*EVIDENCE FOR A PROPOSED INITIATION COMPLEX FOR PROTEIN SYNTHESIS IN RETICULOCYTE POLYRIBOSOME PROFILES**

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Abstract.—Polysomes consisting of 40*S* ribosomal subunits attached to monosomes, disomes, trisomes, and tetrasomes have been isolated for the first time by the use of isokinetic sucrose density gradients. They are formed upon incubation of rabbit reticulocyte lysate in the presence of 10 mM NaF. It is proposed that these structures represent transient initiation complexes for protein synthesis.

Many of the details of the mechanism of protein synthesis have been established for procaryotic cells. Of particular interest is the proposed mechanism of initiation which has been shown to involve the binding of a 30*S* ribosomal subunit and initiator tRNA (fMet-tRNA) to a specific initiator codon, usually AUG or GUG, on the 5′ terminus of a messenger RNA, followed by the subsequent addition of a 50*S* subunit.[1–3] In addition, soluble protein factors[4] and GTP are required.[5, 6]

In contrast to these studies with *E. coli*, much less is known about the mechanism of initiation in eucaryotic cells. The most thoroughly investigated mammalian systems have been the HeLa cell,[7–9] reticulocyte,[10–12] and rat liver.[13–16] Although some insight into peptide bond formation and elongation of polypeptide chains has been gained, very little has been learned about initiation. Experiments by Joklik and Becker[7] suggest that in HeLa cells ribosomal subunits enter polysomes before they combine to form monosomes. Similar observations have been made with ascites tumor cells by Hogan and Korner,[17] who proposed that subunits are directly involved in the formation and breakdown of polysomes, and that 80*S* ribosomes merely constitute a pool of subunits. Stimulation of initiation has been observed in reticulocytes upon addition of ribosomal subunits.[18] Other experiments,[19] however, suggest that monosomes can reaggregate directly to form active polysomes, thereby eliminating the necessity to invoke ribosomal subunits in polysome formation.

It has been proposed that, in the cell-free reticulocyte system, fluoride specifically inhibits initiation of new globin chains, but not elongation or termination of preexisting polypeptide chains.[20, 21] This suggestion has been strengthened by the studies of Hunt *et al.*[22] on the distribution of ribosomes along the messenger RNA in NaF-treated reticulocytes. We have examined this effect in more detail.

We wish to report here the first evidence of a new kind of polysome, in eucaryotic cells, consisting of 40*S* subunits attached to monosomes, disomes, trisomes, and tetrasomes, which accumulates in a reticulocyte lysate synthesizing protein when NaF is present. These complexes have been demonstrated by the use of an isokinetic sucrose density gradient.[23] We propose that they represent transient

intermediates in protein synthesis and that fluoride blocks initiation of new polypeptide chains by inhibiting the subsequent association of the 60S subunit, thus preventing the formation of a complete ribosome.

Methods.—Preparation of reticulocytes: Reticulocytes were obtained from white rabbits (8–9 lb.) made anemic by four daily injections with 1 ml of a 2.5% phenylhydrazine solution.[24] On the sixth day, 50-ml blood was withdrawn by heart puncture. Under these conditions, 90–95% of the red cells were reticulocytes.

In vivo labeling: Fifteen mc $^{32}P_i$ was injected intravenously into a rabbit on the fourth day of phenylhydrazine injection. This procedure gave an RNA preparation with a specific activity of approximately 700,000 cpm per milligram.

Incubation of whole cells: The cells were first washed in cold Krebs-Ringer bicarbonate buffer (KRB)[25] and then incubated in two volumes of KRB medium containing a mixture of 18 L-amino acids in 1.6 times the concentration described by Borsook *et al.*,[24] 0.3 mg/ml Fe $(NH_4)_2$ $(SO_4)_2$, 3.6 mM glucose, 0.6 mM inosine, and 4% plasma (v/v). After incubation the cells were chilled and washed twice with KRB.

Lysis: The reticulocytes were lysed by adding 1 vol of a dilute salt solution (5 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$) to 1 vol packed cells, and isotonicity was restored after 1 min by addition of .1 vol 1.5 M KCl.[26] The mixture was centrifuged for 20 min at 30,000 *g*, to remove the stroma. The supernatant served as lysate and was stored in liquid nitrogen.

Cell-free incubation: One ml of the incubation mixture contained 0.8 ml lysate and 0.2 ml of the following solution: 5 mM ATP, 1.25 mM GTP, 25 mM phosphoenolpyruvic acid (PEP) (adjusted to pH 7), and 250 μg/ml PEP-kinase, 3 mM $MgCl_2$, 5 mM Cleland's reagent, 50 mM Tris, pH 7.4, 18 L-amino acids except leucine in $^1/_2$ the concentration used by Borsook *et al.*[24] Leucine-^{14}C was added at a final concentration of 0.1 mM and specific activity 5 μc/μmole.

