PRECISION OF INITIATION OF GLOBIN SYNTHESIS IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM

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Summary

The strictness of the requirement for Met-tRNA$_F$ (the natural initiator tRNA) in the initiation of protein synthesis in eukaryotic cells has been examined by determining if N-blocked aminoacyl-tRNAs can force false initiation to occur when added to a cell-free protein synthesizing system.

N-acetyl-^{14}C-valyl-tRNA prepared by acetylation of rabbit reticulocyte valyl-tRNA was added to a cell-free system without perturbing its high efficiency in synthesizing hemoglobin and at a three-fold excess over the level of endogenous Met-tRNA$_F$. After one hour of incubation at 37°, less than 0.2% of the added N-acetyl-valine had been transferred into N-terminal sites of completed globin chains. N-acetyl-^{14}C-valyl-tRNA, nearly equal in amount to that of endogenous Met-tRNA$_F$, could be recovered intact at the end of the incubation period. Similar results were obtained with N-acetylated aminoacyl-tRNA of leucine, serine, histidine and phenylalanine.

In explaining these results mechanisms involving inactivation of the N-acetyl-aminoacyl-tRNA during acetylation or during the incorporation experiment were considered and rejected in favor of one whereby the protein synthesizing apparatus is able to discriminate to a high degree against N-acetylated aminoacyl-tRNA.

Introduction

The mechanism of initiation of protein synthesis in eukaryotic cells is not as well understood as that in bacteria[1]. The cytoplasm of eukaryotic cells contains at least two species of methionyl-tRNA but it has no transformylase of the type found in bacteria. One of these tRNAs can be converted to fMet-tRNA by the transformylase enzyme of *E. coli*. In a cell-free system (prepared from mouse ascites tumor cells) in which

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the peptide bond formation is directed by synthetic polynucleotides, this species of methionyl-tRNA (Met-$tRNA_F$) transfers methionine into *N*-terminal positions of peptides whereas the other species (Met-$tRNA_M$) transfers methionine residues into internal positions[2].

In eukaryotic cells very few of the completed proteins have methionine at the *N*-terminus whereas 40% of bacterial proteins have methionine as the *N*-terminal amino acid[3]. This suggests that a very efficient mechanism exists in eukaryotes for removing the *N*-terminal methionine prior to release of the completed chains from the ribosomes. This has been verified in the case of globin synthesis in the rabbit reticulocyte[4].

Methionine has also been reported to be the *N*-terminal residue in nascent chains of proteins isolated from other eukaryotic cells, such as proteins in *Neurospora crassa*[5], protamine in trout testis[6] and isocytochrome C in yeast[7]. Nevertheless, it is far from certain that all proteins are initiated with methionine. For one thing the role of *N*-acetyl-aminoacyl-tRNAs in eukaryotic cells has yet to be excluded. For example, *N*-acetyl-methionine has been found at the *N*-terminal site of α-crystallin[8] and of proteins from the thorax of the honey bee (*Apris mellifica*)[9]; nascent chains of f2a histones synthesized in regenerating rat liver contain the *N*-terminal *N*-acetyl-serine residue[10]; and *N*-acetyl-glycine has been also suggested as a possible initiator because of its presence at the *N*-terminus of nascent peptides from hen oviduct minces[11].

The strictness of the requirement for Met-$tRNA_F$ in the initiation of hemoglobin synthesis has been examined in this paper by determining if *N*-blocked aminoacyl-tRNAs, such as valyl-tRNA, can force initiation in hemoglobin chains to occur at valine sites rather than at the natural methionine site, when added under conditions which did not perturb normal hemoglobin syuthesis. Preparations of biologically active *N*-modified aminoacyl-tRNA species and a cell-free system capable of initiating many rounds of polypeptide synthesis are also described.

Methods and materials

Preparation of aminoacyl-tRNA

tRNA was extracted from rabbit reticulocyte lysates by a phenol procedure and partially purified on a BD-cellulose column as reported in detail elsewhere[12]. The tRNA was further purified by passage over a DEAE-cellulose (Whatman DE-32) column previously washed with a buffer solution of 10 mM sodium acetate at pH 5.4, containing 0.2 M NaCl and 10 mM $MgCl_2$. tRNA was eluted with a buffer solution of 10 mM sodium acetate at pH 5.4, containing 2.0 M NaCl and 10 mM $MgCl_2$, and precipitated with 2.5 volumes of ethanol overnight at 4°, redissolved in water and stored at –20°.

Crude aminoacyl-tRNA synthetase preparations were obtained from fresh rabbit reticulocyte lysates (from which ribosomes had been removed by sedimentation) and stored in the liquid state (in 50% (v/v)glycerol) at –20°[13].

Enzymatic aminoacylation of tRNA was conducted in 0.1 M glycyl-glycine at pH 7.0 containing 20 mM $MgCl_2$, 0.5–5.0 mM ATP, 15 μM amino acid of appropriate specific activity and 0.25–2.0 A_{280} units of synthetase preparation and 1.0–20 A_{260} units of tRNA per ml of solution, as previously desribed[12]. The aminoacylated tRNA was separated from free amino acids and unreacted tRNAs by chromatography on a small BD-cellulose column (0.4 cm x 7.5 cm). Unreacted amino acids, nucleotides and protein components were removed from the column with 10 mM sodium acetate at pH 4.5, containing 0.3 M NaCl and 10 mM $MgCl_2$ (Buffer A) and tRNA species were fractionated with a linear salt gradient (total of 300 ml) from 0.3 M to 1.0 M NaCl, containing 10 mM $MgCl_2$ and 10 mM sodium acetate at pH 4.5 Radioactive fractions were pooled, diluted with an equal volume of Buffer A and placed on another BD-cellulose column (0.4 cm x 3 cm). tRNA was eluted with 3 ml of 10 mM sodium acetate at pH 4.5, containing 1.0 M NaCl, 10 mM $MgCl_2$ and 15% ethanol, thus effecting a concentration of tRNA. tRNA was precipitated and stored as described above.

