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The Attachment of Polyuridylic Acid to Reticulocyte Ribosomes

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The attachment of polyuridylic acid to reticulocyte ribosomes was studied by using polyadenylic acid, which inhibits the attachment reaction only, while permitting translation of polyuridylic acid bound to ribosomes. After addition of polyadenylic acid the amount of polyphenylalanine synthesized under standard conditions was taken as a measure of the bound polyuridylic acid. In this way certain parameters of the attachment reaction and the subsequent translation of attached polyuridylic acid were defined: (1) polyuridylic acid–ribosome interaction at 37° requires only Mg^{2+} at an optimum concentration of 8mM; (2) K^+ (required for translation) is a non-competitive inhibitor of the attachment reaction; (3) optimum polyphenylalanine synthesis directed by attached polyuridylic acid occurs at 5mM-Mg^{2+} concentration; (4) from kinetic studies single ribosomes appear to participate in the attachment reaction.

PolyU* directs the synthesis of polyphenylalanine by reticulocyte ribosomes (Arnstein, Cox & Hunt, 1962; Arlinghaus & Schweet, 1962). The cell-free system, which was described in detail by Williamson, Hausmann, Heintz & Schweet (1967), showed similar requirements to the system for the synthesis of haemoglobin by reticulocyte ribosomes except that the optimum Mg^{2+} concentration was higher for polyphenylalanine synthesis. In this paper I describe an attachment reaction involving polyU and reticulocyte ribosomes and evidence is given that it is the attachment step which requires a high Mg^{2+} concentration. Subsequent to this attachment taking place the initiation and growth of the polyphenylalanine chain appear to be optimum at 5mM-Mg^{2+} concentration, which is within the optimum range for haemoglobin synthesis. To study the attachment reaction use was made of polyA, which, as shown by Williamson *et al.* (1967), inhibits only the attachment of polyU to ribosomes and not the translation of polyU already attached; thus attachment was allowed to proceed under chosen conditions, terminated by addition of polyA, and the attached polyU measured by allowing its translation to polyphenylalanine under standard conditions. This procedure allowed a kinetic study of the attachment reaction from which the following points have emerged: (1) the only requirement for the attachment of polyU to ribosomes at 37° is Mg^{2+} at an optimum concentration of 8mM; (2) under these conditions ribosomes appear to participate singly in the attachment reaction; (3) K^+ is a non-competitive inhibitor of this attachment reaction.

*Abbreviations: polyU, polyuridylic acid; polyA, polyadenylic acid; t-RNA, transfer RNA; m-RNA, messenger RNA.

MATERIALS AND METHODS

Materials. Glutathione and nucleoside triphosphates were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Creatine phosphate and creatine kinase were products of C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. [U-^{14}C]Phenylalanine and [U-^{14}C]valine were purchased from The Radiochemical Centre, Amersham, Bucks. PolyU and polyA were supplied by Miles Laboratories Inc., Elkhart, Ind., U.S.A.

Assay procedures for attachment of ribosomes to polyU and for polyphenylalanine synthesis. PolyU-directed polyphenylalanine synthesis was measured as described by Williamson *et al.* (1967); the reaction mixture was scaled down to a final volume of 0·5ml., except where stated.

The attachment reaction was studied in a mixture containing ribosomes and polyU at the concentrations indicated, together with any, or none, of the other components of the complete system for polyphenylalanine synthesis. After incubation the attachment reaction was terminated by chilling and addition of polyA [1·2–1·4-fold (w/w) excess relative to polyU present]. The mixture was then supplemented to contain all of the constituents of the complete system for polyphenylalanine synthesis and incubated for 120min. at 37°, except where stated. Endogenous incorporation of [^{14}C]phenylalanine by ribosomes unattached to polyU was measured after addition of polyA at zero time; this background (see Table 1, line 2) was

determined in every experiment and subtracted from all results to obtain a measure of attached polyU.

Derived ribosomes, prepared as described by Williamson *et al.* (1967) by incubation in the complete cell-free system of Allen & Schweet (1962), consist almost entirely of 80s monomeric ribosomes and have a low endogenous incorporation. Samples were prepared for counting by precipitation with 5% (w/v) trichloroacetic acid and filtration on Oxoid membranes (Williamson & Askonas, 1967).

