Articles

Inhibition of Protein Synthesis in CHO Cells by Actinomycin D: Lesion Occurs after 40S Initiation Complex Formation†

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ABSTRACT: Inhibitors of RNA synthesis such as actinomycin D, 2-mercapto-1-(β-4-pyridylethyl)benzimidazole, and cordycepin progressively inhibit the initiation of protein synthesis in intact nucleated mammalian cells independent of their effect on mRNA synthesis. The mechanism of this effect is unknown. The activity of cell-free lysates is not directly affected by these inhibitors, suggesting that their effect is indirect and requires an intact cell. However, lysates prepared from L-cells or CHO cells treated with the inhibitors do exhibit a decrease in initiation activity corresponding in magnitude to the effect seen in intact cells. Mixing experiments with lysates isolated from untreated or treated cells provide no evidence for a translational inhibitor. However, experiments analyzing the incorporation of [^{35}S]methionine and [^{35}S]Met-tRNA$_f$ into initiation complexes showed that while the level of labeled 40S initiation complex in lysates from treated cells was the same or higher than in control lysates, the rate or efficiency of formation of the 80S initiation complex was inhibited. These results imply that the transcriptional inhibitors do not affect the level or charging of the initiation tRNA^{Met}, the activity of the eIF-2 initiation factor needed for ternary complex formation, and the availability of active 40S ribosomal subunits. Thus, this site of action is different from that observed in other translational control systems such as the hemin response in reticulocytes and the interferon-induced translation inhibition in virally infected cells. This effect may reflect the cell's coordination of nuclear transcription and cytoplasmic translation.

Since a significant proportion of the messenger RNA of many mammalian cells has a turnover time on the order of a cell generation (Perry & Kelly, 1973; Singer & Penman, 1972), translational control would appear to be the primary means for short-term regulation of the rate of protein synthesis. Transcriptional control is usually more important for long-term or major changes in gene expression such as the activation of the globin genes in erythroblasts. Because there are homeostatic regulatory mechanisms present in multicellular organisms, short-term regulation of genetic expression is not as crucial to the survival of the individual cell as it is in microbial cells. Nevertheless, it is clear that translational control is still important for a cell's response to alterations in its environment. These alterations include nutritional, energetic, ionic, and developmental changes which can occur in normal growth and development as well as in various disease or deficiency states [reviewed in Jagus et al. (1981)].

There are several mechanisms of translational control which have been intensively studied. These include the hemin-dependent synthesis of globin in reticulocytes (Jagus et al., 1981), the effect of interferon on translation in virus-infected cells (Farrell et al., 1978), and the virus-induced turnoff of host cell translation in nonerythroid cells (Trachsel et al., 1980; Tahara et al., 1981; Ray et al., 1983). However, another control mechanism which appears to be different from these deals with the effect of transcription on translation. We and others have shown, using several cell types (Singer & Penman, 1972; Craig, 1973, 1979; Cooper & Braverman, 1977), that actinomycin D, MPB,[1] and cordycepin which are primarily known for their inhibition of RNA synthesis also progressively inhibit translation. The cell types affected include mouse L-cells, mouse myeloma cells, HeLa cells, CHO cells, and human lymphocytes. The inhibition occurs at the level of initiation and does not involve any significant decrease in the level or activity of the normally stable ribosomes, tRNA, and mRNA. Inhibition does not occur in rabbit reticulocytes which lack a nucleus or in cytochalasin B enucleated lymphocytes (Cooper & Braverman, 1977; Craig, 1979). This phenomenon is somewhat of an enigma at the present time, since there is nothing in the currently accepted mechanism of protein synthesis that can explain it.

The experiments described in this paper are concerned with establishing the biochemical basis for the effect of inhibition of RNA synthesis on translational initiation. The results show that actinomycin D and MPB do not act directly on the translational system but induce, in intact cells, some defect in an initiation step that occurs after the formation of the [40S·eIF2·Met-tRNA$_f$·GTP] complex.