Preparation of N-acetyl-aminoacyl-tRNA

The procedure involves preparing *N*-acetoxy-succinimide and reacting it with the appropriate aminoacyl-tRNA which had been partially puritfied as described above.

N-acetoxy-succinimide was synthesized according to the method of Gillam *et al.*[14]. 460 mg of *N*-acetoxy-succinimide and 0.238 ml of glacial acetic acid were dissolved in 40 ml of dry dioxane; 824 mg of dicyclohexyl-carbodiimide was then added and the solution was left standing overnight at room temperature. Dicyclohexylurea (a by-product) was filtered off and the filtrate evaporated to an oil. *N*-acetoxy-succinimide (melting range 126–127°) was trice crystallized from isopropyl alcohol. 20–50 A_{260} units of aminoacyl-tRNA was shaken overnight at room temperature in a solution containing 0.4 mmole of *N*-acetoxy-succinimide in 2.5 ml of dimethylformamide (DMF) and 0.02 M potassium acetate at pH 4.5[15]. tRNA was sedimented by centrifugation, washed twice with cold DMF, once with cold ethanol and dissolved in water.

The extent of acetylation was determined by treating an aliquot of the preparation with 0.4 M KOH at 37° for 30 min. Under these conditions the aminoacyl bond is hydrolyzed but not the bond between the acetyl residue and the amino group. The released amino acids were then separated by paper chromatography (Whatman No. 1) in a solvent system of *n*-butanol-glacial acetic acid-water (78:5:17). The dried paper was cut into 1 cm strips which were counted for radioactivity.

Procedure for incubation in a cell-free system

Preparation of lysates from rabbit reticulocytes and the conditions for cell-free synthesis of hemoglobin have been described in detail elsewhere[16]. For each preparation of radioactive *N*-aminoacyl-tRNA it was necessary to ascertain the optimum amount of tRNA that could be added to the lysate without perturbing the system. The rate and extent of hemoglobin synthesis in incubations containing varying amounts of acetylated aminoacyl -tRNA were monitored by measuring the incorporation of free amino acid

(0.5 μCi L- ^{14}C-leucine added per 200 μl of incubation mixture) at 37° for 60 min. The amount of radioactivity transferred from the added acetylate aminoacyl-tRNA into hemoglobin is insignificant compared to that of the free amino acid.

Analysis of products synthesized in the cell-free system

A. By Sephadex G-75 chromatography

Upon completion of an incubation the reaction mixture (3 ml) was placed directly on a Sephadex G–75 column (3.5 cm x 108 cm), equilibrated with 0.01 M sodium phosphate at pH 6.8 containing 0.05 M KCl. Elution was carried out with the same buffer solution at a constant flow rate. Absorbancy of the fractions collected was monitored at 260, 280 and 410 nm. Aliquots were removed and either placed in a toluene based scintillator mixture (5 g Omnifluor (New England Nuclear)/1 toluene containing 25% (v/v) ethanol) and counted in a Nuclear Chicago Scintillation Counter, Isocap 300, or spotted directly on Whatman 3 MM paper filter discs. The discs were then treated with cold 10% trichloracetic acid (TCA), followed by hot 10% TCA, dried and counted in a Nuclear Chicago Gas-Flow Planchet Counter.

B. By DEAE-cellulose chromatography

Following completion of an incubation the ribosomes were removed by centrifugation at 105,000 x g for 3 hr in a Beckman Spinco Ultracentrifuge L2–65. The postribosomal supernatant was placed on a DEAE-cellulose column (0.9 cm x 6 cm) equilibrated with 0.01 M Tris-HCl at pH 7.2 containing 0.025 M KCl; hemoglobin does not bind to the resin under these conditions and can be eluted with the equilibrating buffer solution.

C. Preparation of globin

Globin was obtained from hemoglobin prepared by method A or B above by drop-wise addition of the solution to 25 volumes of ice-cold acidified acetone (0.2% (v/v) HCl). Globin was sedimented by centrifugation and dissolved in water.

D. Preparation and chromatography of the tryptic peptides

Globin was aminoethylated and digested with trypsin (trypsin: protein, 1:100 (w/w) at 37°, pH 8.0 for 2 hr) as previously described[12]. The tryptic peptides were separated on a column of Dowex 50–X8 (0.9 cm x 16 cm kept at 50°) utilizing a 500 ml gradient of pyridine acetate (0.2 M pyridine at pH 3.1 to 2.0 M pyridine at pH 5.0). 3.0 ml fractions were collected and aliquots were removed for radioactivity counting.

Materials

Radioactive amino acids were purchased from either Amersham Searle, Illinois or from New England Nuclear, Boston. All compounds used were of reagent grade unless otherwise specified.

Results

Purity of N-acetyl-^{14}C-valyl-tRNA

The extent of acetylation of valyl-tRNA was determined by estimating the amount of radioactive amino acids released by alkaline hydrolysis of the *N*-acetyl-amino-acyl-tRNA preparation. The results obtained for two samples (Table I) showed that the acetylation reaction was from 93 to 97 % complete.

Effect of added tRNA on globin synthesis in the lysate cell-free system

The time course of incorporation of radioactive leucine into globin in a lysate cell-free system in the presence and absence of *N*-acetyl-valyl-tRNA is shown in Fig. 1. In both cases synthesis is linear for approximately 10 min and ceases by 60 min. The results can also be expressed in terms of the number of chains synthesized per ribosome (Table II). In the presence of *N*-acetyl-valyl-tRNA (10 A_{260} units per ml of lysate) the initial rate and extent of protein synthesis range from 85 to 90 % of the control value.