RESULTS

Attachment assay. It has been shown that addition of polyA to the polyU-directed cell-free system blocks only attachment of polyU to ribosomes and does not impair the reading of polyU already attached to ribosomes (Williamson *et al.* 1967). This is illustrated by the time-course of polyphenylalanine synthesis when polyA is added to the complete system after a 5min. incubation; the rate of incorporation is slightly enhanced compared with a control without polyA, but in the presence of polyA polyphenylalanine synthesis is completed much sooner and there is less total incorporation (Fig. 1). The plateau of incorporation in the presence of polyA can be taken as a measure of the amount of polyU that becomes bound to the ribosomes during the 5min. incubation before the addition of polyA.

To establish that the attachment of ribosomes to polyU during preincubation was the limiting step for subsequent polyphenylalanine synthesis, some of the parameters were examined. The results are shown in Table 1. The incorporation observed after addition of polyA at zero time (line 2) represents the background endogenous incorporation by derived ribosomes that must be subtracted from each result to estimate the polyU-stimulated incorporation. The attachment reaction is strongly dependent on the temperature of preincubation, very little attachment occurring during 10min. at 0° (line 3) relative to 10min. at 37° (line 1). If polyU is omitted during preincubation, and added after polyA, only background incorporation occurs during the subsequent incubation (line 4). The presence of Mg^{2+} during preincubation appears to be essential for ribosome–polyU interaction (line 5); indeed, incubation of ribosomes in the absence of Mg^{2+} is deleterious, as judged by the decrease in background endogenous incorporation.

Varying the time of preincubation before addition of polyA and measurement of the subsequent plateaux of incorporation gives us an apparent time-course for the attachment of polyU to ribosomes (Fig. 2). However, in this initial experiment, both the attachment reaction and subsequent translation of bound polyU are occurring during preincubation in the complete system. Consequently the absolute values of polyU attachment

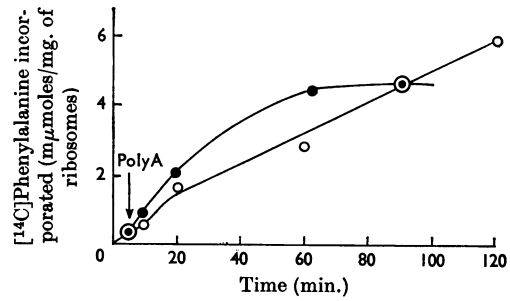

Fig. 1. Time-course of polyU-directed polyphenylalanine synthesis in the presence and absence of polyA. The complete system for polyphenylalanine synthesis (see the Materials and Methods section) was used with 120μg. of polyU and 1·0mg. of derived ribosomes in a final volume of 1·4ml. ●, PolyA (150μg.) added after 5min.; ○, control.

Table 1. *Requirements for attachment of ribosomes to polyU*

The complete preincubation mixture consisted of the complete system for polyphenylalanine synthesis (see the text), with 2mg. of derived ribosomes and 120μg. of polyU per tube. After preincubation, polyA (150μg.) was added and also any components not present so that each tube contained the complete system in a final volume of 1·4ml. Incorporation was measured after 120min. at 37°.

Attachment preincubation conditions	Extent of attachment of polyU (mμmoles of [14C]phenylalanine incorporated/mg. of ribosomes)
Complete system, 10min., 37°	3·1
Complete system, 0min., 0°	0·2
Complete system, 10min., 0°	0·3
PolyU omitted, 10min., 37°	0·2
Mg^{2+} omitted, 10min., 37°	0·03

may be too high and the time-course shown in Fig. 2 may not truly reflect the attachment reaction. The components of this assay mixture were therefore varied to determine the minimal requirements for optimum attachment of polyU to ribosomes. The time-course for the attachment reaction was measured under various conditions of incubation of ribosomes with polyU (Fig. 3). Omission of all components except K^+, tris–hydrochloric acid buffer and Mg^{2+} has little effect on the initial rate of attachment, showing that only ribosomes and polyU are involved in this reaction and that phenylalanyl-t-RNA is not required. When the univalent cations K^+ and tris are omitted the initial rate of attachment is enhanced. The initial attachment of ribosomes to polyU at 37° was maximal in the presence of Mg^{2+} alone. This raises two ques-

