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Treatment of Chinese Hamster Ovary Cells with the Transcriptional Inhibitor Actinomycin D Inhibits Binding of Messenger RNA to Ribosomes†

Matthew Kostura* and Nessly Craig
Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Maryland 21228
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ABSTRACT: Inhibitors of RNA synthesis such as actinomycin D, MPB, and cordycepin progressively inhibit the initiation of protein synthesis in intact, nucleated mammalian cells. This inhibition is not dependent on the levels of mRNA, ribosomes, or tRNA. Lysates prepared from CHO cells treated with actinomycin D do not incorporate labeled globin mRNA or ovalbumin mRNA into 80S initiation complexes at the rates of untreated control extract. The ability of the extracts to produce and accumulate 48S preinitiation complexes was assessed using the 60S subunit joining inhibitors edeine and 5′-guanylyl imidodiphosphate. Control extracts were able to accumulate both the 48S preinitiation complexes and the migration-related intermediates in the presence of both inhibitors. However, lysates derived from CHO cells treated with actinomycin D were unable to produce these complexes. This was also true at low temperature, a condition that does not inhibit mRNA binding but prevents migration of the 43S complex along the mRNA. Mixing experiments with extracts from untreated control or AMD-treated CHO cells provided no evidence for a translational inhibitor. Thus, our data are consistent with the hypothesis that treatment of whole cells with actinomycin D inhibits protein synthesis initiation at the level of mRNA binding and not at migration or 60S subunit joining.

A number of investigators have determined that the rate and extent of protein synthesis in nucleated eukaryotic cells is dramatically inhibited by treatment with transcriptional inhibitors such as actinomycin D (AMD),[1] MPB,[1] and cordycepin. The inhibition has been observed in numerous cell types including HeLa cells (Singer & Penman, 1972, 1973),

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* Address correspondence to this author at The Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

[1] Abbreviations: MPB, 2-mercapto-1-(α-4-pyridylethyl)benzimidazole; AMD, actinomycin D; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; eIF, eukaryotic initiation factor; $Gpp(NH_2)p$, 5′-guanylyl imidodiphosphate; pCp, cytidine 5′,3′-bisphosphate; m^7GTP, 7-methylguanosine triphosphate; CHO, Chinese hamster ovary.

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mouse L-cells and CHO cells (Craig, 1973 and unpublished observations), lymphocytes (Cooper & Braverman, 1977), and chicken erythrocytes (Banerji et al., 1984). Typically protein synthesis can decline as much as 75% over 6–8 h with only a 10% loss of mRNA (Craig, 1973; Singer & Penman, 1972). As a rule, the extent of inhibition is not correlated to the levels of tRNA, mRNA, or ribosomes, but to some translational process.

A variety of experiments have demonstrated that the blockade of protein synthesis in nucleated cells treated with transcriptional inhibitors is not a result of a direct inhibition of translation by the drugs. Concentrations of AMD that were found to inhibit translation in nucleated cells did not lower the rates of protein synthesis in enucleated lymphocytes or sea urchin merogenes (Cooper & Braverman, 1977; Sargent & Raff, 1976) or in cultured rabbit reticulocytes (Craig, 1979). Subsequently, experiments in cell-free extracts derived from wheat germ or mouse L-cells demonstrated that neither AMD, MPB, nor cordycepin inhibited translation, even at levels 10 times greater than those that inhibit protein synthesis in vivo (Leinwand & Ruddle, 1977; Craig & Kostura, 1983). Thus, the inhibition is not caused by a direct action of these drugs on the translational machinery. These observations suggest that ongoing transcription is required for optimal translation in nucleated mammalian cells.

We recently performed experiments designed to elucidate the site of protein synthesis inhibition caused by treatment of CHO cells with AMD (Craig & Kostura, 1983). Our experiments were designed to determine whether the supply of the 43S initiation complex (40S subunit: Met-tRNA$_f$) was limiting in cell-free extracts prepared from CHO cells that had been pretreated with AMD. Our results suggested that the supply of 43S complex in AMD-treated cell lysates was equivalent, if not greater, than in lysates prepared from untreated CHO cells. However, we observed that these same complexes could not be utilized efficiently in a globin mRNA-dependent formation of the 80S initiation complex. The net result was a loss of [^{35}S]Met-tRNA$_f$ associated with the 43S complex. These results suggested that a defect in translation initiation occurred in AMD-treated CHO cells between 43S complex formation and 80S complex assembly, possibly at the level of mRNA binding. However this previous work could not establish the precise point in the pathway affected by the transcriptional inhibitors because the experiments followed the binding of [^{35}S]Met-tRNA$_f$ and did not directly analyze the ability of the extracts to bind mRNA. In order to resolve this ambiguity, we have now directly analyzed mRNA binding using 3′ end-labeled mRNAs. The experiments described in this paper were designed (1) to measure the levels and kinetics of mRNA binding and formation of 80S complexes; (2) to determine whether initiation complex intermediates containing mRNA can form in cell-free extracts derived from AMD-treated CHO cells; and (3) to compare the ability of messages with known differences in primary and secondary structure in the 5′ region to bind to ribosomes of either control cell extracts or AMD-treated cell extracts. This last set of experiments compared the relative binding ability of globin mRNA and ovalbumin mRNA. Our results are consistent with the hypothesis that treatment of CHO cells with the transcriptional inhibitor actinomycin D results in the loss of the ability of the small ribosomal subunit to bind mRNA efficiently.