Chromatography of a lysate incubation on Sephadex G-75

A lysate in which *N*-acetyl-^{14}C-valyl-tRNA was incubated in the presence of ^{3}H-leucine and ^{3}H-valine was was fractionated on a Sephadex G-75 column to separate ribosomes, hemoglobin and tRNA from each other and from other components of the reaction mixture. The A_{260} profile (Fig. 2a) reveals 5 main components which have been identified by their positions of elution and by their spectral ratios (A_{260}/A_{280} and A_{410}/A_{280}) (Table III) as ribosomes (Peak A), tRNA (Peak B), hemoglobin (Peak C), globin (Peak D), and low molecular weight compounds (Peak E). The position of elution of tRNA is a little surprising in view of its molecular weight (25,000). It appears that tRNA aggregates under the conditions of elution used here. This was verified by examining the elution of a sample of purified tRNA under the same conditions.

The radioactivity profiles are shown in Fig. 2b, c and d. Tritium is found in the fractions containing ribosomes, tRNA, hemoglobin, globin and small molecular weight compounds (the salt front). There is no evidence of radioactive material of molecular weight between that of globin and the salt front.

The large amount of tritium present in the fractions collected precluded detection of carbon-14 by liquid scintillation counting. ^{14}C-radioactivity was determined by treating each fraction with cold TCA and counting the precipitated material in a gas-flow planchet counter which has a much higher efficiency for carbon-14 (30 %) than for tritium (<0.01 %) (Fig. 2c). In order to determine how much of the ^{14}C-radioactivity is associated with tRNA (as *N*-acetyl-^{14}C-valyl-tRNA) and how much is incorporated into peptide linkages of protein, each fraction was further treated with hot TCA. This treatment hydrolyzes RNA and makes it soluble but has no effect on peptide bonds. The results show that *N*-acetyl-^{14}C-valine is largely in the form of *N*-acetyl-valyl-tRNA (note the disappearance of Peak B in Fig. 2d).

Hence, the radioactivity in Peaks A, C and D (Fig. 2b) is largely due to ^{3}H-leucine and ^{3}H-valine which have been incorporated into peptide linkages. The counts in Peak A are from nascent globin chains attached to ribosomes. At most only 5% of the original ^{14}C-label added to the lysate appears in Peaks A, C and D.

Analysis of tryptic peptides from globin synthesized in the presence of N-acetyl-^{14}C-valyl -tRNA

A lysate was incubated as in Fig. 1. After one hour of incubation the ribosomes were removed by sedimentation and the supernatant was fractionated on a DEAE-cellulose column to clearly separate hemoglobin from tRNA and other components of the reaction mixture. The hemoglobin purified in this way was precipitated in acid-acetone, aminoethylated and digested with trypsin. The peptides were then separated on a Dowex 50-X8 column.

Table I

Purity of N-acetyl-^{14} C-valyl-tRNA

An aliquot of a solution of *N*-acetyl-^{14}C-valyl-tRNA was combined with 1/10 volume 4 M KOH and incubated for 30 minutes at 37°. The solution was then spotted on a Whatman No. 1 paper and submitted to descending chromatography in a solvent system of *n*-butanol: glacial acetic acid: water (78:5:17) overnight. Standards were ^{14}C-valine and *N*-acetyl-valine. 1 cm strips were cut out and counted. The Table shows the results of two preparations.

Compound	R_f	Cpm	% Total
N-Acetyl-^{14}C-valyl-tRNA #1			
^{14}C-Valine	0.43	257	5
N-acetyl-^{14}C-valine	0.93	4667	93
^{14}C-valyl-tRNA	0.00	79	2
N-Acetyl-^{14}C-valyl-tRNA #2			
^{14}C-Valine	0.42	658	1
N-acetyl-^{14}C-valine	0.92	61688	97
^{14}C-valyl-tRNA	0.00	1202	2

Table II

Synthetic activity of lysates

The incubation conditions are those given for Fig. 1. The amount of globin synthesized are the levels after 1 hour at 37°. Calculations are based on the following assumptions: 1) 17.5 leucine and 14.5 valine residues per globin chain. 2) 1.5 mg ribosome (MW=4.5 x 10^6) per ml of lysate. This has been determined in a number of lysates and ranges from 1.3–1.8 mg ribosome per ml. 3) Counting efficiencies of 60% and 10% for ^{14}C and ^{3}H, respectively.

Lysate	Addition	nmoles globin per ml lysate	chain globin per ribosome	% synthesis
7–28–B[a]	+water	2.9	9.6	100
	+*N*–acetyl–^{14}C-valyl-tRNA #1	2.5	8.3	86
11–11–D[b]	+water	2.4	8.0	100
	+*N*-acetyl-^{14}C-valyl-tRNA #2	2.2	7.3	91
1971–1972[c]	—	1.9–7.3	6.5–27.6	—

[a]with ^{14}C-leucine (specific activity of 11 μCi/μmole)
[b]with H^3-leucine and ^{3}H-valine (specific activity of 1250 μCi/μmole)
[c]based on Table 2, reference 15

Table III

Identification of the components from a lysate fractionated on Sephadex G–75

Peak[e]	A_{260}/A_{280} This Study	A_{260}/A_{280} Literature	A_{410}/A_{280} This Study	A_{410}/A_{280} Literature	Component
A	1.7	(1.8)[a]			Ribosome
B	1.3	2[b]			tRNA
C	0.9	0.8[c]	3	3.4[c]	Tetrameric Hemoglobin
D	0.8	(0.7)[d]			Globin
E	1.8–4.0				Low MW Compounds

a. Determined experimentally using a preparation of free ribosomes.
b. Miles Laboratories Inc. Catalog D (1971) p. 80.
c. Prankerd, T.A.J. (1961) "The Red Cell" p. 30., Charles C. Thomas, Springfield, Illinois.
d. Determined experimentally using a preparation of purified rabbit globin.
e. As designated in Fig. 2.

