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Initiation Factor IF-3-Dependent Binding of *Escherichia coli* Ribosomes and *N*-Formylmethionine Transfer-RNA to Rabbit Globin Messenger

(puromycin/AUG codon/valyl-tRNA binding/protein synthesis/hemoglobin)

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ABSTRACT **An initiation complex has been formed in high yields from *E. coli* ribosomes, 9S messenger RNA for rabbit hemoglobin, and *N*-formylmethionine-tRNA. Initiation factor IF-3 is required for the binding and puromycin is required for the release of fMet. Valyl-tRNA fails to bind to the second codon, whereas a mixture of 15 aminoacyl-tRNAs promotes incorporation. Together with quantitative data, the findings suggest that IF-3 directs the ribosomes to an AUG codon on one of the two globin messengers, at a site that is different from the normal starting point for globin synthesis.**

The mechanism by which ribosomes recognize the AUG start signal at the beginning of a message is not understood. The two most obvious possibilities are: (*i*) the secondary structure of the messenger exposes only the starting triplet and masks internal AUG triplets, regardless of whether they are in or out of phase; or (*ii*) the ribosomes respond to an additional signal, for example a specific nucleotide sequence present only near the initial AUG triplet. The second possibility is attractive, because it could explain translational control if we assume that the signal is specific for certain classes of messengers, and if we further postulate specific protein adaptors that allow ribosomes to recognize only their cognate messengers (1–7). Favored candidates for such a role in specificity have been initiation factors required for the correct attachment of ribosomes to natural messengers.

Although partial discrimination has been reported with different IF-3 fractions and bacteriophage messengers (2–8), the basis of the specificity has not been established, and the nature of the signal is elusive (9). A more decisive answer could be expected by testing a bacterial translation system with a known messenger from the other end of evolution. The development of a purified system from *Escherichia coli* in which nearly all the ribosomes are active in initiation with R17 RNA (10) and the availability of messenger RNA for rabbit globin (11) have made such an approach feasible. Here, we describe the formation with high efficiency of an initiation complex of globin messenger with *E. coli* ribosomes, *N*-formylmethionine-tRNA, and initiation factors IF-1, IF-2, and IF-3.

MATERIALS AND METHODS

Rabbit Globin Messenger-RNA. Globin mRNA was prepared from polysomes by sodium dodecyl sulfate extraction according to Staehelin *et al.* (12), as described by Lingrel *et al.* (13, 14).

Ribosomes were prepared from S-30 extracts (15) by sedimentation through a sucrose gradient in a B-29 zonal rotor. "Native" 30S subunits were isolated by adjusting the Mg^{++} concentration in the gradient to 6 mM and pooling the fractions corresponding to the 30S peak. The particles were then centrifuged to form a pellet in an angle-head rotor, dissolved in buffer A (60 mM NH_4Cl–10 mM $MgAc_2$–20 mM Tris·HCl pH 7.5–10 mM 2-mercaptoethanol) and stored at −65°. The 50S subunits, produced by centrifugation of vacant 70S ribosomes‡ through a sucrose gradient at 1 mM Mg^{++}, were fractionated from the gradient and concentrated as above after the Mg^{++} concentration was raised to 10 mM. Vacant 70S ribosomes, free of subunits, were obtained from the S-30 extract by two zonal centrifugations (10).

Other Components. The aminoacylation of tRNA, as well as the preparation of *N*-formyl-[^{3}H]methionyl-tRNA and crude initiation factors, were described.§ R17 amB2 RNA was prepared on a large scale by a modification of the procedure of Gesteland and Spahr (16). Both the amber mutant and the CR-63 host cells were a gift from Dr. R. Gesteland. Details of this method will be published elsewhere (Muller and H. Noll, in preparation). Purified initiation factor IF-1 was prepared according to Lee–Huang *et al.* (17), IF-2 as described by Remold-O'Donnell and Thach (18), and IF-3 by the method of Dubnoff and Maitra (19). Each preparation gave only one major band upon acrylamide gel electrophoresis; all three factors were required for translation of R17 RNA.