Experimental Procedures

Materials. 5′-[^{32}P]pCp was prepared according to the method of England and Uhlenbeck (1978). [γ-^{32}P]ATP was synthesized according to the procedure of Walseth and Johnson (1979) from carrier-free [^{32}P]orthophosphoric acid (New England Nuclear). Sparsomycin was obtained from Dr. John Douros, Natural Products Branch, National Cancer Institute; edeine, from Calbiochem; pactamycin, from Dr. Gary Neill, Upjohn Co.; actinomycin D, from Sigma; Gpp(NH$_2$)p, from Sigma; and T4 RNA ligase, from P. L. Biochemicals. All other reagents and biochemicals were the highest quality obtainable from Sigma, P. L. Biochemicals, or Boehringer-Mannheim.

The Chinese hamster ovary (CHO) cell line was grown in suspension in α-MEM with 10% horse serum (KC Biologicals or Gibco) as described by Thompson and Baker (1973). For experiments using lysates from cells treated with AMD (1.0–2.0 μg/mL), exponentially growing cultures of CHO cells were divided into two equal portions and the inhibitor was added to one portion for 4–8 h (see each experiment for particular values). This was the protocol previously described by Craig (1973) for L-cells and results in a very rapid inhibition of RNA synthesis (>90% within 1 h) and a progressive exponential decay in translation and polysome levels. Typically, CHO extracts prepared from cells treated in such a manner exhibited a 70–80% reduction in the ability to incorporate labeled amino acids into acid-precipitable material relative to untreated controls. Nevertheless, lysates prepared from AMD-treated cells still had a similar percentage of specific initiation activity as determined by pactamycin sensitivity. In all lysates used, of both types, initiation accounted for at least 50–70% of all total protein synthesis activity.

Globin mRNA was prepared from a phosphocellulose flow-through fraction (in 100 mM KCl, 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT) containing the unbound material from 4 L of total rabbit reticulocyte lysate (generously provided by Dr. Brian Safer, NIH). The flow-through material was brought to 40% saturation with ammonium sulfate (BRL, ultrapure, RNase free) at 4 °C and the precipitate collected by centrifugation at 10000g for 20 min. The precipitate contained greater than 90% of the total RNA found in whole reticulocyte lysate (Kostura, M., unpublished observation). Oviduct poly(A+) RNA was purified from the oviduct magnum of freshly killed egg-laying hens. RNA from both sources was purified by extraction with 8 M guanidine hydrochloride in 25 mM NaOAc, pH 5.0. Poly(A+) RNA was isolated by oligo-dT chromatography according to the method of Kryostek et al. (1974). Globin mRNA was further purified by centrifugation through 5–20% sucrose gradients, spun at 35000 rpm at 4 °C for 18 h in an SW-40 rotor.

3′ end-labeled globin and ovalbumin mRNA were prepared according to the method of England and Uhlenbeck (1978). Purification of the labeled product involved removal of free labeled nucleotide by Sephadex G-25 filtration and electrophoresis through 1.5% acid–urea agarose gels according to the method of Rosen et al. (1975). The labeled RNA was purified from the gels by slicing out the bands corresponding to the globin or ovalbumin RNA, crushing with a Dounce homogenizer, and then extracting the RNA from the gel with 1.0 mL of 0.3 M NaOAc, pH 6.0, with continuous shaking for 1 h. The crushed agarose was pelleted by centrifugation at 10000g, and the supernatant, which contained >70% of the labeled RNA, was filtered through a 0.45-μm nylon filter. The RNA containing filtrate was concentrated by precipitation with ethanol using 10 μg of mouse liver tRNA as carrier. Typically the labeled globin RNA had a specific activity of (5.5–6.0) $\times$ 10^5 cpm/μg. Ovalbumin mRNA had a specific activity of (6–7.0) $\times$ 10^4 cpm/μg.

Preparation of CHO Cell-Free Extracts. Extracts from CHO cells were prepared essentially as described by Fischer (1980) except that the final buffer concentration in the lysate was 135 mM KOAc, 25 mM Hepes, pH 7.4, 3.0 mM Mg-$(OAc)_2$, and 4 mM DTT. The cell-free translation reactions for mRNA binding contained 100 μL of lysate in a final volume of 200 μL with the following final concentrations of components: 30 mM Hepes, pH 7.4, 67.5–150 mM KOAc (see figure legeneds for exact concentration), 1.5 mM Mg-$(OAc)_2$, 10 mM creatine phosphate, 20 units/mL of creatine phosphokinase, 2 mM DTT, and the 20 unlabeled amino acids at 100 μM each. Incubations were carried out at 30 °C for the desired times.

Analysis of 3′ End-Labeled mRNA Binding to CHO Ribosomes. Analysis of mRNA binding to CHO ribosomes was accomplished by the complete cell-free translation system with 0.1–0.2 μg of globin mRNA or 0.25–0.35 μg of ovalbumin mRNA and various inhibitors of initiation. The inhibitors were sparsomycin (0.1 mM), an elongation inhibitor; edeine (10 μM), an inhibitor of 60S subunit joining; and Mg^{2+}Gpp-(NH_2)p (1 mM), which also prevents 60S subunit joining. The use of these compounds has been described previously (Craig & Kostura, 1983; Kozak & Shatkin, 1978; Benne & Hershey, 1978). After incubation of the CHO extracts, the reactions were quenched with an equal volume of ice-cold 20 mM Tris-HCl, pH 6.8, 50 mM KCl, 2 mM MgCl, and 0.5 mM Gpp(NH_2)p. Sucrose gradients were prepared, centrifuged, and fractionated as described previously (Craig & Kostura, 1983). All gradients were eluted from the top and passed through an Isco flow cell that recorded a continuous UV trace of the gradient. All sedimentation values were estimated by using the sedimentation of the ribosomal subunits and monomers found in the gradients as standards. All gradients were fractionated, and 0.4-mL fractions were collected directly into water. The amount of ^{32}P in each fraction was determined by quantitating the Czerenkov radiation activity using liquid scintillation spectrometry.