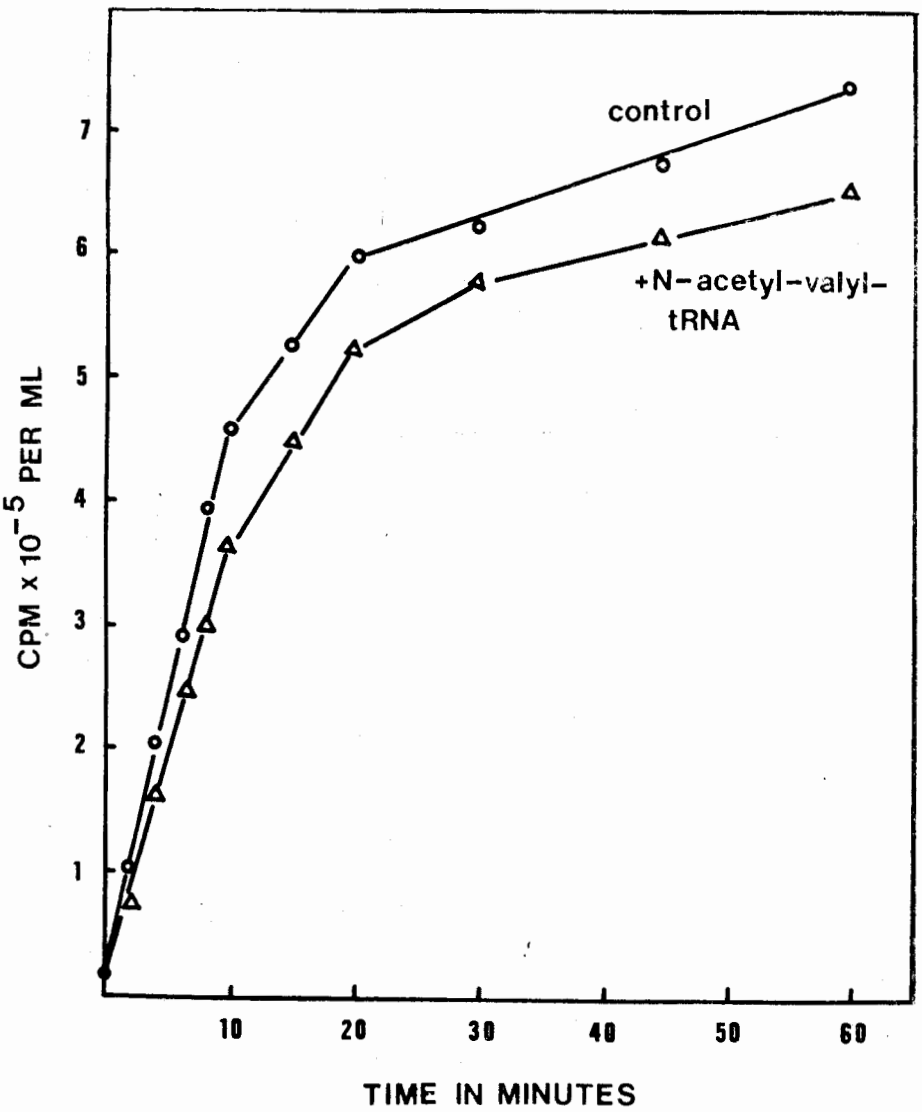


Fig. 1 Globin synthesis in a cell-free lysate system

From a 2.2 ml lysate (7–28–B) incubation mixture, 400 µl was removed to which was added 25 µl water together with 0.5 µCi ^{14}C-leucine and the resulting solution incubated at 37° for 60 minutes. At varying times 20 µl aliquots were removed, spotted on paper filter discs (Whatman No. 1, 24 mm) and counted (see Methods and Materials Section). From the remaining original reaction mixture to which was added 160 µl *N*-acetyl-^{14} C-valyl-tRNA#1 (11.8 A260, 6.5 x 10^5 cpm), another 400 µl aliquot was removed and was incubated with 0.5 µCi ^{14}C-leucine and assayed in the same manner as control.