Analytical Procedures. Isokinetic sucrose gradients (20) with a top concentration of 5% and a volume of 3.4 ml were prepared six at a time in an automatic machine (21, 22). The gradients contained 100 mM NH_4Cl–15 mM $MgAc_2$–20 mM Tris·HCl, pH 7.5. Centrifugation was for 1.3 hr at 60,000 rpm and 5° in the SB-405 rotor of an IEC B-60 ultracentrifuge. The gradients were scanned at 260 nm and processed for radioactivity measurement with the automatic equipment of the Molecular Instruments Co. (MICO, P.O. Box 1652, Evanston, Ill. 60204), in combination with a Gilford 222 spectrophotometer. The MICO equipment consists of a precision tube-

‡ Vacant ribosomes are defined as ribosomes that are not programmed for protein synthesis and contain neither mRNA nor tRNA (ref. 26 and §).

§ Noll, M. L., Hapke, B., Schreier, M. H. & Noll, H. (1972) *J. Mol. Biol.* submitted.

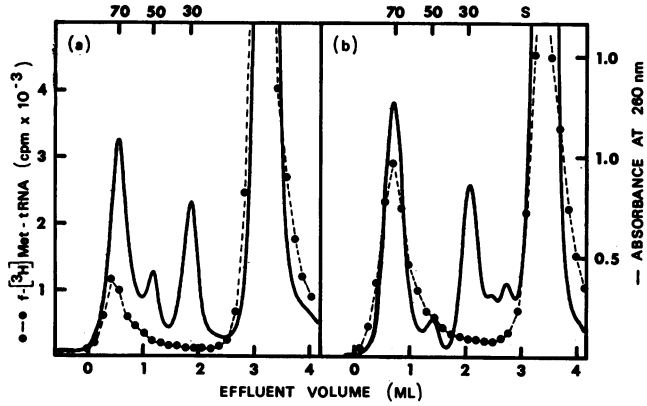

FIG. 1. Sedimentation patterns that illustrate the formation of initiation complex from 50S subunits and "native" 30S particles without initiation factors. (*a*) Standard reaction mixture + 0.13 A_{260} units of globin mRNA; (*b*) control with 0.90 A_{260} units of R17 amB2 RNA as messenger. The incubation mixtures (100 μl) contained 0.60 A_{260} units of 50S subunits; 0.65 A_{260} units of "native" 30S subunits, and the following standard components [concentrations in mM]:80,000 cpm of f[^{3}H]Met-tRNA$_f$ (87% pure from Oak Ridge, 75% formylated; 3.27 Ci/mmol); GTP [1.0]; NH_4Ac [50]; $MgAc_2$ [5]; 2-mercaptoethanol [10]; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) buffer, pH 7.6 [50]. After incubation for 20 min at 37°, the reaction mixture was layered over the gradient. The radioactivity under the 70S peak corresponds to (*a*) 4.5 pmol of fMet and (*b*) 14 pmol, after correction for "wall effect."

puncturing device, a convection-free flow cell of novel design and very low optical noise, and a programmable fraction collector (Radiograd MR-747) with a microdispensing head capable of delivering fractions as small as 50 μl without losses directly into liquid scintillation vials, with simultaneous addition of liquid scintillation fluid (23). The gradient fractions were counted with MICO Radiomix as scintillation fluid. The optical scan of the gradients at 260 nm was recorded simultaneously on a strip chart and, with the help of a frequency converter, on magnetic tape. The curves stored on magnetic tape were reproduced with a Hewlett-Packard X-Y plotter at the desired size. Quantitative measurements of absorbance peak areas were made with a Du Pont model 310 Curve Resolver. Recoveries from gradients were corrected for losses along the side wall of the tube due to the radially directed force of centrifugation. The magnitude of this correction was found to be dependent on (*i*) input, (*ii*) distance migrated in tube, and (*iii*) effective mass of particle. The corrections were made with the aid of empirical calibration curves (H.N. and M.L.N., to be published). The counting efficiency was 21% for ^{3}H.

