added, one can calculate that 1 molecule of fMet-tRNA is attached per 70 S RNA chain present in the assay.

However, it is not yet known whether each RNA molecule is involved in initiation complex formation or whether a proportion of the molecules bind more than one fMet-tRNA. The 35 S species obtained by denaturation of the 70 S RNA could accomodate 2–3 additional fMet-tRNA molecules per chain. Although we have not yet examined whether the 35 S–ribosome complexes thus formed are all equivalent in their capacity to react with puromycin, this latter finding can be accounted for by the unmasking of initiation regions that would be inaccessible to *E. coli* ribosomes due to the 70 S RNA secondary structure. It would be of particular interest to compare the stoichiometry of 70 S and 35 S directed fMet-tRNA binding in the presence of eukaryotic factors and 80 S ribosomes with that observed here, and to examine whether the initiation regions involved are the same.

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