Results

Binding of Globin mRNA and Ovalbumin mRNA to Ribosomes in CHO Cell-Free Extracts. The ability of CHO cell-free extracts to bind mRNA was assessed with 3′ end-labeled globin mRNA as a probe. Binding assays were performed with sparsomycin both to sequester endogenous, polysome bound RNA as well as to allow the accumulation of globin mRNA-containing 80S initiation complexes. As can be observed in Figure 1, control extracts were able to accumulate a significantly greater amount of 3′ end-labeled globin mRNA into 80S initiation complexes compared to extracts derived from AMD-treated cells. It should be noted that a mRNA–43S complex ("48S preinitiation complex"; Safer et al., 1978) is not seen under these experimental conditions in either extract at any time of incubation. This was not due to an instability of the complex during centrifugation, since we have been able to observe the complex when 60S subunit joining has been made rate limiting (see below). Therefore, the rate-limiting step of translation initiation in extracts derived from both untreated and AMD-treated CHO cells is not at the level of 60S subunit joining but is at an earlier step—probably mRNA binding to the small ribosomal subunit or migration of the subunit to the AUG codon.

Binding experiments were also performed using ovalbumin mRNA. Ovalbumin mRNA was chosen because (1) earlier reports suggested that the presence of AMD will augment translation of this message in estrogen-treated hen oviduct magnum explant (Palmiter & Schimke, 1973), (2) the primary

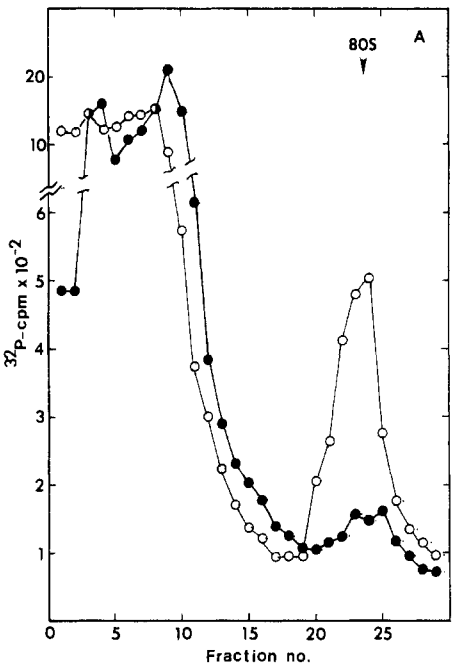

FIGURE 1: Incorporation of globin mRNA into 80S complexes in the presence of the elongation inhibitor sparsomycin. (A) A 0.2-μG portion of ^{32}P 3′ end-labeled globin mRNA was added to each reaction and incubated at 30 °C for 5 min and then centrifuged through sucrose gradients as described under Experimental Procedures. The material in fractions 20–25 represent labeled mRNA sedimenting at 80S. The top of the gradient is at the left of the panel. Sedimentation is from left to right; (○) control lysate; (●) AMD lysate.

sequence and secondary structure of the message around the 5′ end have been determined and found to be rather unstructured and weak (McReynolds et al., 1978; Schroeder et al., 1979), and (3) ovalbumin mRNA translation has been shown to be highly resistant to the presence of the cap analogues in wheat germ systems and in rabbit reticulocyte lysates (Schroeder et al., 1979; M. Kostura, unpublished observation). This last phenomenon has been previously correlated with both a lowered mRNA secondary structure and a decreased requirement for the cap-binding proteins for efficient translation (Sonnenberg et al., 1981).

A comparison of the binding abilities of both globin and ovalbumin mRNA in the CHO extracts indicates that globin mRNA was incorporated into 80S initiation complexes at a noticeably greater rate in both control or AMD lysates (see Figure 2). Assuming that differences in levels and rates of binding reflect the differences in affinity of the messages for the various translation components, it is clear that, with equimolar concentrations of input mRNA, extracts derived from CHO cells show a greater ability for binding globin mRNA compared to ovalbumin mRNA. Extracts derived from AMD-treated cells do not exhibit a greater ability to bind ovalbumin mRNA. These data suggest that the AMD-induced inhibition of translation in CHO cells does not result in an extract that exhibits a preference for binding ovalbumin mRNA.

Binding of mRNA to the Small Ribosomal Subunit Affected by RNA Synthesis Inhibition. Since the 80S complex is the end result of the initiation pathway, quantifying the incorporation of labeled mRNA into 80S complexes only allows an assessment of the total rate of protein synthesis initiation. However, this method cannot precisely determine the step in the initiation pathway at which protein synthesis may be altered and made rate limiting in AMD-treated CHO cells. Therefore, we designed experiments in which 48S