RESULTS

In tests designed to examine specificity between ribosomes and messengers, the quality of the ribosomes is critical. It has been shown elsewhere (10)§ that the following preparations of ribosomes and factors from *E. coli* will form initiation complexes with fMet-tRNA and R17 RNA in essentially stoichiometric amounts: (*a*) pure vacant 70S ribosomes (regardless of whether or not they have been washed with 1 M NH_4Cl) in the presence of crude or purified initiation factors, (*b*) washed or unwashed active 50S subunits in the presence of active, washed 30S subunits and crude or purified initiation factors; and (*c*) active 50S subunits and unwashed "native" (10) 30S subunits, without addition of crude or purified initiation factors. Vacant 70S ribosomes are always active, but require initiation factors that they lack. A stringent test for activity of subunits is their ability to form couples: only subunits that are capable of forming strongly interacting couples during incubation at 37° are active in initiation with R17 RNA. Although the presence of initiation factors on native 30S particles prevents their interaction with 50S subunits, addition of an excess of 50S subunits will nevertheless convert them nearly quantitatively into vacant couples by displacing the equilibrium (10)

$$30S \cdot IF \rightleftharpoons 30S + IF$$

in favor of the association

$$30S + 50S \rightarrow 70S,$$

where IF indicates the presence of unspecified initiation factor(s).

Requirement of initiation factors for formation of initiation complex

We first tested native unwashed 30S subunits for formation of an initiation complex with 50S subunits, globin mRNA, and labeled fMet-tRNA, because this combination requires no crude initiation factors and, hence, is very low in RNase. The sedimentation diagram in Fig. 1*a* shows that fMet-tRNA was bound to the 70S peak. From the total radioactivity under the 70S peak, and the known specific activity of fMet-tRNA,

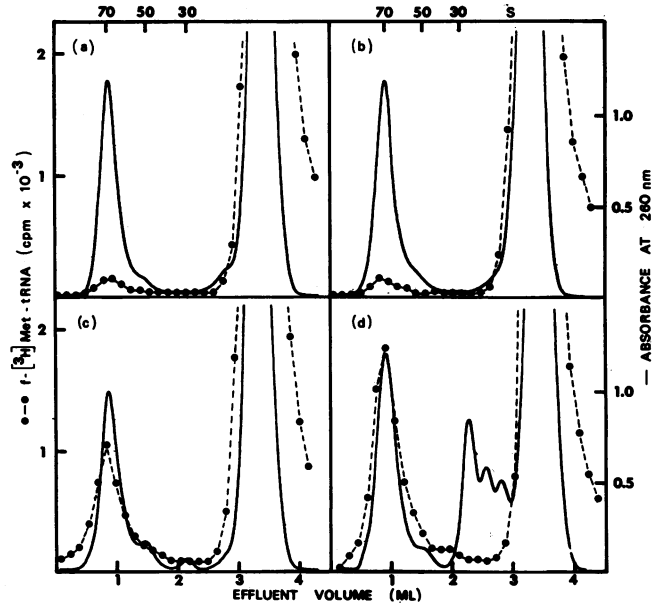

FIG. 2. Sedimentation patterns that show the effect of initiation factors on the formation of initiation complex from pure vacant 70S ribosomes. The incubation mixtures (100 μl) contained the standard components, ribosome couples (0.85 A_{260} units; globin mRNA (0.13 A_{260} units); plus the following additions: (*a*) none; (*b*) initiation factors IF-1 (0.7 μg) and IF-2 (0.3 μg); (*c*) IF-3 (1.4 μg) plus IF-1 and IF-2 [all determined by the Lowry assay with bovine serum albumin as standard]; (*d*) R17 amB2 RNA (0.9 A_{260} units). Other conditions are as in Fig. 1. The radioactivity under the 70S peak corresponds to (*a*) 0.60 pmol; (*b*) 0.60 pmol; (*c*) 4.4 pmol; (*d*) 7.4 pmol, after correction for "wall effect."

we calculate that the amount of fMet-tRNA bound corresponds to 4.5 pmol, or about 29% of the vacant ribosome couples present. By comparison, the yield of initiation complex with R17 RNA under the same conditions (14 pmol of fMet bound) is about thrice as large; about 65% of the 50S particles (or 85% of the active 50S particles) added appear in the 70S initiation complex (Fig. 1*b*).