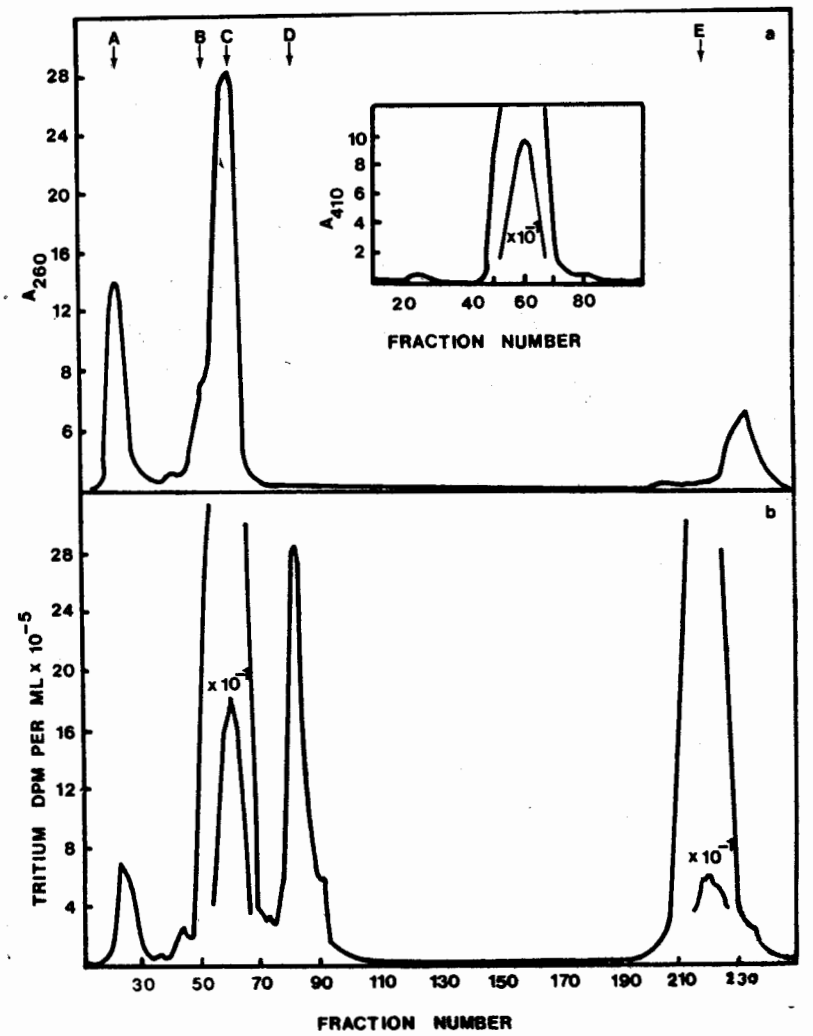
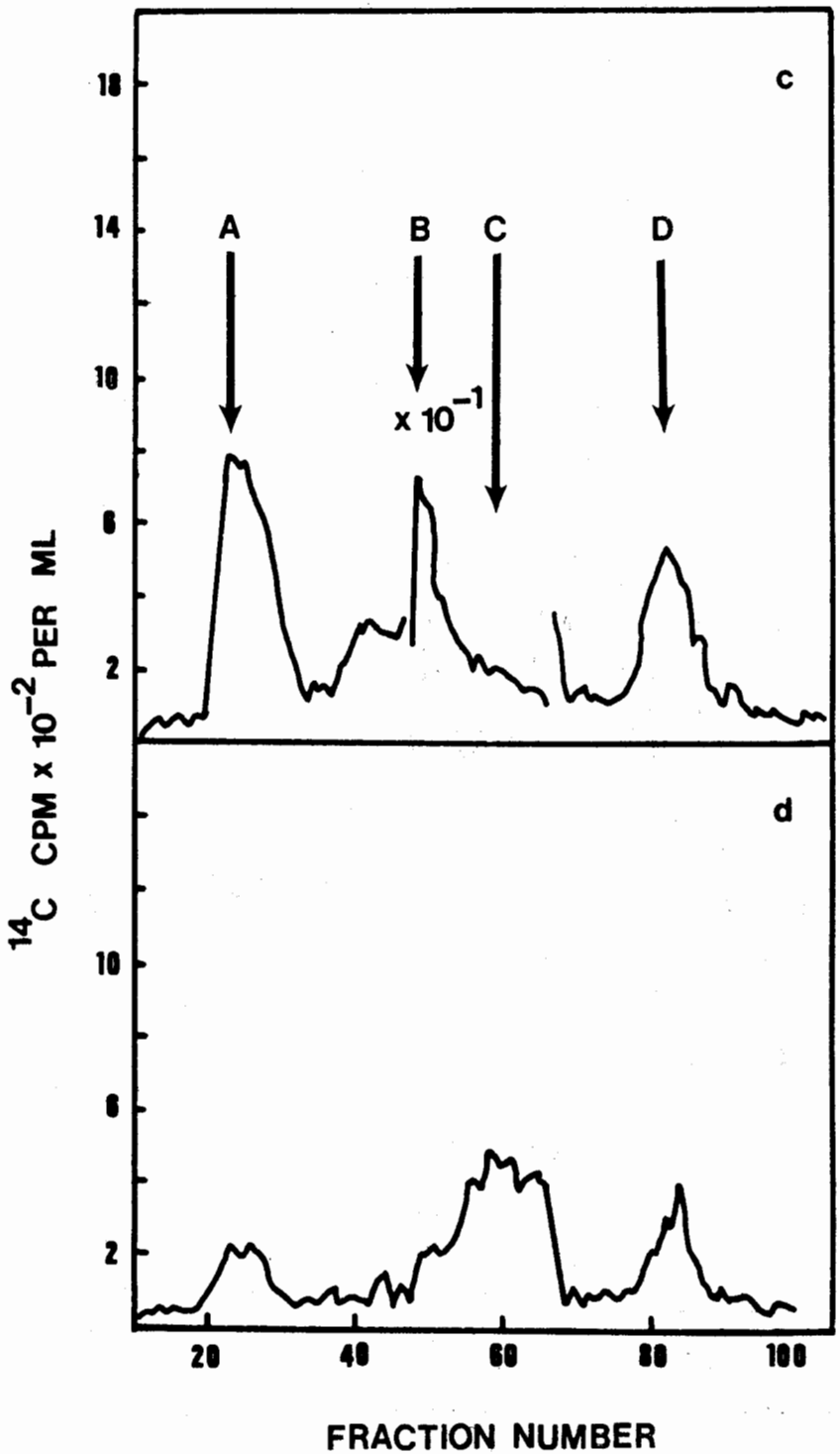


Fig. 2 Chromatography of lysate on Sephadex G–75

500 µl of *N*-acetyl-^{14}C-valyl-tRNA #2 (94 A_{260}, 1.6×10^6 cpm) were added to a 8.5 ml lysate (11.–11–D) incubation mixture, containing 500 µCi each of ^{3}H-leucine and ^{3}H-valine (final specific activity = 1250 µCi/µmole). The resulting mixture was incubated at 37° for 60 minutes. The solution was then separated on a column (3.5 cm x 108 cm) of Sephadex G-75 with an eluting buffer of 0.01 M sodium phosphate at pH 6.8, containing 0.05 M KCl. 2.8 ml fractions were collected.

a) Absorbance measured at 260 and 410 nm.
b) Total radioactivity measured directly in an ethanol-toluene scintillator (^{3}H efficiency = 32%).
c) Cold 10% TCA precipitable radioactivity measured in a gas-flow counter (^{14}C efficiency = 30%, ^{3}H efficiciency < 0.01%)
d) Radioactivity remaining after treatment with hot followed by cold 10% TCA, measured as in (c).