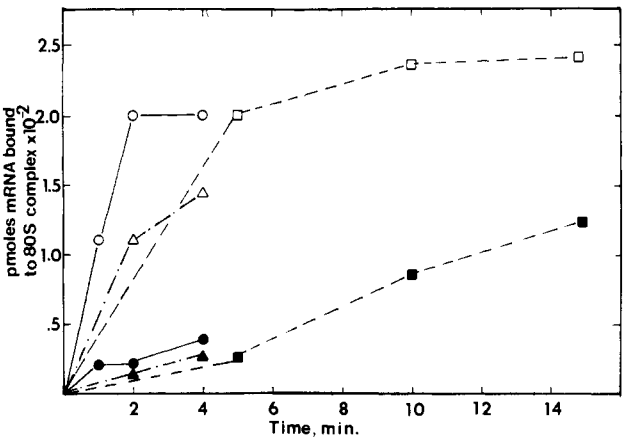

FIGURE 2: Comparison of globin mRNA and ovalbumin mRNA binding ability in CHO extracts. This graph represents a composite of the absolute amount of labeled globin or ovalbumin mRNA incorporated into 80S complexes as a function of time in the presence of the elongation inhibitor sparsomycin. Binding assays were performed according to methods: (○) globin mRNA bound to control lysate ribosomes at 150 mM K^+ concentration; (●) globin mRNA bound to AMD lysate ribosomes at 150 mM K^+ concentraton; (□) ovalbumin mRNA bound to control lysate ribosomes at 150 mM K^+ concentration; (■) ovalbumin mRNA bound to AMD lysate ribosomes at 150 mM K^+ concentrations; (△) ovalbumin mRNA bound to control lysate ribosomes at 80 mM K^+ concentrations; (▲) ovalbumin mRNA bound to AMD lysate ribosomes at 80 mM K^+. The input concentrations of mRNA for each reaction were 0.70 pmol of message for the globin mRNA; 0.62 pmol of ovalbumin mRNA at 150 mM K^+; and 0.62 pmol of input ovalbumin mRNA at 80 mM K^+.

preinitiation complexes as well as other migration-related intermediates of initiation could be identified and quantitated. In order to isolate the 48S initiation complex we used the 60S subunit joining inhibitor edeine. This inhibitor has been found to block 60S subunit joining by not allowing the proper recognition of the AUG start codon (Kozak & Shatkin, 1978). Figure 3 represents a typical gradient profile of globin mRNA binding in CHO extracts in the presence of edeine. The control extracts form an identifiable 48S initiation complex as well as heavier sedimenting complexes at 66S and 90–100S. As reported by Kozak using wheat germ extracts (1978, 1979) and confirmed by us (Craig & Kostura, 1983; Kostura and Safer, unpublished observations) in both CHO and rabbit reticulocyte lysates, the complexes represent protein synthesis initiation intermediates containing one, two, or three small subunits on a single mRNA. However, extracts derived from AMD-treated cells do not form these complexes. Since these initiation intermediates in the initiation pathway occur before the 60S subunit joins and since the formation of these intermediates is greatly reduced or lacking in the lysates derived from AMD-treated cells, aberrant 60S subunit joining is not directly involved in the inhibition of translation induced by AMD. We have confirmed this by determining that recognizable 48S complexes are not produced in AMD extracts in the presence of the nonhydrolyzable GTP analogue Gpp-(NH_2)p. Under our conditions the 60S subunit joining process is not completely inhibited with the concentrations of the analogue we used, and so we are able to observe a number of intermediates in the initiation pathway up to and including 80S complex formation. The presence of the intermediate 66S complex in these analogue experiments most likely represents events whereby joining of the 60S subunit has been sufficiently slowed so that more than one 43S complex may bind to the mRNA before the 80S complex is formed. This is consistent with observations that the formation of the migration-related intermediates is not dependent on the presence of edeine but can occur in the presence of the GTP analogue. This compound allows migration of the 43S complex to the AUG codon but no further (Kozak, 1980). Even in these experiments with this entirely different inhibitor the extracts derived from AMD-treated cells form neither the 48S nor the 66S intermediates (see Figure 4C,D).

The failure of the AMD extracts to incorporate mRNA into 48S complexes could be due to an inability to bind mRNA and form a stable 48S complex or to a defect in the migration of the 43S complex along the mRNA. In principle we can determine which of these two events were occurring if it were possible to experimentally separate mRNA binding from mRNA migration. This has been accomplished by Kozak