If vacant couples free of subunits are used, the formation of the initiation complex depends strongly on the addition of either crude or purified initiation factors. Addition of IF-1 and IF-2 without IF-3 produces no stimulation above the small amount of complex formation observed in the absence of factors (compare Fig. 2*a* and *b*). Addition of IF-3 with both IF-1 and IF-2, however, causes a dramatic stimulation (Fig. 2*c*). Because of the limited amount of globin mRNA available, we have not been able to test IF-3 alone, or in combination with either IF-1 or IF-2.

Yield of initiation complex as a function of mRNA input

The yield of initiation complex depends both on the globin mRNA input and the level of RNase present in the system. When crude initiation factors were used instead of purified factors, about 40% less fMet-tRNA was bound (not shown), apparently because the RNase present in crude factors (10) degrades a significant fraction of the mRNA before it has formed an initiation complex. This result suggested that under the conditions of these experiments the mRNA was limiting. Indeed, the binding of fMet-tRNA was proportional to the mRNA input in this concentration range, and only at higher input was there an indication of saturation (Figs. 3 and 4). From the slope of the linear portion of the dose–response curve in Fig. 4, it may be calculated that about 18% of the input mRNA has been bound. The fraction of ribosomes bound near the saturation level, on the other hand, reaches

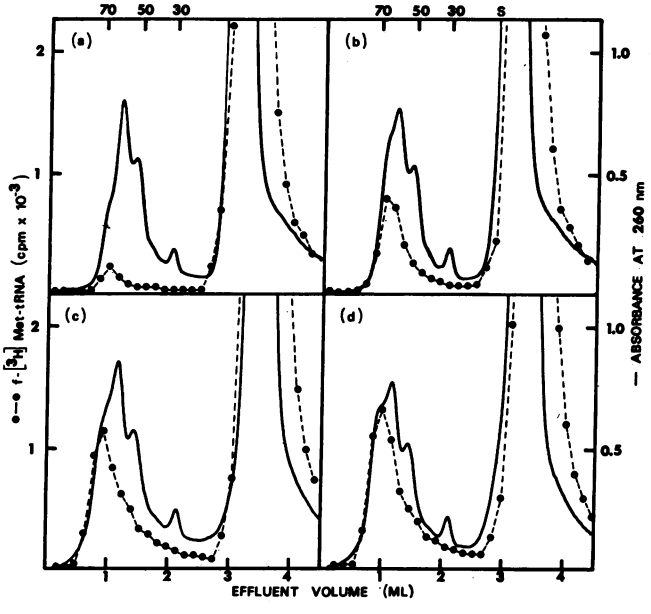

FIG. 3. Sedimentation patterns showing the effect of mRNA concentration on yield of initiation complex. The incubation mixtures contained the same components as indicated in Fig. 2*c*, except that the ribosome input was 0.68 A_{260} units and mRNA input was zero (*a*), 0.068 (*b*), 0.13 (*c*), and 0.27 A_{260} units (*d*). Mg^{++} concentration in the gradients was 6 mM.

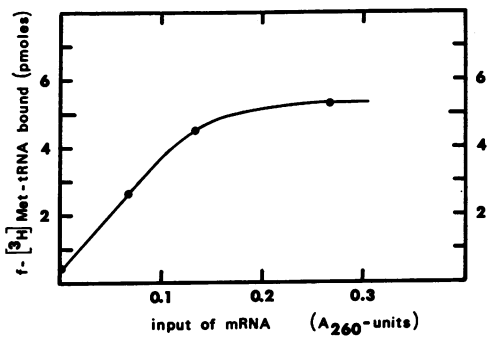

FIG. 4. Plot of f[^{3}H]Met-tRNA bound to 70S initiation complex as a function of rabbit globin mRNA input. The values have been taken from Fig. 3 and corrected for the "wall effect."

about 30%. Under similar conditions, R17 RNA added in a molar ratio of two per ribosome will bind as many as 90% of the ribosomes used in this preparation.