For polysome profiles, aliquots were diluted with 3 vol medium A[27] minus sucrose and layered directly on isokinetic sucrose density gradients[23] (15–32.8% w/w sucrose) containing 3 mM magnesium acetate, 50 mM KCl, 5 mM Tris-HCl, pH 7.5. Centrifugation was performed in a SW 25.3 rotor at 22,000 rpm for 4 or 7 hr. Protein synthesis was assayed with the Mans and Novelli filter disk technique,[28] as described previously.[27]

Analysis of the RNA: Ribosomal fractions were collected from polysome gradients with NaCl replacing KCl after centrifugation for 7 hr at 22,000 rpm. The release of RNA was accomplished by addition of sodium dodecyl sulfate (SDS) (1% final concentration) and EDTA (10 mM final conc) at room temperature. After addition of unlabeled carrier RNA, the RNA was precipitated with 2 vol of cold ethanol, redissolved in SDS buffer (0.3% SDS, 5 mM EDTA, 50 mM sodium acetate, pH 5.1) and analyzed on a 5–28.4% isokinetic sucrose density gradient[23] (0.1 *M* NaCl, 0.01 *M* sodium acetate, pH 5.1) at 22,000 rpm for 22 hr. Determination of radioactivity was carried out as previously described.[27]

Results.—Reticulocyte lysates were incubated in the cell-free protein synthesizing system. Aliquots of the incubation mixture were withdrawn, assayed for protein synthesis, and the polysome profiles determined (Fig. 1). The time course of protein synthesis was similar in all cases. After an initial sharp rise, incorporation reached a plateau after 15 to 20 minutes. Counts incorporated after 20 minutes, under various conditions of incubation, are presented in Table 1. In the presence of 10 mM NaF, protein synthesis reached 30 per cent of the control level, and in the polysome profiles additional peaks with intermediate sedimentation coefficients were observed, as shown in Figure 1*d*, *e*. If protein synthesis was inhibited by the addition of 1 mM cycloheximide to the incubation mixture or by incubating at 0°, the formation of the intermediate peaks was prevented (Fig. 1*f*). Sodium fluoride at a concentration of 1 mM