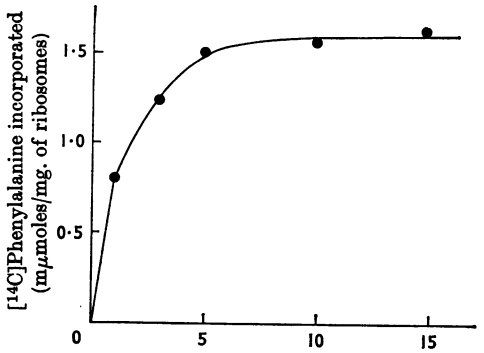

Fig. 2. Apparent time-course of attachment of polyU to ribosomes in the complete polyphenylalanine-synthesizing system. The bulk preincubation contained the complete system including [^{14}C]phenylalanine; the concentration of ribosomes was 1·0 mg./ml. and that of polyU was 240 μg./ml. Samples (0·42 ml.) were removed at zero time and at various times during preincubation at 37°, and polyA (160 μg.) was added, giving a final volume of 0·5 ml. Incubation was continued in the presence of polyA at 37° for 120 min. The amount of polyphenylalanine synthesis was then measured and plotted against the time of preincubation indicated on the abscissa. Correction was made for the endogenous [^{14}C]phenylalanine incorporation of derived ribosomes by subtraction from each point of the radioactivity incorporated after addition of polyA at zero time.

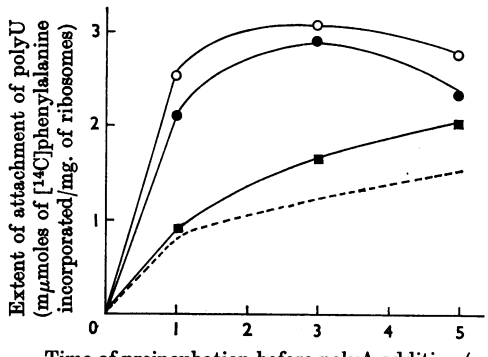

Fig. 3. Time-course of attachment of polyU to ribosomes in the absence of components required for translation. The time-courses were obtained as described for Fig. 2 except that polyU and ribosomes were preincubated in the presence of only the indicated components; the complete polyphenylalanine-synthesizing system was reconstituted at the time of addition of polyA. Preincubation conditions: ■, 67 mM-KCl, 33 mM-tris–HCl, pH 7·5, 6·7 mM-$MgCl_2$; ○ and ●, 8 mM-$MgCl_2$. Incubation conditions for translation of attached polyU: ■ and ●, usual complete synthesizing system with Mg^{2+} concentration 8 mM; ○, complete system with Mg^{2+} concentration 5 mM. For comparison the initial part of the apparent time-course of attachment in the complete system (Fig. 2) is illustrated by the broken line. Attachment was measured as described for Fig. 2.

tions: (1) what is the optimum Mg^{2+} concentration for the attachment reaction?; (2) after the attachment reaction has taken place what is the optimum Mg^{2+} concentration for polyphenylalanine synthesis?

These two questions are interrelated since the assay used here for measurement of the attachment reaction involves translation. A preliminary experiment showed that the optimum Mg^{2+} concentration for polyphenylalanine synthesis by ribosomes that are already attached to polyU was less than the normal optimum of 7 mM-Mg^{2+} for the complete polyU-stimulated system in which no preliminary attachment reaction has taken place. The influence of the Mg^{2+} concentration on the translation reaction in the resulting apparent time-course of attachment is shown in Fig. 3. Measurement of attached polyU by translation at 5 mM-Mg^{2+} gives somewhat higher values than measurement at 8 mM-Mg^{2+} (the concentration of Mg^{2+} in the attachment reaction). The lower Mg^{2+} concentration was therefore used in the assay of attached polyU.

Optimum Mg^{2+} concentration for attachment of polyU to ribosomes. The extent of attachment of polyU to ribosomes was measured as a function of Mg^{2+} concentration at 37° (Fig. 4). PolyU (120 μg.)