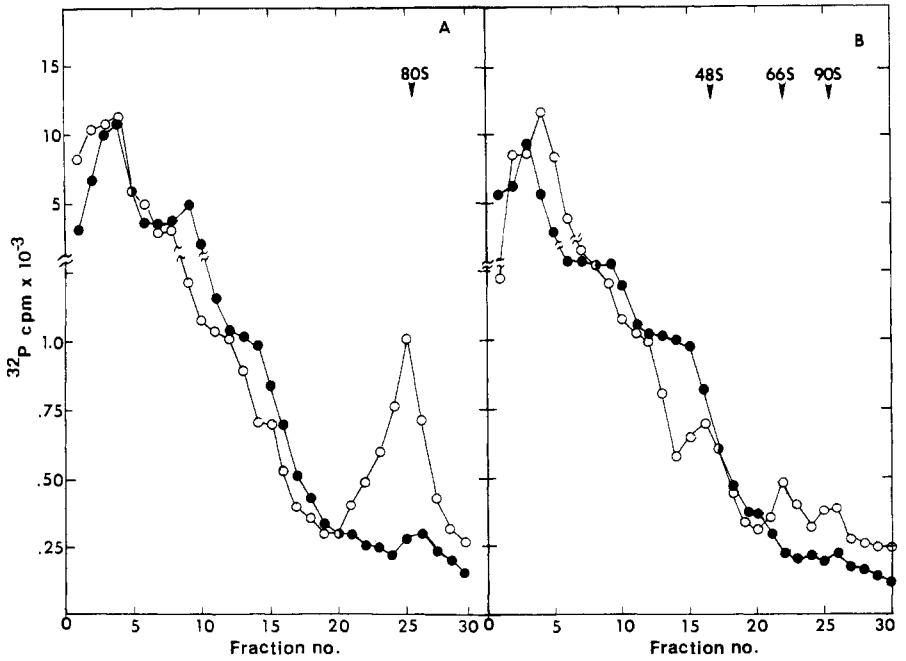

FIGURE 3: Formation of 48S complex preinitiation intermediate and migration complexes in the presence of the initiation inhibitor edeine. Binding of globin mRNA to 43S complexes was performed at 70 mM potassium in the presence of (A) 100 μM sparsomycin and (B) 10 μM edeine. All reactions were quenched after 5-min incubation at 30 °C: (○) binding of 3′ end-labeled globin mRNA to control ribosomes and ribosomal subunits; (●) binding of labeled globin mRNA in lysates derived from AMD-treated cells.

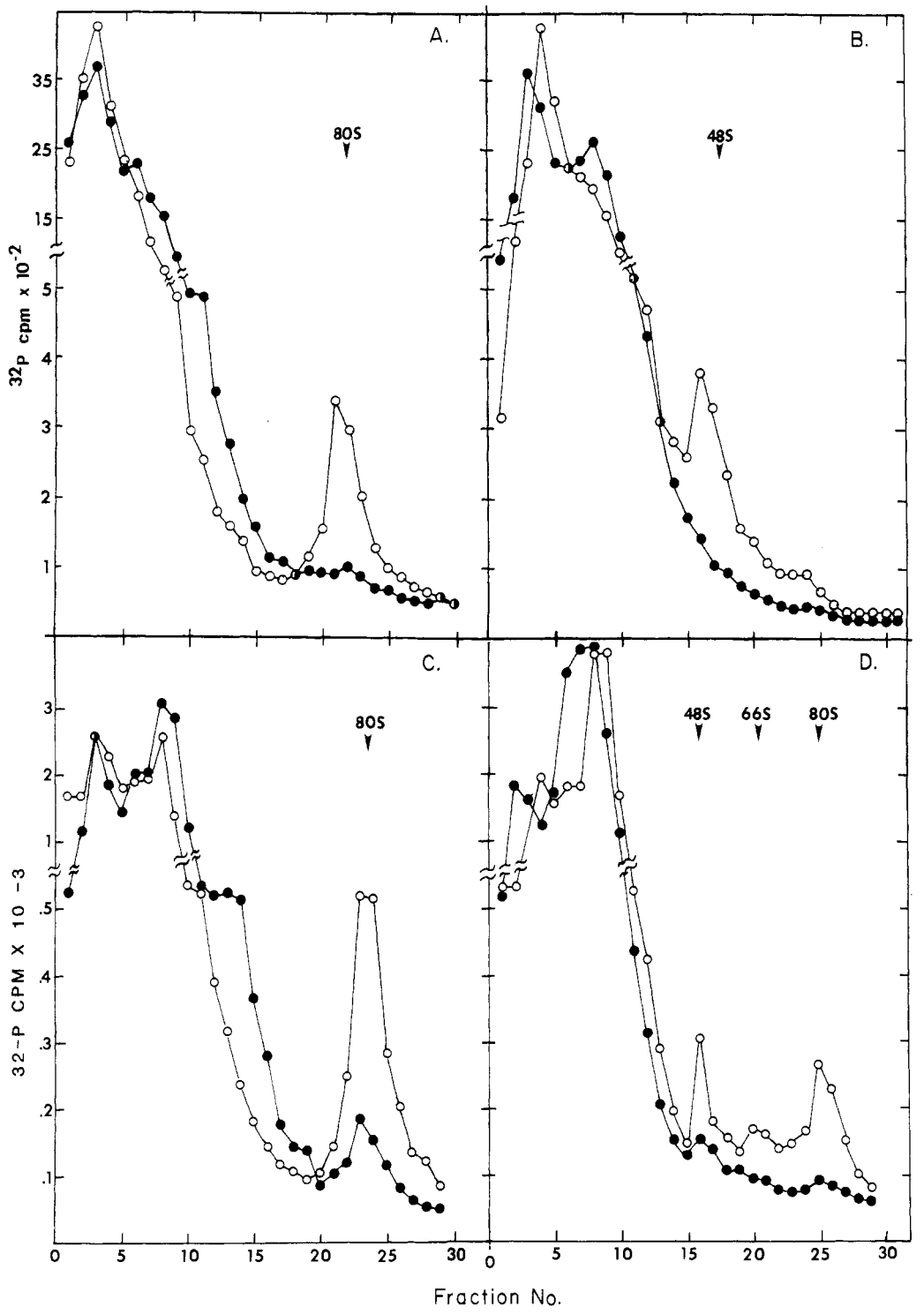

FIGURE 4: Globin mRNA binding to CHO ribosomes at low temperatures and in the presence of GTP analogue. CHO cell-free extracts were incubated for 5 min in the presence of 70 mM potassium acetate and 0.1 μg of 3′ end-labeled globin mRNA with (A) 100 μM sparsomycin at 30 °C and (B) sparsomycin at 0 °C: (○) binding of mRNA in control lysate; (●) binding of mRNA in AMD lysate. The peak of material at fraction numbers 20–25 is 80S sedimenting material. Fractions 15–19 represents material sedimenting at 48S. Figure 4C,D represents binding of globin mRNA to CHO ribosomes in the presence of the 60S subunit joining inhibitor GppNp. The experiments were performed seperatley from those shown in parts A and B. Therefore, an exact correspondence between sedimentation values is not observed. (C) Binding of mRNA in the presence of sparsomycin and 1 mM Mg^{2+}GTP. (D) Binding of mRNA in the presence of 1 mM Mg^{2+}GppNp. (○) Binding of mRNA to control lysate ribosomes; (●) binding of globin mRNA to AMD lysate ribosomes.