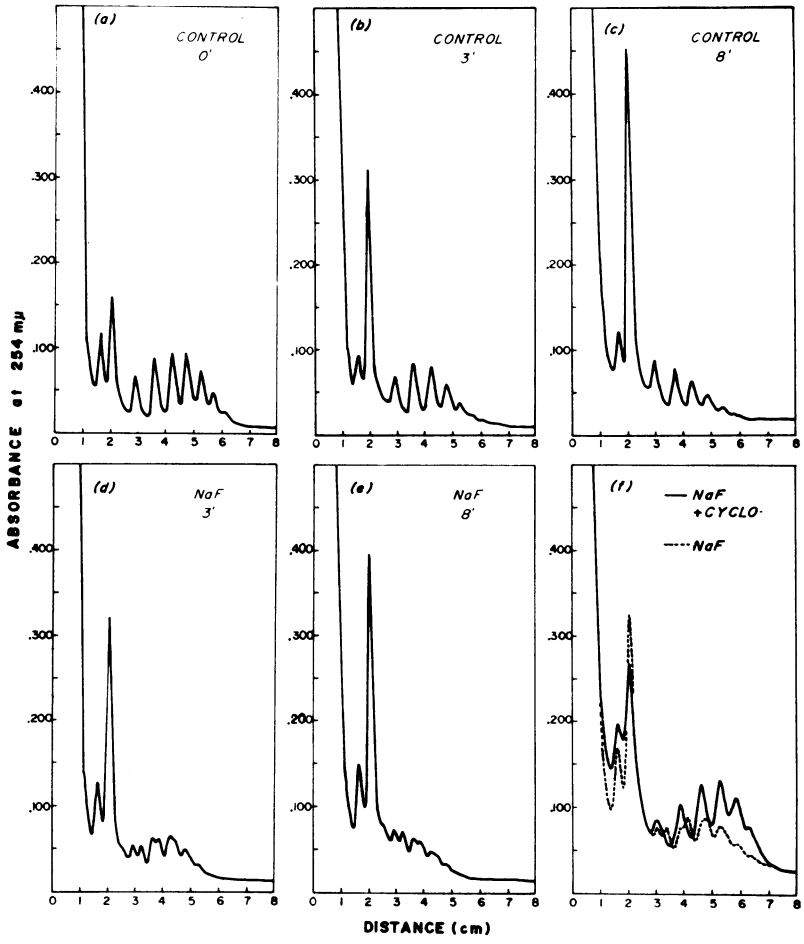

FIG. 1.—Lysate was incubated in the complete cell-free system. Aliquots were layered directly on sucrose density gradients after 0, 3, 8 min, and sedimented for 4 hr at 22,000 rpm. (*a*), (*b*), (*c*) Control incubation; (*d*), (*e*) incubated with 10 mM NaF; (*f*) 10-min incubation with 10 mM NaF (- - -) and 10 mM NaF together with 1 mM cycloheximide (——). Hemoglobin sedimented at the top of the gradient.

was found to have no effect on protein synthesis or polysome profiles, in agreement with the observation of Hardesty *et al.*[20]

If the lysate was incubated without ATP, GTP, PEP, and PEP-kinase, the intermediate peaks became even more prominent almost to the complete exclusion of the normal polysome profile (Fig. 2*b*). It seemed important to assess sedimentation coefficients of the intermediate peaks and to that end, lysates incubated without an energy source in the absence (control lysate) and presence of NaF, were centrifuged separately (Fig. 2*a*, *b*) and cosedimented (Fig. 2*c*), the control lysate serving as an internal marker. The sedimentation values are listed in Table 1.

The intermediate peaks were less stable and disappeared almost completely

TABLE 1. *Sedimentation coefficients and RNA ratios of ribosomal complexes.*

	Sedimentation coefficient	RNA total recovery (cpm)	28S RNA (cpm)	18S RNA (cpm)	Ratio 28S/18S
Monosome	80	3 570	2 068	739	2.80
Disome	120	4 500	2 380	957	2.49
Intermediate peak	132	3 120	1 618	816	1.98
Trisome	150				
Intermediate peak	162				
Tetrasome	182				
Pentasome	204				

when the polysomes were pelleted at 105,000 *g* for 2.5 hours through 0.5 *M* sucrose and resuspended in Medium A minus sucrose.

Reformation of polysomes *in vitro* was investigated by the use of a lysate prepared from cells that had been previously incubated with 10 mM NaF for 20 minutes. This procedure converted polysomes almost entirely to monosomes, Figure 3*a*. During the subsequent *in vitro* incubation of the lysate under the same conditions as in Figure 1*a*, polysomes reformed to some extent, Figure 3*b*, and protein synthesis attained 50 per cent of the control level. When 10 mM NaF was added to the incubation mixture, however, protein synthesis reached only 10 per cent of the control, essentially no polysome reformation occurred as anticipated, and the intermediate peaks once again were evident.

To characterize further the intermediate peaks, their RNA composition was determined. ^{32}P-labeled lysate was incubated with NaF for eight minutes in the absence of an energy source and centrifuged at 22,000 rpm on an isokinetic sucrose gradient for seven hours. The fractions containing monosomes, disomes, and the 132*S* complex were collected from a total of six gradients and pooled, as shown in Figure 4*a*. The disomes were contaminated with 20–30 per cent material from the 132*S* peak. The RNA was prepared as described under *Methods* and fractionated on a sucrose gradient. The distribution of radioactivity is illustrated in Figure 4*b*, *c*, and *d*. Note that 90 per cent of the initial radioactivity

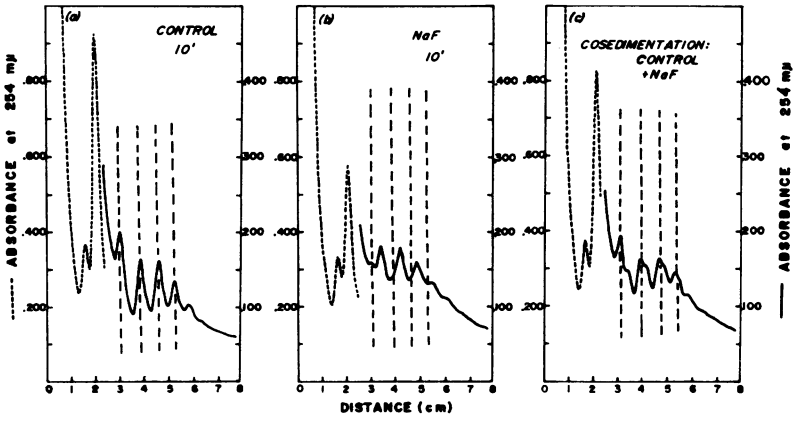

FIG. 2.—Lysate was incubated in the cell-free system without an exogenous energy source and layered directly on sucrose density gradients as in Fig. 1. (*a*) Control incubation, (*b*) incubated with 10 mM NaF, (*c*) samples from (*a*) and (*b*) cosedimented.