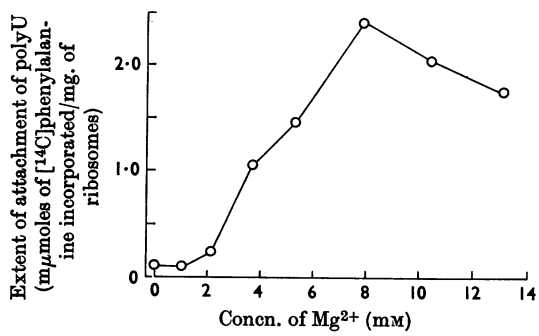

Fig. 4. Extent of attachment of polyU to ribosomes as a function of Mg^{2+} concentration. The attachment reaction mixture contained ribosomes (590 μg.) and polyU (120 μg.) at various concentrations of $MgCl_2$ in a final volume of 0·19 ml.; preincubation was for 5 min. at 37°. The reaction mixture was then chilled, polyA (160 μg.) and the complete-system components were added, and the concentration of $MgCl_2$ was adjusted to 5 mM in each case (final volume 0·5 ml.). Incubation was then continued for 90 min. at 37°. The extent of attachment during preincubation was measured as described for Fig. 2.

and ribosomes (590 μg.) were incubated at different Mg^{2+} concentrations in a volume of 0·19 ml. for 5 min. at 37°. After chilling, polyA (160 μg.) and the components of the complete incorporation

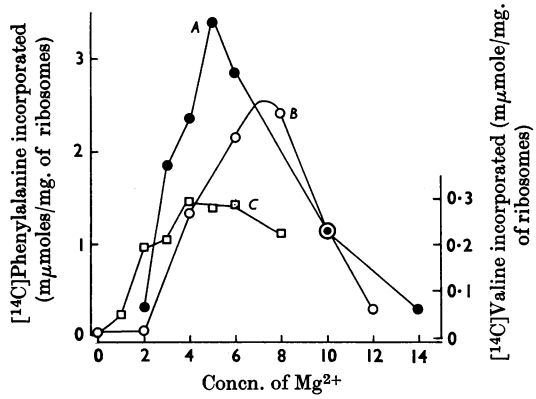

Fig. 5. Effect of Mg^{2+} concentration on translation. Curve *A* (●), polyphenylalanine synthesis directed by polyU already attached to ribosomes; curve *B* (○), polyphenylalanine synthesis directed by polyU without prior attachment reaction; curve *C* (□), haemoglobin synthesis directed by endogenous m-RNA. For curve *A*, attachment of polyU (1·2mg.) to ribosomes (5·0mg.) was carried out at 8mM-$MgCl_2$ in final volume 1·2ml. for 5min. at 37°. After chilling, polyA (1·6mg.) was added (final volume 2·0ml.). Samples (0·2ml.) were supplemented with complete-system components at various final $MgCl_2$ concentrations (final volume 0·5ml.) and incubation was continued for 60min. at 37°. For curve *B*, the complete polyU-stimulated system was as described in the Materials and Methods section with incubation for 90min. at 37°. For curve *C*, the complete cell-free system of Allen & Schweet (1962) was used. The final volume of the reaction mixture was 0·5ml.; incorporation of [14C]valine was measured after incubation for 60min. at 37°.

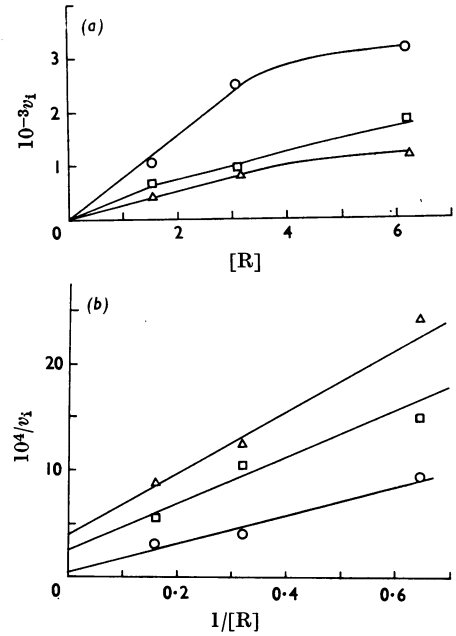

Fig. 6. Initial velocity, v_1, of attachment of polyU to ribosomes as a function of ribosome concentration, [R] and K^+ concentration. (*a*) v_1 plotted as a function of [R]; (*b*) $1/v_1$ plotted as function of 1/[R]. The time-course of the attachment reaction under various ionic conditions and at various concentrations of ribosomes was measured by using the procedure described for Fig. 4 with the addition of polyA at 0, 1, 3 and 5min. during preincubation. Ionic conditions of attachment were: ○, 8mM-$MgCl_2$, no KCl; □, 8mM-$MgCl_2$, 52·5mM-KCl; △, 8mM-$MgCl_2$, 105mM-KCl. The initial slope of the time-course of attachment was taken as a measure of v_1 (units: counts/min. per min.) and is plotted against the concentration of ribosomes, [R], in the attachment reaction (units: mg./ml.).