(1980) in wheat germ extracts but involved hexokinase treatment of the extracts as well as the use of an mRNA modified to contain unusual bases. We have devised conditions under which 48S initiation complexes can be formed in the apparent absence of migration using native globin mRNA. Performing the binding assays at 0 °C in ice–water, in the presence of creatine phosphate and creatine kinase and at low salt concentrations, allows production of the 48S complex to occur without the formation of 66S, 80S, or 90S complexes (see Figure 4A,B). Since we are only forming the 48S complex at low temperature, it would appear that we are measuring mRNA binding in the absence of subunit migration. Under these conditions it is clear that the control lysate is able to form the 48S complex but that no complex formation is observed in the extracts derived from AMD-treated cells. Therefore, lysates derived from AMD-treated cells appear to be deficient

in mRNA binding. This is supported by the observation that the 66S complex can only be formed by migration of the 43S complex along the mRNA followed by a subsequent binding of a second 43S complex at the 5′ end of the mRNA (Kozak, 1978).

The data that we have obtained are consistent with the hypothesis that mRNA binding to the 43S complex is lower in extracts derived from AMD-treated cells. Nevertheless, it is possible that the affinity of the mRNA for the 43S complex is similar in both types of extracts but that the extracts derived from AMD-treated cells lack some component or activity that increases the stability of the resultant 48S complex. At present we are unable to determine which of the two possibilities is correct.

No Dominant Inhibitors of Translation Initiation Are Observed in the AMD Extracts. One possible explanation of our results is that the treatment of CHO cells with AMD induces the formation of some inhibitor of mRNA binding. One possibility is that the mRNA are sequestered into inhibitory mRNP structures within the cell. The presence of proteins that bind to mRNA exclusively in AMD-treated cells has been recently demonstrated. Results have indicated that a protein of 32K molecular weight is found to associate with HeLa cell mRNA shortly after exposure of HeLa cells to AMD (Dreyfuss et al., 1984). Thus, it was of interest to determine whether the AMD extracts contained any components that could bind to the exogenous mRNA or mRNP and, in so doing, form a stable inhibitory structure or a complex incapable of efficiently forming an 80S initiation complex. To test this hypothesis, we performed mixing experiments in which varying amounts of control and AMD extracts were present together in the standard binding reaction. The experiments were performed such that globin mRNA was exposed to AMD extract at 0 °C and then additional control extract and master mix were added followed by subsequent incubation at 30 °C. The results of these experiments suggested that preincubation of the mRNA with the AMD extract did not lead to the formation of a stable structure that was unable to bind to control extract ribosomes (Figure 5). Total binding activity was strictly proportional to the amount of control extract present in the incubation. Reciprocal mixing experiments were performed whereby the globin mRNA was added to the control extract first followed by all other components. Identical levels of binding and the additive nature of the effect were still observed (data not shown). Thus, the AMD lysate does not contain a dominant translational inhibitor that will block the formation of the 80S initiation complex. These results support our previous work that suggests that no dominant inhibitor of translation is found in AMD extracts (Craig & Kostura, 1983). Whether the mRNP structure in the lysates derived from AMD-treated cells is associated with translation inhibition is still unclear. It is possible that the associated proteins are exchanged readily in the mixed extracts and that the resultant distribution of mRNP, and translatable mRNA, would lead to the data shown.

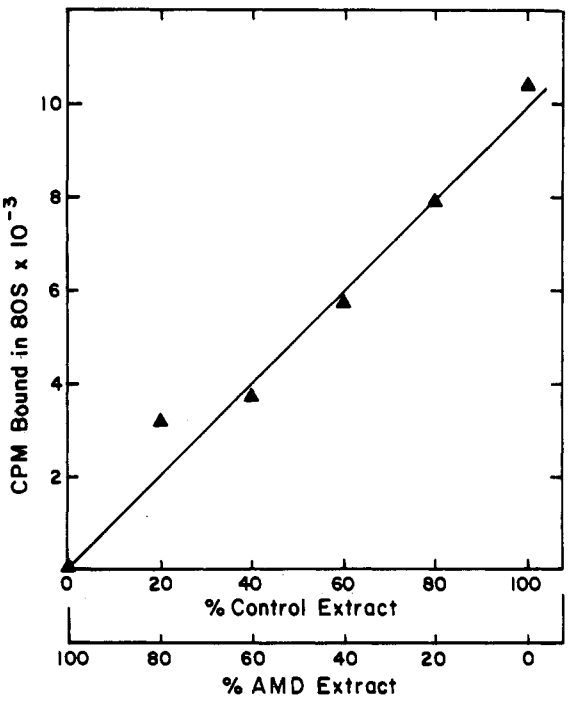

FIGURE 5: Mixing experiments with control lysates and lysates derived from AMD-treated CHO cells. Control lysate and lysate derived from CHO cells treated for 5 h, with 2 μg/mL AMD were mixed in the indicated proportions to yield 100 μL of lysate in each binding reaction. Binding assays were performed such that 0.1 μg of 3′ end-labeled globin mRNA was first mixed with a quantity of AMD lysate in the absence of master mix at 0 °C and allowed to incubate for 5 min at that temperature. Control extract was then added followed by the remaining reaction components and sparsomycin. The mixture was then incubated at 30 °C for 10 min, quenched, and analyzed on sucrose gradients according to methods. The values plotted represent material incorporated into an 80S complex. All values were derived by assessing binding to ribosomes in a reaction containing no control lysate (100% AMD extract). This was used as a control to assess the proportion of material bound at any other concentration of AMD lysate. The normalized value was subtracted from the total to determine the net binding to ribosomes due to control lysate activity. The line represents the values expected if no inhibitors were present in the AMD lysate, which would depress binding to a value lower than would be expected due to simple dilution of the control lysate.