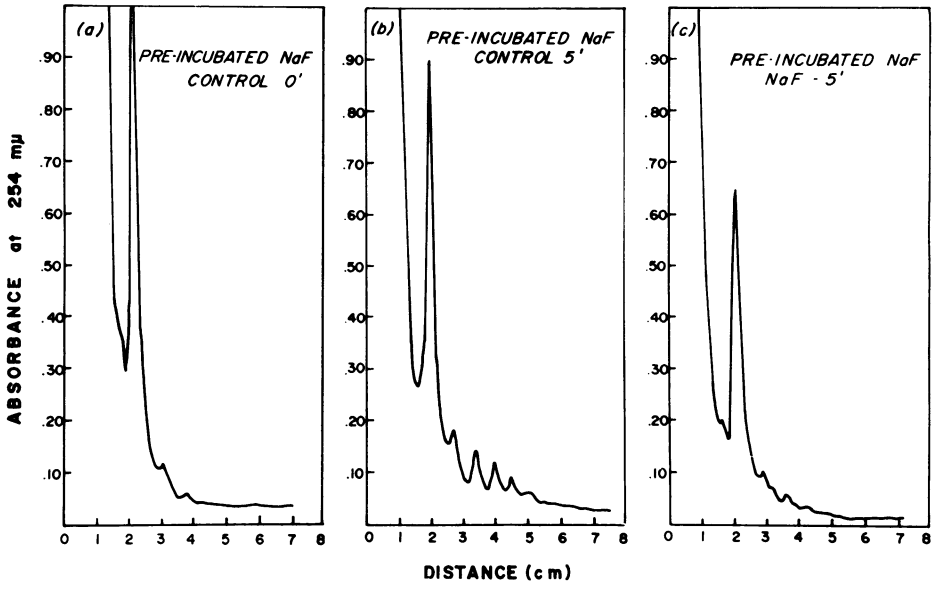

FIG. 3.—Lysate was prepared from cells that had been preincubated for 20 min in the presence of 10 mM NaF. (*a*) Lysate was fractionated at zero time as in Fig. 1. (*b*) Lysate incubated in the complete system for 5 min was fractionated as in Fig. 1. (*c*) Lysate incubated in the complete system with 10 mM NaF for 5 min was fractionated as in Fig. 1.

was recovered from these gradients; the ratios of 28*S*/18*S* RNA are presented in Table 1. The ratios, together with the sedimentation behavior, indicate that the 132*S* peak represents a disome with an additional 40*S* subunit (Table 1).

Discussion.—It is a general observation that the primary deficiency of fractionated cell-free protein-synthesizing systems from mammalian cells is a partial or complete failure in the reinitiation of new polypeptide chains.[11, 29, 30] Thus, protein synthesis in the reticulocyte cell-free system, reconsituted from ribosomes and supernatant factors by Schweet, Lamfrom, and Allen in 1958,[31] consisted almost exclusively of the completion of preexisting chains. Later, Lamfrom and Knopf[32] reported that unfractionated reticuloycte lysates were more active in initiating peptide chains, although the rate of protein synthesis still declined precipitously after an incubation of five to ten minutes at 37°C, concomitant with a conversion of polysomes to monosomes. This is in agreement with our results (Fig. 1*a*, *b*, *c*). It is for this reason that the unfractionated lysate system was selected as best suited for these investigations of the effect of fluoride on initiation. In addition to a 70-per cent inhibition of protein synthesis, the appearance of split polysome peaks was observed (Fig. 1*d*, *e*). The isokinetic gradients employed in this study[23] permit the assignment of fairly accurate sedimentation coefficients for the intermediate peaks (Table 1). It should be emphasized that the isolation of these peaks in sufficient quantity for further analysis was difficult because protein synthesis must occur for their formation (Fig. 1*f*) with the result that, even after a short period of incubation, all polysome structures were rapidly converted to monosomes and subunits.