system were then added, the Mg^{2+} concentration was adjusted to 5mM and the final volume made up to 0·5ml. The incorporation of [14C]phenylalanine after 90min. incubation was measured in the usual way and plotted as a measure of the extent of attachment. The optimum Mg^{2+} concentration for the attachment reaction is 8mM (Fig. 4); very little attachment occurs below 2mM-Mg^{2+}, attachment being proportional to Mg^{2+} concentration between 2 and 8mM and being inhibited at higher Mg^{2+} concentrations.

Optimum Mg^{2+} *concentration for translation of polyU by attached ribosomes.* The attachment of polyU to ribosomes was accomplished by preincubation at 8mM-Mg^{2+} concentration as described in the preceding section and then translation was measured as a function of the Mg^{2+} concentration under normal complete-system conditions. The results illustrated in Fig. 5 (curve *A*) show that the optimum Mg^{2+} concentration for the steps involved in polyphenylalanine synthesis subsequent to polyU–ribosome interaction is 5mM. The optimum is quite sharp, with little phenylalanine incorporation below 2mM-Mg^{2+} concentration; proportionality between Mg^{2+} concentration and incorporation exists between 2 and 5mM and marked inhibition of incorporation occurs above 5mM-Mg^{2+} concentration. Also illustrated in Fig. 5 for comparison are the Mg^{2+}-dependency of polyphenylalanine synthesis in the complete cell-free system in the absence of prior attachment of ribosomes to polyU (curve *B*) and the Mg^{2+}-dependency of haemoglobin synthesis by reticulocyte ribosomes in the cell-free system (curve *C*). Haemoglobin synthesis shows a broad optimum over the range 4–6mM-Mg^{2+}, in agreement with the findings of Allen & Schweet (1962). Polyphenylalanine synthesis shows a sharp optimum at 7mM-Mg^{2+}, as noted by Williamson *et al.* (1967).

Kinetic studies on the attachment reaction. By using the assay described above to obtain the time-

course of attachment, the initial velocity of this reaction could be measured as a function of the various parameters of the system. The dependence of initial velocity of attachment on ribosome concentration is illustrated in Fig. 6(*a*); reciprocal plots of these data are shown in Fig. 6(*b*). Under optimum conditions (8mM-Mg^{2+} concentration) the dependency of initial velocity of attachment on ribosome concentration is a curve of the shape expected if monomeric ribosomes attached singly to polyU (Fig. 6*a*); this conclusion is derived from the linear double-reciprocal relationship (Fig. 6*b*), which indicates that the ribosome–polyU interaction is first-order with respect to ribosomes at constant polyU concentration. The inhibitory effect of K^+ on the attachment reaction is clearly seen by the decrease in initial velocity, but the shape of the curve relating initial velocity and ribosome concentration remains substantially unaltered (Fig. 6*a*); the reciprocal plots of these data are linear and show that K^+ is a non-competitive inhibitor of attachment (Fig. 6*b*).

DISCUSSION

The use of polyA to prevent attachment of ribosomes to polyU has allowed me to study the nature of this initial step in some detail. Inhibition of the attachment reaction is due to polyA–polyU complex-formation. Subsequent to this polyA-sensitive reaction the translation of polyU by attached ribosomes proceeds normally, even in the presence of polyA, so I have also been able to study separately the peptide-bond-forming reaction.

The initial polyA-sensitive step appears to be the formation of a ribosome–polyU complex without the participation of phenylalanyl-t-RNA. The only requirement for this temperature-dependent interaction of ribosomes and polyU is Mg^{2+} at an optimum concentration of 8mM. Translation of the attached polyU then occurs at an optimum Mg^{2+} concentration of 5mM, which approximates to the optimum for haemoglobin synthesis at a similar K^+ concentration. The increased Mg^{2+} requirement for the complete polyU-stimulated cell-free system (optimum concentration 7mM-Mg^{2+}) thus appears to be a compromise between the different optima for the attachment of ribosomes to polyU and the subsequent steps in the translation of this polyU to polyphenylalanine. The relatively sharp Mg^{2+} concentration optimum of the polyU-stimulated system can be seen to stem from inhibition of peptide-bond formation at Mg^{2+} concentrations above 7mM and from the inefficiency of attachment of ribosomes to polyU at lower Mg^{2+} concentrations.