Discussion

Treatment of nucleated cells with the transcriptional inhibitor AMD results in the loss of protein synthesis activity. Our present results suggest that the association of mRNA with the small ribosomal subunit is deficient in cells treated with AMD and that the defect resides at the level of the initial binding event. Using 3′ end-labeled globin or ovalbumin mRNA, we have demonstrated that the rate and extent of mRNA incorporation into 80S complex in the presence of sparsomycin is much less in extracts derived from AMD-treated CHO cells. In addition, we were unable to observe the accumulation of 48S complexes under identical experimental conditions. Therefore, we are not forming stable, but inactive, mRNA-containing preinitiation complexes in the AMD extracts. Subsequent experiments using the 60S subunit joining inhibitors edeine and GppNp also failed to result in significant accumulation of labeled mRNA in 48S or migration-related preinitiation intermediates. Therefore, the inability to bind mRNA in lysates derived from AMD-treated cells appears to be centered at the mRNA binding and/or the migration of the 43S complex along the mRNA to the initiation codon. We developed conditions under which we could observe mRNA binding where migration of the 43S complex along the mRNA is limiting. Such conditions involve the incubation of the full extract, using low concentrations of potassium (<70 mM), at the temperature of ice–water. When labeled globin mRNA was added to the extracts, a 48S complex was formed in the control but not in the AMD extract. We did not observe any complexes that would be formed by the migration of the 43S complex to the interior of the message followed by subsequent binding of addition 43S complex to the same message. The apparent inability of the AMD extracts to form a stable mRNA-containing intermediate under these conditions would suggest mRNA binding to the 43S complex is deficient. However, we are unable to ascertain at

this point whether this phenomenon is related to a lowered affinity of the message for the 43S complex or whether the resultant 48S complex is intrinsically unstable and is rapidly degraded.

We have previously determined that the 43S complexes formed in extracts derived from AMD-treated cells are inefficiently utilized in mRNA-dependent shift assays designed to quantitate the levels and rates of 80S complex formation in the presence of the elongation inhibitor sparsomycin. Similar results were observed when the levels of 48S complex formed in the presence of the 60S subunit joining inhibitor edeine (Craig & Kostura, 1983) were determined. The net result in both cases is an mRNA-dependent loss of [^{35}S]-Met label from 43S complexes in extracts derived from AMD-treated CHO cells. The loss is dependent on the concentration of mRNA in the reaction and is independent of 60S subunit joining. Thus, these results suggested that the potential for an interaction between 43S complex and mRNA, albeit an unstable one, is rather high. It is conceivable that the loss of Met-tRNA$_f$ associated label, and subsequent dissolution of 43S complex, is due to deacylation of the 48S complex. Nevertheless we feel that this explanation does not adequately explain our results for a number of reasons. First, deacylation has been observed in other systems (most notably in hemin-deprived rabbit reticulocyte lysates), but it is always associated with the formation of an intermediate containing nonstoichiometric amounts of deacylated tRNA and mRNA in the 48S intermediate (Safer et al., 1978; Gross, 1979). Deacylation has also only been observed during the formation of the 80S initiation complex. The 43S-associated Met-tRNA has been shown to be relatively stable provided 60S subunit joining is limited (Nygard & Hultin, 1977; Kostura, M., unpublished observations). Finally it has been demonstrated that any deacylation associated with 60S subunit joining is effectively inhibited by edeine (Balkow & Rabinowitz, 1973; Safer & Jagus, 1978; Gross, 1979). Therefore, the lack of mRNA binding in AMD extracts in the presence of edeine cannot be attributed to a defect in 60S subunit joining activity, which may lead to deacylation, and subsequent lower mRNA levels in the nascent 48S complex. Thus, our data are consistent with the hypothesis that mRNA binding or subunit migration is defective in extracts derived from AMD-treated cells.

In contrast to cell-free extracts derived from AMD-treated cells, in vivo experiments have suggested that poliovirus mRNA, encephalomyocarditis mRNA, and ovalbumin mRNA can be translated at the same or greater rates in the presence of AMD (Jen et al., 1980; Palmiter & Schimke, 1973). In the case of ovalbumin this enhancement of translation in hen oviduct magnum (called "superinduction") has been determined to reside at the level of translation inhibition, although effects on elongation were noted (Palmiter & Schimke, 1973). We monitored the capacity of the CHO extracts to incorporate ovalbumin mRNA into 80S complexes. The results suggested that ovalbumin mRNA per se does not contain any particular structure or sequence that will allow the message to be incorporated into 80S complexes to a greater extent than a globin control. This observation is consistent with other in vitro work that suggests that extracts derived from AMD-treated HeLa cells do not exhibit a preference for messages that are able to be translated in a cap-independent manner (encephalomyocarditis mRNA) relative to globin mRNA (Jen et al., 1980; Detjen et al., 1981). The reason for the apparent increase in translation from a few particular mRNAs in vivo is not readily apparent from our in vitro mRNA-binding data. However, we have observed a preferential translation of both globin and ovalbumin mRNA in the AMD extracts. This phenomenon is not associated with an increase in total translation. Rather the output of the translation system is redirected around the high quantity of exogenous message added. Therefore, it is possible that the superinduction phenomenon is a function of the intrinsic translatability of a particular message and its ability to compete for rate-limiting translational components.