Experimental Procedures

Materials. The Chinese hamster ovary (CHO) cell line (from Dr. Sharon Krag of Johns Hopkins University) was grown in suspension in α-MEM with 10% horse serum (KC Biologicals) as described by Thompson & Baker (1973). Mouse L-cells were grown as previously described (Craig, 1973). For experiments using lysates prepared from cells treated with inhibitors of RNA synthesis (1.0 μg/mL actinomycin D or 50 μg/mL MPB), exponentially growing cultures of either L-cells or CHO cells were divided into two portions, and the inhibitor was added to one portion for varying

† From the Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Maryland 21228. *Received April 15, 1983.* Supported by American Cancer Society Grant CD-55.

[1] Abbreviations: MPB, 2-mercapto-1-(β-4-pyridylethyl)benzimidazole; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; eIF, eukaryotic initiation factor; Gpp(CH$_2$)p, guanosine 5′-(β,α-methylenetriphosphate); SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/83/0422-6064$01.50/0

times (see each experiment for particular values). This was the protocol previously described by Craig (1973) for L-cells and results in a very rapid inhibitor of RNA synthesis (>90% within 1 h) and a progressive exponential decay in translation and polysome levels (half-life of 4–6 h). The response to CHO cells was essentially identical with that of L-cells (unpublished data).

Reticulocyte poly(A)+ RNA ("globin mRNA") was prepared as described by Krystosek et al. (1974). Total mouse liver tRNA was prepared, and the $tRNA^{Met}$ charged, with [^{35}S]methionine as described by Stanley (1979). [^{3}H]Leucine (59 Ci/mmol), [^{35}S]methionine (1084 Ci/mmol), [8-^{14}C]ATP (49.3 mCi/mmol), [8-^{14}C]GTP (48.4 mCi/mmol), [2,8-^{3}H]adenosine (34.2 Ci/mmol), and [8-^{3}H]guanosine (6.15 Ci/mmol) were from New England Nuclear. Sparsomycin was obtained from Dr. John Douros, Natural Products Branch, National Cancer Institute, MPB from Midland Tar Distillers Ltd., Birmingham, England, pactamycin from Dr. Gary Neil, Upjohn Co., edeine from Calbiochem, and actinomycin D from Sigma. The nitrocellulose filters (HAWP, 0.45 μm pore size) were from Millipore. All other chemicals and biochemicals were the highest grade obtainable from Sigma or J. T. Baker.

Cell-Free Systems for Protein Synthesis. CHO lysates were prepared as described by Fischer et al. (1980) except that in some experiments the lysates were centrifuged for only 10 min at 10000*g* before being gel-filtered through Sephadex G-25. L-cell lysates were prepared as previously described (Craig & Fahrman, 1977). In some cases the proportion of incorporation activity due to initiation was increased by preincubating these L-cell lysates in a complete translational system with an energy source and all of the amino acids for 20 min at 30 °C. These preincubated lysates were then gel filtered to remove small molecules such as amino acids which would decrease the specific activity of the labeled amino acids to be added later. The cell-free translation reactions contained 18–25 μL of lysate in a final volume of 50 μL with the following components: 25 mM Hepes, pH 7.3, 150 mM KCl or KOAc, 2.9 mM $Mg(OAc)_2$, 1 mM ATP, 0.2 mM GTP, 6 mM creatine phosphate, 20 units/mL creatine phosphokinase, 5 μCi of [^{3}H]leucine, the other unlabeled 19 amino acids at 100 μM, and 1 mM dithiothreitol. Incubations were at 30 °C, and 5-μL aliquots, taken at varying times, were spotted onto Whatman No. 1 filter paper and processed as previously described (Craig & Fahrman, 1977). This cell-free translational system was suitable for the analysis of the basis for the inhibition of protein synthesis by actinomycin D, MPB, and cordycepin since this system had a significant initiation activity. This was seen in its response to 1 μM pactamycin which is a specific inhibitor of translational initiation (Goldberg et al., 1973) (see Figures 1 and 2). This system also synthesized globin in response to added reticulocyte poly(A)+ mRNA (unpublished data).