Earlier studies in mammalian systems have suggested an initial polyU–ribosome interaction consistent with that described here. The resistance to polyA inhibition of polyphenylalanine synthesis when microsomes from Ehrlich ascites-tumour cells were 'activated' by preincubation with polyU was noted by Pedersen & Hultin (1963); in their experiments preincubation of polyU and microsomes required neither energy nor cell sap for subsequent incorporation to be refractory to polyA. Thus, although they did not define the parameters further, Pedersen & Hultin (1963) appear to have observed the same attachment reaction that is described in the present paper.

In studies with rat liver ribosomes, Revel & Hiatt (1965) have shown that high Mg^{2+} concentration (15mM) is required for an early step in polyU-directed polyphenylalanine synthesis; this step they defined as the formation of a ribosome–t-RNA–polyU complex. After the formation of this complex incorporation of phenylalanine was higher at 7·5mM-Mg^{2+}, the optimum for endogenous incorporation with liver ribosomes, than at 15mM-Mg^{2+}. These authors were probably observing the combination of two reactions, both of which require a high concentration of Mg^{2+}: first, the polyU attachment to ribosomes described in the present paper, and, secondly, the non-enzymic binding of phenylalanyl-t-RNA to the complex. With reticulocyte ribosomes an enzymic GTP-requiring binding of phenylalanyl-t-RNA has been shown to take place at a lower Mg^{2+} concentration than non-enzymic binding. The ribosome–polyU complex described in the present paper should be an intermediate in the enzymic binding reaction described by Arlinghaus, Shaeffer & Schweet (1964). It has been shown that the rate of the binding reaction is slightly but definitely enhanced by prior formation of the ribosome–polyU complex (J. Shaeffer, personal communication).

The kinetic studies described here are consistent with the idea that ribosomes attach singly to polyU. In this respect the reaction resembles the attachment of monomeric ribosomes to m-RNA contained in normal haemoglobin-synthesizing polyribosomes, as demonstrated in the cell-free system (Goodman & Rich, 1963; Hardesty, Hutton, Arlinghaus & Schweet, 1963; Williamson & Schweet, 1965). However, the latter reaction occurs only under the optimum conditions for peptide-bond formation in the complete cell-free system.

There have been numerous studies on the binding of m-RNA to bacterial ribosomes. Gilbert (1963), using an *Escherichia coli* system, showed the formation of multiple ribosome–polyU complexes at 0°, but it was not demonstrated that such structures were identical with those engaged in and formed during active protein synthesis. Dahlberg & Haselkorn (1966, 1967) have carried out an extensive study on the interaction of RNA of turnip

yellow-mosaic virus with *E. coli* ribosomes. This interaction appears to involve specific ribosome-binding sites on molecules of RNA of turnip yellow-mosaic virus, but, unlike the attachment of reticulocyte ribosomes to haemoglobin m-RNA mentioned above, the attachment of *E. coli* ribosomes to RNA of turnip yellow-mosaic virus occurs in the absence of energy, t-RNA or supernatant protein. The binding of RNA of turnip yellow-mosaic virus to *E. coli* ribosomes is stabilized by Mg^{2+} and destabilized by K^+ at high Mg^{2+} concentrations. This is consistent with the present studies.

It is noteworthy that from competition experiments with polyU and RNA of turnip yellow-mosaic virus, Dahlberg & Haselkorn (1967) concluded that these messengers compete for the same ribosomes and that the equilibrium constants for their association with ribosomes are of the same order of magnitude, suggesting that these two very different messengers interact with bacterial ribosomes in a similar way. The polyU–reticulocyte ribosome interaction described here shows a greater similarity to the attachment of m-RNA to bacterial ribosomes than to the attachment reaction involving haemoglobin m-RNA and reticulocyte ribosomes. It is not known whether a specific mechanism is operating in the latter reaction or whether only the initiation of haemoglobin chains is necessary to stabilize this attachment. One could speculate that perhaps the attachment of ribosomes to polyU at high Mg^{2+} concentration does not involve a specific reaction concerned in the attachment of reticulocyte ribosomes to natural m-RNA. The facility with which ribosome–polyU reaction occurs may explain why this synthetic polynucleotide functions so efficiently as a messenger in the cell-free system.

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