(As no TCA precipitable radioactivity was found in tubes 100–250, these fractions were not represented in Figures (c) and (d)).


c
d
A
B
C
D
x 10^{-1}
18
14
10
6
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2
20
40
60
80
100
^{14}C CPM x 10^{-2} PER ML
FRACTION NUMBER

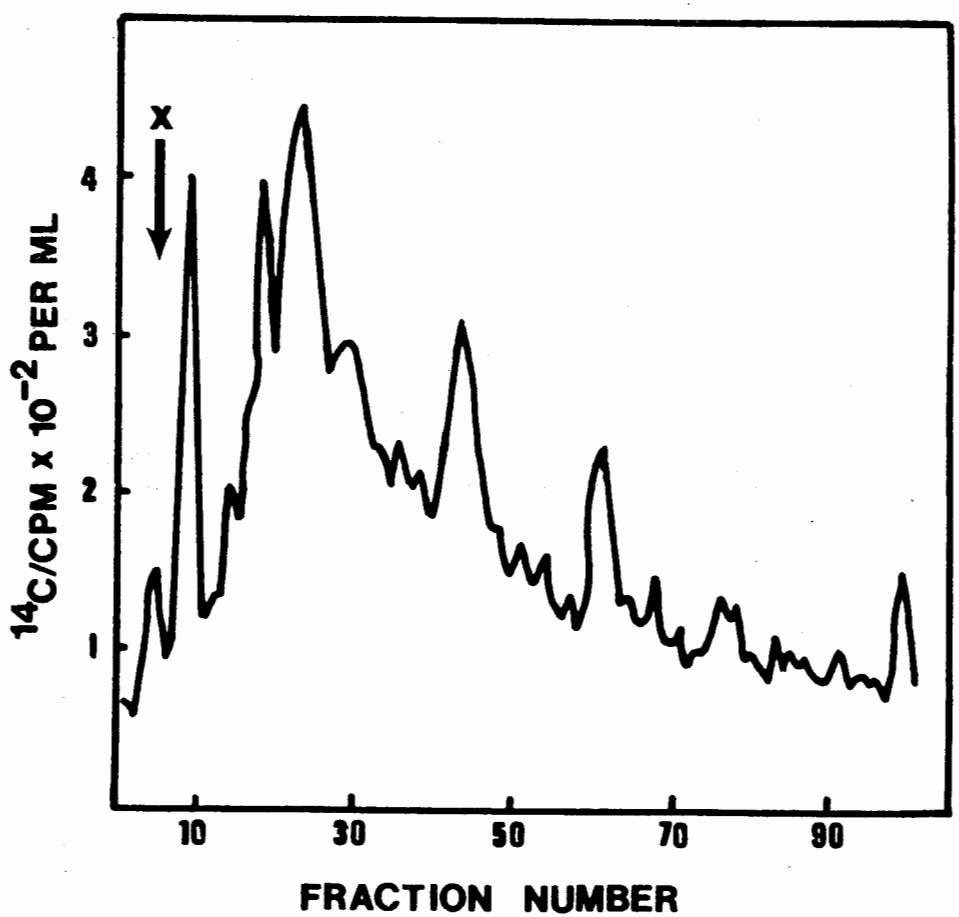

Fig. 3 Globin tryptic peptides obtained from a lysate incubation of *N*-acetyl-^{14}C-valyl-tRNA

The incubation mixture described in Fig. 1 was centrifuged to remove ribosomes and then chromatographed on a DEAE-cellulose column to yield a partially purified hemoglobin. Globin (90 A_{280}, 32980 cpm) was prepared by acid-acetone precipitation, followed by aminoethylation and tryptic digestion. Tryptic peptides (24560 cpm) were separated on a Dowex 50–X8 column; 3.0 ml fractions were collected from which 1.0 ml aliquots were removed for counting (^{14}C efficiency = 90%). Recovery from the column was 91%.

A large number of peptides were generated (Fig. 3) and a comparison of this "fingerprint" with that obtained with globin which had been uniformly labelled with valine[17] (not shown) indicates that this pattern arose from the transfer of ^{14}C-valine into internal sites. Peptides that have a blocked *N*-terminal residue would not have been retained by the resin under the conditions of elution used. Only a small peak (X) is seen at the start of the gradient, constituting 4% of the total radioactivity recovered from the column and could contain peptides with *N*-acetylated ^{14}C-valine.

Discussion

We have attempted to examine the preciseness of initiation of protein synthesis in eukaryotic cells by examining the effects of addition of various *N*-blocked aminoacyl-tRNAs (in particular, *N*-acetyl-valyl-tRNA) on globin synthesis in a rabbit reticulocyte cell-free system. The choice of *N*-acetyl-valyl-tRNA as the modified aminoacyl-tRNA was dictated by the following considerations.

1. From inspection of the *N*-terminal amino acid sequence of the α- and β-chains of globin[18], valine is the *N*-terminal amino acid in the completed α- and β-chains.