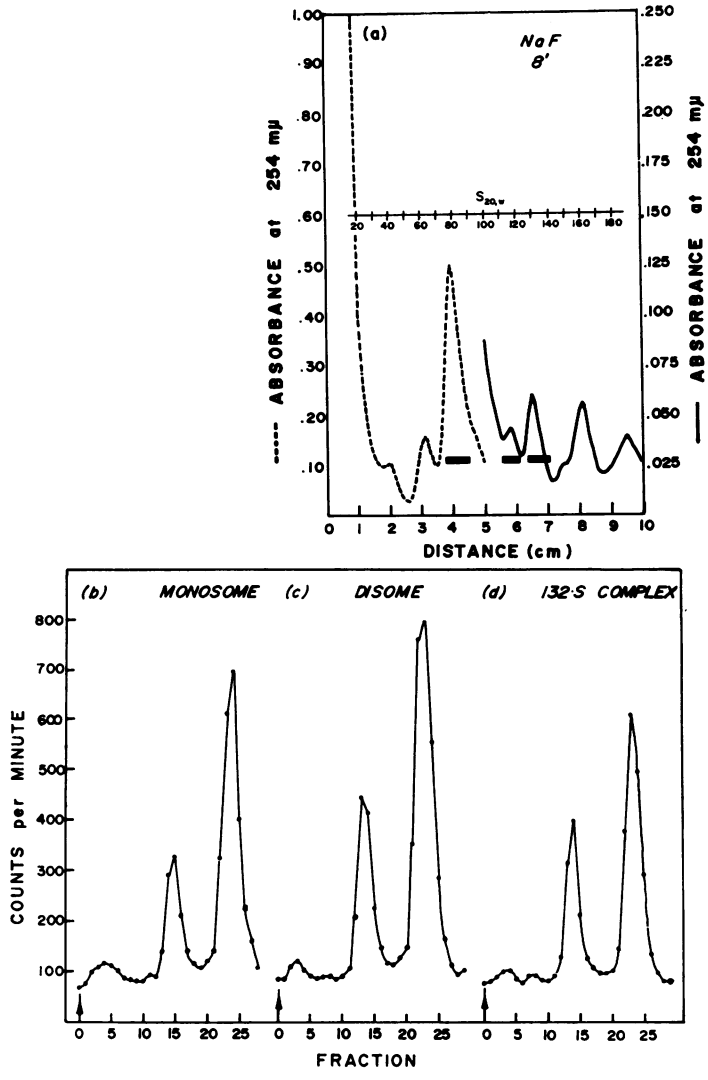

FIG. 4.—(*a*) ^{32}P-labeled lysate was incubated as in Fig. 2(*b*), layered directly on a sucrose density gradient, and sedimented for 7 hr at 22,000 rpm. The bars mark the monosome, disome, and 132*S* fractions collected from the gradient. (*b*), (*c*), (*d*) The RNA that was released from monosomes, disomes, and the 132*S* complex was sedimented for 22 hr at 22,000 rpm. The ^{32}P count distribution is shown.

However, by withholding exogenous energy from the system, we were able to arrest protein synthesis specifically at a stage where these intermediate subribosomal complexes are the predominant species. Under these conditions the patterns were not appreciably altered between 3 and 15 minutes of incubation.

The monosome and disome fractions and the 132*S* complex were characterized in terms of the ratios of 28*S*/18*S* ribosomal RNA. Since each 40*S* and 60*S* subunit is known to contain only one molecule of 18*S* and 28*S* ribosomal RNA,

respectively, the addition of a 40*S* subunit to a disome would result in a 28*S*/18*S* RNA ratio of two thirds of the value for an 80*S* ribosome. For this reason, both the monosome fraction and the intermediate 132*S* complex were collected from the same gradients with the values of the monosomes serving as an internal control. The disome fraction was contaminated with an estimated 20–30 per cent of the intermediate peak which, as collected, was about 90 per cent pure with a small amount of disome present. The results were in agreement with the predicted values, which are 1.87 for disomes plus an equal amount of 40*S* subunits and 2.44 for disomes contaminated with 30 per cent of the 132*S* complex. The calculated sedimentation coefficients (Table 1) confirm these conclusions.

We interpret our results as indicative of the addition of a 40*S* subunit to polysomes. From the known effect of fluoride on initiation,[20, 21] it is tempting to speculate that under physiological conditions initiation of new globin chains in reticulocytes starts with the addition of a 40*S* followed by a 60*S* subunit analogous to bacterial systems.[2] Fluoride may specifically prevent the addition of the 60*S* subunit. The general significance of these studies is emphasized by the fact that we have observed small amounts of these intermediate structures, by their optical density and radioactivity, in polysome patterns from ^{32}P-labeled whole cells incubated even in the absence of NaF. In parallel incubations, NaF was shown to increase their relative amounts, but never as drastically as in cell-free incubations.

These structures are highly labile, transient intermediates requiring the use of gradients with superior resolution for their demonstration. This may explain why they have not been reported previously.

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