Analysis of 40S and 80S Initiation Complex Formation. The formation of the "40S" initiation complex containing the 40S ribosomal subunit with eIF-2, GTP, and $Met\text{-}tRNA_f$ was analyzed on sucrose gradients by using modifications of the procedures of Safer et al. (1979), Lenz & Baglioni (1979), and Pain et al. (1980). In brief, either [^{35}S]methionine or [^{35}S]$Met\text{-}tRNA_f$ was added for 1–10 min at 30 °C to complete cell-free systems that had been preincubated for 2 min with the appropriate inhibitor. Sparsomycin (1 mM) was used to inhibit elongation (Goldberg et al., 1973), the nonhydrolyzable GTP analogue ($Gpp(CH_2)p$)[1] (1 mM) to prevent the addition of the 60S ribosomal subunit to form the 80S complex (Benne & Hershey, 1978), and edeine (5 μM) to block 60S subunit joining without blocking the movement of the 40S initiation complex along the mRNA (Kozak & Shatkin, 1978). At the end of the incubation the reaction mixture of 200 μL was quenched with an equal volume of 0.5 mM $Gpp(CH_2)p$ in 25 mM Hepes, pH 7.4, and layered onto a 12-mL sucrose gradient ranging from 15% to 40% (w/w) sucrose in 20 mM Tris-HCl, pH 6.8, 50 mM KCl, and 2 mM $MgCl_2$. The gradients were spun at 40000 rpm in a Beckman SW40 rotor at 3–4 °C for 4–4 1/2 h. Each gradient was pumped, beginning at the top, through a Gilford 250 spectrophotometer to monitor absorbancy at 260 nm and collected as separate 0.4-mL fractions into an equal volume of 10 mM sodium acetate, pH 5.3, 5 mM $MgCl_2$, and 1 mg/mL methionine. Each fraction was passed through a Millipore filter, and the filter was washed with 5 mM $MgCl_2$, dried, and then counted in a scintillation counter. The counting efficiency was about 33%. Control experiments with either cold 5% trichloroacetic acid precipitation or precipitation with cetyltrimethylammonium bromide (Darnbrough et al., 1973) showed that the Millipore filtration retained the same amount of the [^{35}S]Met-tRNA bound to ribosomal particles.

The formation of the 80S initiation complex or the movement of the 40S complex along mRNA was analyzed in exactly the same way, with the exception that it was necessary to add reticulocyte poly(A)+ RNA at 0.1–15 μg/200 μL because the majority of the endogenous mRNA was tied up in polysomes and not available. As reported by Kryostek et al. (1974) and confirmed by electrophoresis in acid urea–agarose gels, this RNA preparation contains about 25–35% 9S RNA (presumably globin mRNA) with the remainder as 18S.

Analysis of ATP and GTP Levels. A comparison of the levels of ATP and GTP found in control cells and in cells treated with the three inhibitors of RNA synthesis were determined by using two different techniques. ATP was directly determined by using the luciferin–luciferase assay as described by Emerson & Humphreys (1971). There were three to five separate experiments for each of the inhibitors, and each involved two to three incubation times ranging from 1 to 5 h. However, because there was no significant difference between the results for the various incubation times, these results were pooled. The level of ATP in untreated cells in 5 experiments with 27 assays was 11.1 + 1.2 (SEM) nmol/10^6 cells. The method described by Vaughan & Hansen (1973) was used in two experiments to measure the relative values for both ATP and GTP between the treated and untreated cells. This involved incubating the cells with [^{3}H]adenosine and [^{3}H]-guanosine to label their ribonucleotide pools, isolating the cellular ribonucleotides in the presence of a known amount of [^{14}C]ATP and [^{14}C]GTP added as internal controls for recovery and as normalization factors, and then separating the ribonucleotides on a Dowex–formate column for analysis. This method does not give abolute values of ATP and GTP per cell but values relative to the ^{14}C standards which can then be compared to determine whether or not exposure of the cells to the inhibitors of RNA synthesis had altered the ATP and GTP levels.

Results

Transcription Inhibitors Do Not Affect Cell-Free Translation. Previous experiments with several cell types (Singer & Penman, 1972; Craig, 1973, 1979; unpublished experiments) showed that inhibitors of RNA synthesis such as actinomycin D, MPB, and cordycepin progressively inhibited the initiation of protein synthesis. To determine whether these inhibitors affected translation initiation directly by interacting with some