As in similar experiments with poly(U) and R17 RNA, the formation of the initiation complex leads to a strengthening of the interaction between subunits. This effect manifests itself in an increased resistance to dissociation by hydrostatic pressure at high centrifugal forces. Thus, the sedimentation patterns obtained at 6 mM Mg^{++} in the gradient clearly show that the binding of radioactive fMet-tRNA in response to increased concentrations of mRNA is associated with a parallel growth of the 260-nm absorbance peak sedimenting at 70S. This increase takes place at the expense of the 60S and 50S peaks, which correspond to the subunits of pressure-dissociated 70S couples§. The differential sedimentation behavior of complexed ribosomes and vacant ribosome couples is abolished if the vacant couples are stabilized by elevation of the Mg^{++} concentration in the gradient from 6 to 15 mM (see Fig. 1) or by diminution of the velocity of centrifugation.

Release of *N*-formylmethionine by puromycin

A characteristic property of IF-3-dependent chain initiation is the binding of fMet-tRNA to the P-site in the 70S complex, and the release of *N*-formylmethionine by reaction with puromycin. The results in Fig. 5 show that incubation with puromycin completely released fMet radioactivity from the 70S peak. We conclude that the complex formed with globin-mRNA is not an unspecific artefact, but the result of specific interactions associated with AUG-dependent chain initiation.

Involvement of 9S messenger

In order to ascertain that the stimulatory activity was associated with homogeneous 9S messenger, rather than with some impurity rich in AUG or GUG triplets, the messenger preparation was examined with respect to sedimentation behavior and biological activity. The sedimentation pattern in Fig. 6*a* shows that over 80% of the material absorbing at 260 nm sedimented as a sharp peak, with a rate corresponding to 9.0 S. When a narrow cut (Fig. 6*a*) containing the 9S peak was collected and tested in the initiation reaction, it was found to contain all the activity (Fig. 6*b*), i.e., the specific activity of the 9S material collected from the gradient had increased by 15%, in proportion to the removal of low molecular weight, UV-absorbing material at the top of the gradient (36 against 31 pmol of fMet bound per $A_{260\,nm}$). In view of the high resolution of our sedimentation analysis (we would detect major con-

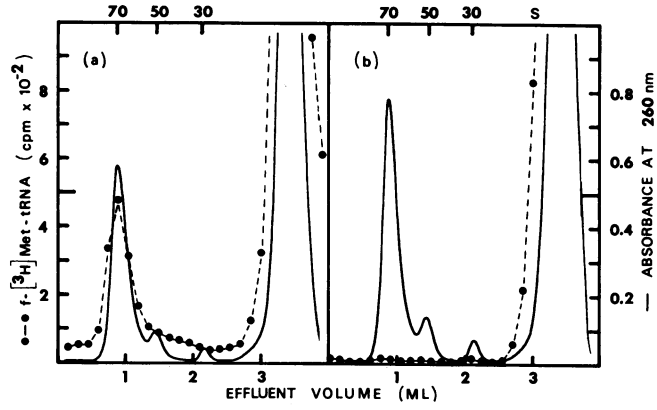

FIG. 5. Sedimentation patterns showing release by puromycin of fMet-tRNA bound to an *E. coli* ribosome–globin-mRNA initiation complex. (*a*) The incubation mixture (100 μl) was identical to that shown in Fig. 3*c;* after incubation for 20 min at 37°, a 45-μl sample was withdrawn, diluted with 50 μl of incubation buffer, and analyzed on the gradient. (*b*) To the remaining portion, 5 μl of 0.01 M puromycin was added, and the incubation was continued for an additional 5 min. The sample was then treated for gradient analysis.

taminants with S values differing from 9.0 S by at least ±0.55 S) and the apparent homogeneity of the 9S peak, we consider it unlikely that the agent responsible for the binding of fMet-tRNA is not 9S globin mRNA.