2. An *N*-blocked amino acid would have to be incorporated at an *N*-terminal site in a peptide thus precluding peptide bond formation with a prior amino acid and simplifying the characterization of the product.

3. An acetyl group is the blocking group (aside from a formyl residue) that is least likely to perturb the protein synthetic apparatus in a cell-free system. Further more, *N*-acetyl-valyl-tRNA (from *E. coli*) has been shown to be necessary for translation of globin mRNA in an *E. coli* cell-free system[19].

A reticulocyte cell-free system[16] was used since the rate of chain initiation is the same in this system as in the intact cell for periods of 10 to 15 minutes. (Most other cell-free protein synthesizing systems do not carry out chain initiation but only complete chains already started in the intact cell). In addition, the product of synthesis, hemoglobin, can be easily purified and the α-and β-globin chains can be digested with trypsin to yield peptides that are readily separated from one another by standard chromatographic techniques (see Methods and Materials Section).

It was first necessary to determine the level of tRNA that could be added without perturbing the cell-free system. Fig. 1 shows that N-acetyl-valyl-tRNA at 10 A_{260} units per ml of lysate does not perturb the initial rate or the extent of protein synthesis to any great degree. When the results are expressed in terms of the number of globin chains synthesized per ribosome (Table II) it can be seen that each ribosome is capable of synthesizing many chains *de novo* indicating that the system has a high capacity for carrying out chain initiation.

If globin chains can be initiated by *N*-acetyl-valyl-tRNA in competition with endogeneous Met-$tRNA_F$ (the natural initiator tRNA present in the lysate), does initiation with *N*-acetyl-valine lead to chain abortion during synthesis or are the chains completed in the same way as chains initiated with methionine?

To answer this question a lysate cell-free system was incubated with *N*-acetyl-^{14}C-valyl-tRNA in the presence of ^{3}H-leucine and ^{3}H-valine as described in Fig. 2. The amount of *N*-acetyl-valyl-tRNA added is 3 times the level of endogenous Met-$tRNA_F$ estimated to be present in the lysate mixture[20]. The radioactive amino acids were added in order to measure the rate of protein synthesis and to facilitate detection of small peptides that might result from aborted synthesis. The lysate was then fractionated on a Sephadex G-75 column (Fig. 2a, b, c and d) to separate ribosomes (Peak A), hemoglobin (Peak C), globin (Peak D) and tRNA (Peak B) from each other and from the small molecular weight components of the reaction mixture (Peak E, the salt front). Tritium found in Peaks A, C and D (Fig. 2b) are from ^{3}H-leucine and ^{3}H-valine that have been transferred into peptide chains (Peak A as nascent globin chains attached to ribosomes). There is no evidence of material (such as aborted nascent chains) with a molecular weight between that of globin and salt indicating that nascent peptide chains are not released from the ribosomes as such. ^{14}C-radioactivity is associated with Peak B (tRNA) in the form of *N*-acetyl-^{14}C-valyl-tRNA (compare Fig. 2c with 2d, and see the discussion in the Results Section). It is estimated that only 5% of the original ^{14}C-label added to the lysate appears in Peaks A, C and D.

Thus, few if any, peptides smaller than completed globin chains are present in the non-ribosomal fraction of the lysate indicating that abortive synthesis cannot have occurred to any great extent. The small amount of carbon-14 that is incorporated into globin may be due to *N*-acetyl-valine or valine. *N*-acetyl-valine can be incorporated only into *N*-terminal sites whereas valine can be incorporated into *N*-terminal and internal sites.

A lysate was incubated as in Fig. 1 but the only radioactive component present was *N*-acetyl-^{14}C-valyl-tRNA. A "fingerprint" of the tryptic peptides obtained from the globin chains synthesized (Fig. 3) reveals many labelled peptides, a pattern which is similar to that resulting from the transfer of ^{14}C-valine into internal sites[17]. Out a total of 6.5 x 10^5 cpm of *N*-acetyl-^{14}C-valyl-tRNA added to the lysate, only 5% of the carbon-14 label appears in completed globin chains (3.3 x 10^4 cpm). Under similar incubation conditions, over 80% of valine is transferred from valyl-tRNA into globin chains[23]. Peptides that contain an acetylated *N*-terminal residue would not be retained by the resin; only a small peak (X, representing 4% of the column radioactivity) is obtained at the start of the gradient. If peak (X) consists only of peptides with acetyl-valine as the *N*-terminal residue, this means

that less than 1 out of 1,500 chains syntesized during the incubation period and released into the supernatant fraction were initiated with *N*-acetyl-valyl-tRNA.*

Three explanations have been considered for the low level of chain initiation with *N*-acetylated aminoacyl-tRNAs.

a. Inactivation of the *N*-acetyl-valyl-tRNA during the acetylation reaction.

A number of observations show that the conditions used to acetylate valyl-tRNA leave the tRNA biologically active[21]. Acetylated phenylalanyl-tRNA (from *E. coli*) prepared in the same manner as here is required for the synthesis of polyphenylalanine in the presence of *E. coli* initiation factors, phenylalanyl-tRNA, poly U and ribosomes; almost no reaction occurs in the absence of *N*-acetyl-phenylalanyl-tRNA[22]. The transfer of valine into globin chains from valyl-tRNA present as a contaminant in *N*-acetyl-valyl-tRNA preparations (see below) also indicates that tRNA has not been inactivated by the acetylation conditions. Johnson[23] has recently demonstrated that ε-*N*-acetyl-lysyl-tRNA (from *E. coli*) is biologically active in that the modified lysine residue is transferred quite efficiently into globin chains synthesized in the cell-free system used in this study.

b. Inactivation of the *N*-acetyl-valyl-tRNA during the incorporation experiment.