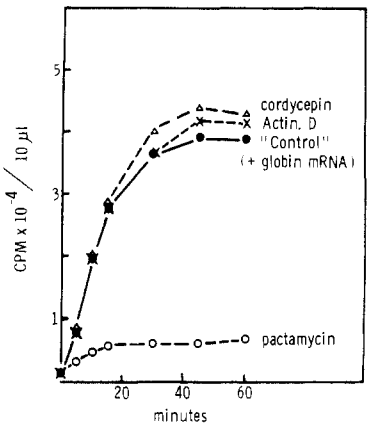

FIGURE 1: Effect of 10 µg/mL actinomycin D and 50 µg/mL cordycepin on [^{3}H]leucine incorporation at 30 °C in L-cell lysates with added rabbit reticulocyte poly(A)+ RNA (globin RNA). The lysates were prepared as described under Experimental Procedures. Pactamycin at 1 µM was added to assay how much of the total incorporation was due to initiation events. Omission of the globin mRNA reduced the incorporation by less than 10%.

component of the translational system or whether the effect was more indirect, they were added to a cell-free system capable of protein synthesis. As can be seen in Figure 1, neither actinomycin D nor cordycepin at concentrations equal to or even high than those capable of inducing translational inhibition in intact cells had any significant effect on either the rate or extent of leucine incorporation. Furthermore, neither actinomycin D nor MPB inhibited the amount of globin synthesized as assayed with SDS gel electrophoresis (data not shown). These experiments suggest that the effect of these inhibitors is indirect and is the result of some alteration in the metabolism of the intact cell.

Transcription Inhibitors Do Not Cause a Depletion of Intracellular ATP and GTP. One possible effect of these inhibitors could be to induce a change in the intracellular levels of either ATP or GTP. A significant decrease in either nucleotide could cause an inhibition of translation. However, measurement of the relative levels of ATP and GTP by two independent procedures did not show any decrease in intact cells treated with the RNA synthesis inhibitors. For example, while control cells had 11.1 ± 1.2 (SEM) nmol of ATP/10^6 cells, cells treated with actinomycin D (2 µg/mL) had 12.2 ± 0.9, with MPB (50 µg/mL), 13.2 ± 0.9, and with cordycepin (45 µg/mL), 12.3 ± 0.8. The percentage change in ATP levels in the treated cells compared to the untreated cells was determined by using the more indirect Dowex column method and found to be +24% for actinomycin D, +22% for MPB, and +56% for cordycepin. There was an increase in GTP levels (+288% for actinomycin D, +129% for MPB, and +250% for cordycepin), but the significance of this is not clear.

Lysates of Cells Preincubated with Transcription Inhibitors Are Deficient in Translation. The hypothesis that actinomycin D and MPB affect protein synthesis indirectly was confirmed by analysis of the translational abilities of cell-free systems isolated from either L-cells or CHO cells treated with either inhibitor at various doses (1–4 µg/mL) for actinomycin D) for varying times ranging from 2 to 10 h. The basic findings were that the level of incorporation and the amount of initiation activity in lysates prepared from treated cells generally correlated with the extent of inhibition observed in intact cells exposed to the same dosage for the same time. A typical example is shown in Figure 2 for CHO cells. These cells are essentially identical in their in vivo and in vitro responses to L-cells which were used in the previously published experiments (Craig, 1973). In this experiment (Figure 2), the

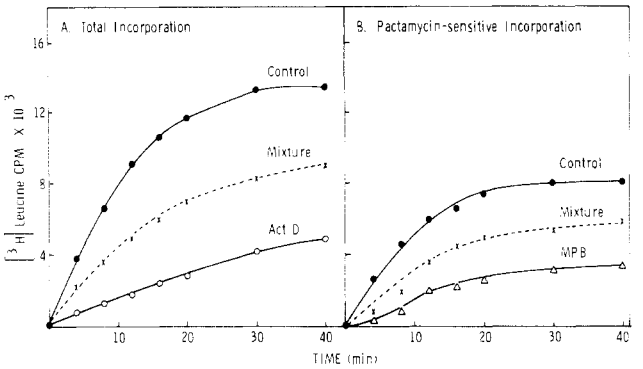

FIGURE 2: Effect of mixing cell-free lysates from control CHO cells and from treated cells on the incorporation of [^{3}H]leucine. Lysates from untreated cells and from treated cells were assayed separately or as a mixture in equal proportions. (A) Total incorporation. Actinomycin D treated cells were exposed to 1 µg/mL for 5 h. (B) Pactamycin-sensitive (="initiation") incorporation. The treated cells were exposed to 50 µg/mL MPB for 5 h. The difference between total incorporation and incorporation in the presence of 2 µM pactamycin is plotted. (●) Lysates from control cells; (○) lysates from actinomycin D treated cells; (△) MPB lysate; (×) mixture of equal parts of control and treated lysates. The dashed line represents the values predicted for strict additivity.

amount of initiation activity as assayed by pactamycin sensitivity (see Experimental Procedures) was only 40% of the control value of the actinomycin lysate (the value expected from the in vivo results was approximately 50%) and 35% for the MPB lysate (expected was approximately 40%). Thus, the lesion in translational initiation caused by these inhibitors in intact cells is still present in the cell-free lysates. This means that the system can be studied and dissected biochemically.