Attachment to a site different from the globin start signal

Since IF-3 is thought to recognize a specific start signal, we did experiments to determine whether the attachment of the ribosomes occurred at the start of the two globin chains or at some other site. As both α and β chains of globin start with N-terminal valine, recognition of the normal initiation site should, in the next step, lead to the binding of valyl-tRNA, in amounts equimolar to that of fMet. Using either [^{3}H]- or [^{14}C]valyl-tRNA, we were unable to detect any binding (Fig. 7*a*). In each case, the identity of the isotopic label with valine was verified with the aid of an amino-acid analyzer. On the other hand, if the initiation complex was incubated with elongation factors EF-T and EF-G and a mixture of tRNA charged with 15 amino acids, substantial incorporation was observed (Fig. 7*b*). These results imply that IF-3 directs the ribosome to a site that is different from the N-terminal start of the globin chains.

Failure of *N*-acetylvalyl-tRNA to promote initiation

The synthesis of rabbit globin in a crude cell-free extract from *E. coli* has been reported. *N*-acetylvalyl-tRNA was suggested to be the chain initiator (24). We were unable to obtain any binding of ribosomes to our mRNA preparation with *N*-acetylvalyl-tRNA under conditions that promoted binding of fMet-tRNA with high efficiency (compare Fig. 7*a* and *c*). This negative result cannot be attributed to a failure of the acetylation procedure, because the efficiency of acetylation was verified by the complete extraction of the radiaoctive product into ethyl acetate.

DISCUSSION

The most important finding of this study is the demonstration that globin mRNA forms an intiation complex with *E. coli* ribosomes and initiation factors at high efficiency. Under the most favorable conditions tested, at least 25% of the added 9S RNA forms a complex with ribosomes and fMet-tRNA. If we assume that α- and β-chain messengers are present in equimolar amounts, and that binding occurs only to one mRNA species, the efficiency would correspond to 50%. This extent is comparable to the efficiency observed with R17 RNA, if we take into account that globin mRNA should be more vulnerable to RNase in proportion to its 5-fold smaller size.

The strong dependence of the formation of this complex on the addition of initiation factor IF-3 was rather surprising, and immediately raised the question of whether initiation took place at the correct starting point. We have tried without success to bind valyl-tRNA to the next position. Binding and incorporation did occur, however, with a mixture of tRNA charged with 15 labeled amino acids. The results make it unlikely that the failure to bind valine is due to the absence in the bacterial tRNA preparation used of that particular valyl-tRNA species that corresponds to the N-terminal triplet of the mammalian messenger. Rather, the results suggest that IF-3 directs the ribosome to an internal AUG triplet. The absence of dimers or larger oligomers and the high efficiency with which the complex is formed at limiting mRNA concentrations argue against multiple attachment sites, and imply that the ribosomes are bound to a single preferred site. The nucleotide sequence of this site is of considerable interest, as it should give a clue to the nature of the signal recognized by IF-3.

The potential of the method for sequencing polypeptides and messenger RNAs by synchronized stepwise translation of a messenger, both in phase and out of phase, is obvious.

We have no direct proof that the messenger involved in the initiation complex studied here is coding for one of the globin chains. However, this conclusion is strongly supported by the homogenous 9S sedimentation rate and high specific activity of the active fraction, as well as by the fact that no other major products are detected in translation experiments with our preparation with mammalian ribosomes (11).

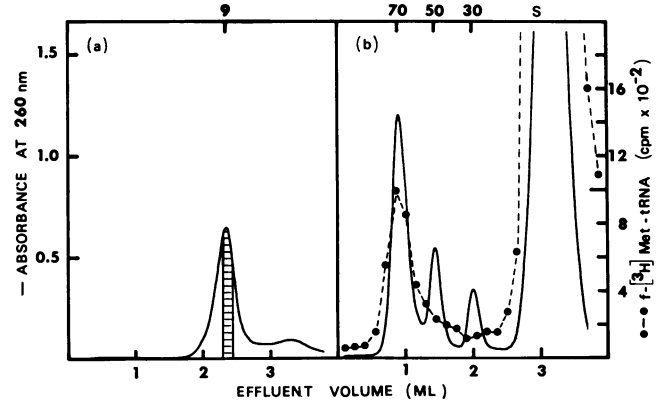

FIG. 6. Association of messenger activity with a 9S component. Rabbit globin mRNA (0.27 A_{260} units) dissolved in 0.1 ml of buffer (50 mM HEPES, pH 7.6–50 mM NH_4Ac) was layered over a standard isokinetic sucrose gradient and centrifuged for 3 hr at 60,000 rpm and 5° (*a*). 0.15 ml, corresponding to the *shaded area* in the sedimentation diagram, was incubated in a total volume of 0.20 ml with the standard components for initiation, as described in Fig. 2*c*. 0.175 ml of the reaction mixture was analyzed on a sucrose gradient (*b*).