There are a number of correlations between RNA synthesis inhibition and protein synthesis inhibition at the cellular level that have been reported. For instance, the onset of mitosis corresponds with a inhibition of transcription as well as translation (Tarnowka & Baglioni, 1979). A number of viral systems are known where the induced decay of host translation follows an inhibition of host transcription; in some cases (e.g., poliovirus), the translation inhibition appears to be an independent affect, but in other cases it is not yet clear. Others have suggested that ongoing transcription defines a population of mRNA capable of translation. This selection would operate at the level of the age of the transcript. Only newly transcribed mRNA would be capable of translation (Thomas & Mathews, 1984). Our data do not support this hypothesis. Highly translatable exogenous mRNA is unable to be incorporated efficiently into translation complexes in extracts derived from AMD-treated cells. AMD-treated mouse L-cells can re-form their polysomes back up to control levels when elongation is slowed with cycloheximide, showing that the rate of initiation rather than the level of translatable mRNA was affected by AMD (Craig, 1973). Therefore, no stable modifications of mRNA structure have occurred that may preclude translation. The existence of altered mRNP structures in AMD-treated HeLa cells (Dreyfuss et al., 1984) may be important in the onset of translational inhibition. However the components that are responsible for this alteration do not appear to be dominant inhibitors of translation nor do they appear to be in great excess in the AMD lysates.

In summary, it is clear that the maintenance of cellular levels of transcription appears to be important in the maintenance of ongoing translation. Whether a particular transcription product is important in the maintenance of translation or whether some, more indirect, coupling exists is unknown. Nevertheless, as shown in this paper, one aspect of this presumably important homeostatic mechanism involves the regulation of mRNA binding to the 43S initiation complex.

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Accuracy of Natural Messenger Translation: Analysis of Codon–Anticodon Recognition in a Simplified Cell-Free System†

Didier Nègre, Alain J. Cozzone,* and Yves Cenatiempo
Laboratory of Molecular Biology, University of Lyon, 69622 Villeurbanne, France
Received February 28, 1986; Revised Manuscript Received May 27, 1986

ABSTRACT: A simplified plasmid-directed coupled system [Robakis, N., Cenatiempo, Y., Meza-Basso, L., Brot, N., & Weissbach, H. (1983) *Methods Enzymol. 101*, 690–706] was used to study the accuracy of natural messenger translation in vitro. In this system, protein synthesis is limited to the formation of the N-terminal di- or tripeptide of the gene product. Such a control is obtained by restricting the supply of aminoacyl-tRNAs in the assay medium to those corresponding specifically to the first two or three triplets in the mRNA coding sequence. We analyzed comparatively the interaction of 6 different codons with their cognate tRNAs and 18 noncognate tRNAs able to recognize triplets differing from the legitimate sequences by one base only. Special attention was paid to the single base errors occurring at the first and second codon positions during ribosomal selection of aminoacyl-tRNA molecules. The noncognate tRNAs were assayed either in the absence of the legitimate tRNAs or under competition conditions. They were chosen so that all the possibilities for misreading any particular base as each of the other three bases could be studied. First, it was mainly observed that translation mistakes can be equally detected in the first and second codon positions; there is no compelling evidence for a most or least accurate site. Second, pyrimidines seem to be read more accurately than purines. In particular, U cannot be read as either C or G, and C can hardly be mistaken for any other base. By contrast, A is easily read as U; also, G can be taken as A, U, or C, at least when the cognate tRNAs are totally missing. Finally, there is no selective misreading of any base of a given class, purine or pyrimidine, by the other base of the same class. Thus, the purine A can be read as the pyrimidine U and vice versa; similarly, G can be mistaken for U or C.

Several types of translational errors occurring in prokaryotes have been described (Hopfield & Yamane, 1980; Abraham, 1983), but there is ample evidence that misreading of the genetic information is mostly due to inaccurate aminoacyl-tRNA selection on the ribosome during protein synthesis (Gallant & Foley, 1980; Yarus & Thompson, 1983; Brakier-Gingras & Phoenix, 1984). To study the process of this selection and to characterize the missense errors that result from anomalous codon–anticodon recognition, a number of in vivo situations have been analyzed. These include, for instance, illegitimate incorporation of cysteine in *Escherichia coli* flagellin (Edelmann & Gallant, 1977), mistranslation of an ochre codon in the *phoA* gene for alkaline phosphatase (Ellis & Gallant, 1982), or modification of the total charge of proteins synthesized during amino acid starvation (O'Farrell, 1978). However, most experiments in the field have been carried out in vitro by analyzing the expression of both natural and synthetic messengers in various cell-free systems (Yarus & Thompson, 1983). Some fundamental patterns of codon–anticodon interaction have thus been elucidated such as the classical wobble rules (Crick, 1966), the more recently proposed "two-out-of-three" hypothesis (Lagerkvist, 1978), or else the notion of "extended anticodon" (Yarus, 1982). The

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