Rapid removal of *N*-acetyl-valine from *N*-acetyl-valyl-tRNA or deacetylation of valine during incubation can also account for a very low rate of *N*-acetyl-valine incorporation. To determine the extent to which these reactions had occurred during the incubation the mixture was fractionated on a DEAE-cellulose column. Hemoglobin was first eluted with 0.05 M KCl, amino acids partially removed with 0.25 M KCl and tRNA eluted with 1.0 M KCl (results not shown). Analysis of the fractions collected indicated that the free amino acids existed only as *N*-acetyl-^{14}C-valine (^{14}C-valine $< 0.5\%$). Aminoacyl--tRNA was found only as *N*-acetyl-^{14}C-valyl-tRNA, constituting 19% of the original amount added to the cell-free incubation.

The presence of *N*-acetyl-valine in the reaction mixture at the conclusion of the incubation period indicates that the acetylated amino acid was removed intact from the tRNA. The source of the small amount of ^{14}C-valine transferred into globin is probably the small amount of ^{14}C-valyl-tRNA which did not react during the preparation of the acetylated valyl-tRNA (see Table I). *N*-acetyl-valine apparently is not hydrolyzed to acetate and valine as no free valine was detected in the incubation mixture.

*The calculation was based on a specific activity of 125 μCi/μmole for ^{14}C-acetyl-valine and a counting efficiency of 90% for ^{14}C. One further assumption was that the acetyl group in the nascent chain was not removed prior to release of the completed chain from the ribosome. It is possible to determine the extent of of deacetylation from knowing the number of *N*-terminal tryptic peptides, but the limited amount of radioactivity precluded purification and identification of the individual peaks.

The hydrolysis of the acyl bond in *N*-acetyl-valyl-tRNA could result from chemical hydrolysis (the half-lives of *N*-acetyl-aminoacyl-tRNA at pH 8.5 in 0.1 M Tris vary from 45 to 300 minutes) or from enzymatic hydrolysis (hydrolase activities have been found in *E. coli* and yeast), or from a combination of these mechanisms[21].

c. Rejection of *N*-acetyl-valyl-tRNA as an initiator tRNA by the synthetic apparatus of the lysate.

At the end of one hour of incubation an appreciable amount (19%) of the *N*-blocked valyl-tRNA is still intact; this is an amount comparable to that of endogenous Met-$tRNA_F$. This indicates that the synthetic apparatus is capable of a very high level of discrimination against false initiation by *N*-acetyl-valyl-tRNA.

The frequency with which *N*-acetyl-valine initiates a globin chain is estimated to be 1 in 1,500 completed globin molecules. This figure is an upper limit (under the present set of conditions) based on the average number of globin chains synthesized per ribosome (Table II). The frequency of insertion of an incorrect amino acid into a growing peptide chains has been estimated to be no greater than 1 in 3,000 with intact cells[24].

Thus, the maximum frequency of false initiation with *N*-acetyl-valyl-tRNA under present conditions is about the same as the frequency of errors encountered in the insertion of incorrect amino acids into proteins. It is conceivable that the frequency of false initiation could be enhanced by increasing the ratio of *N*-acetyl-valyl-tRNA to endogenous Met-$tRNA_F$. We were not able to test this possibility because of the pronounced inhibition of protein synthesis encountered at high levels of tRNA. Hence, the level of false initiation observed here is only for non-inhibitory concentrations of *N*-acetylated valyl-tRNA.

The ability of a reticulocyte lysate to reject analogs of the natural initiator is not restricted to *N*-acetyl-valyl-tRNA. Incubations under similar conditions with *N*-acetylated aminoacyl-tRNA of leucine, serine, histidine and phenylalanine all failed to show incorporation of the *N*-blocked amino acids; only unmodified amino acids were transferred into globin chains (unpublished results). If the acetyl blocking group is replaced with a larger moiety, such as *N*-phenoxyacetyl residue, no detectable amount of false initiation is seen (using *N*-blocked valyl- and phenylalanyl-tRNA) (unpublished results). Apparently, in this case, both the steric and hydrophobic character of the modified amino acids favors a rejection of the *N*-phenoxy-acetyl-aminoacyl-tRNA.

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บทคัดย่อ

เราได้ทดลองว่า นอกจาก Met-$tRNA_F$ ซึ่งเป็น initiator tRNA ที่พบในเซลล์พวก eukaryote แล้ว ยังมี aminoacyl-tRNA ชนิดอื่นหรือไม่ที่สามารถเริ่มต้นการสังเคราะห์โปรตีนได้

เราได้เตรียม *N*-acetyl-^{14}C-valyl-tRNA จาก valyl-tRNA ที่สกัดจากเม็ดโลหิตแดง (reticulocyte) ของกระต่าย และได้นำมาใช้ในระบบ cell-free ซึ่งมีการสร้างโปรตีนฮีโมโกลบินอย่างมีประสิทธิภาพสูง *N*-acetyl-^{14}C-valyl-tRNA ที่นำมาทดลองมีปริมาณเป็นสามเท่าของ Met-$tRNA_F$ หลังจาก incubate ที่อุณหภูมิ ๓๗° ซ เป็นเวลาหนึ่งชั่วโมง แล้วพบว่า *N*-acetyl-valine ที่อยู่ในโปรตีนตอน *N*-terminal มีปริมาณน้อยกว่า ๐.๒ % และยังมี *N*-acetyl-^{14}C-valyl-tRNA ที่ไม่ถูกสลายไปเหลืออยู่

เราได้สรุปผลว่า ในเซลล์พวก eukaryote มีกลไกซึ่งสามารถกันไม่ให้ *N*-acetyl-aminoacyl-tRNA เป็นตัวเริ่มแปลระหัสพันธุกรรม