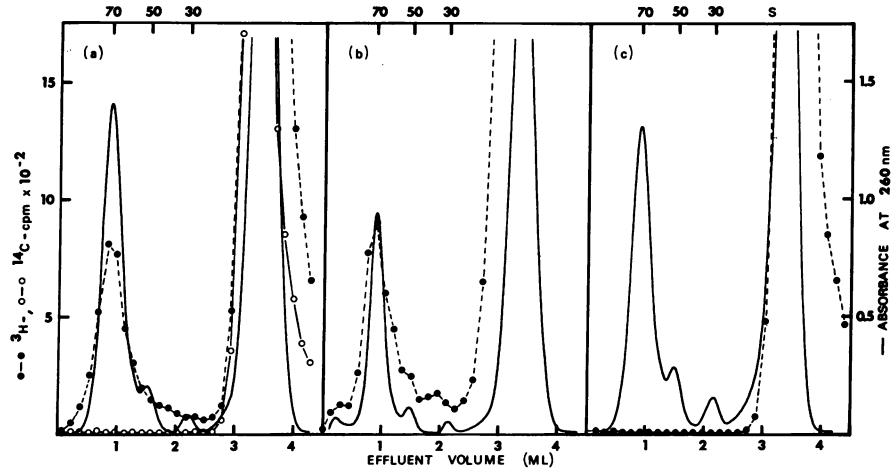

FIG. 7. Failure of valine and *N*-acetylvaline to bind to initiation complex. Standard initiation mixtures (*a* and *c*) were allowed to form an initiation complex as described in the legend to Fig. 2*c*. At the end of 20 min, incubation was continued for 5 min in the presence of elongation factor EF-T and of 0.85 A_{260} units of [^{14}C]valyl-tRNA (210 Ci/mol) containing 17,000 cpm at 62% efficiency (*a*). In (*b*), the first incubation was for 15 min, and the second incubation was for another 15 min in the presence of elongation factors EF-T and EF-G and of tRNA charged with a mixture of 15 [^{3}H]aminoacids (New England Nuclear). The input of tRNA was 0.85 A_{260}, containing 125,000 cpm. In (*c*), the incubation was for 20 min in the presence of *N*-acetyl-[^{3}H]Val-tRNA (0.95 A_{260} units, containing 19,000 cpm) instead of the normal fMet-tRNA as initiator. In the second incubation period, the concentration of Mg was raised to 7.5 mM in order to stabilize the complex for the binding reaction (*a*), and to 9.5 mM to minimize dissociation during the first translocation (*b*) (see ref. 25). (*b*) contained half the volume and amounts, except for GTP (100 nmol) and ribosomes (0.52 A_{260} units). fMet-tRNA was not labeled. The reaction mixtures were analyzed on sucrose gradients for absorbance and radioactivity.

Nevertheless, since ribosomal RNA is the major potential source of contamination, the possibility cannot be ruled out that the effects observed in the *E. coli* system are due to 9S fragments of ribosomal RNA. Such fragments might not be able to form initiation complexes with 80S ribosomes, and may thus escape detection in the mammalian extract. Whatever the exact nature of the RNA, the formation of the complex described here is of interest because there are no precedents in a well defined system for fMet-specific, initiation factor-dependent, binding of bacterial ribosomes to a mammalian RNA. Therefore, if the binding should have been to another RNA species or fragment rather than to globin messenger, the significance of our results with respect to the mechanism of action of IF-3 would not be affected, although such a situation would make it much more difficult to elucidate the site and signal recognized by the factor.

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