A lower translational activity could be the result of a loss or alteration of some component(s) required for optimum synthesis or due to the presence of an inhibitor which blocks protein synthesis. One approach to distinguishing between these possibilities is to perform "mixing experiments" in which lysates from both control and treated cells are mixed together in various proportions and then assayed for their activity. If treated cells contain an inhibitor which is present in excess or capable of acting catalytically, the activity of the combined lysates would be less than that expected on the basis of simple additivity. If one lysate were partially deficient in some necessary component and if the other lysate did not have it in excess, then one would expect additive synthetic activities. Figure 2 shows that in this type of experiment with lysates from either actinomycin D or MPB treated cells, both total incorporation and "initiation" activity appeared to be additive. This was true over a 2-fold range in mixture proportions (not shown). These experiments imply that treatment with actinomycin D or MPB does not produce a translational inhibitor(s) as been seen in other translational control systems (Jagus et al., 1981). The simplest hypothesis is that there may have been a progressive loss or alteration of some factor(s) required for protein synthesis.

Charging of $tRNA_f$ and 40S Initiation Complex Formation Are Unaffected by RNA Synthesis Inhibition. The initial steps in the initiation process of translation involve the charging of $tRNA^{Met}$, the formation of the ternary complex of eIF-2·GTP·Met-$tRNA_f$, and the binding of this complex to the 40S ribosomal subunit (Jagus et al., 1981). To determine whether the formation of the 40S initiation complex is affected by transcriptional inhibitors, we compared the incorporation of [^{35}S]methionine into [^{35}S]Met-$tRNA_f$ bound to the 40S complex in cell-free systems prepared from control cells and from cells treated with the inhibitors. Incorporation of

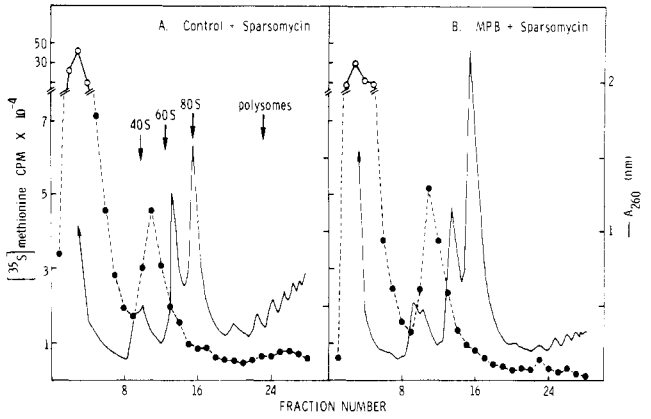

FIGURE 3: Analysis of the formation of the 40S initiation complexes in lysates from control CHO cells and in lysates from cells treated with 50 μg/mL MPB for 4 h. Complete cell-free protein synthesis systems with each lysate were treated for 2 min at 30 °C with 100 μM sparsomycin to inhibit elongation, [^{35}S]methionine at 200 μCi/mL was added for 4 min, and the reaction was then quenched with 200 μM Gpp(CH$_2$)p. The ribosomal particles were analyzed in a 15–40% sucrose gradient in low salt buffer (pH 6.8) and spun for 40000 rpm for 4.5 h. The direction of sedimentation is from left to right. The radioactivity in each fraction was analyzed by using CTAB precipitation. Other experiments using hot (90 °C, 5–10 min) and cold (4 °C) trichloroacetic acid precipitations indicated that essentially all of the 40S associated radioactivity is tRNA bound (presumably Met-tRNA$_f$). (A) Control lysate plus sparsomycin. (B) MPB lysate plus sparsomycin. (—)A_{260} (nm); (●) ^{35}S radioactivity (×10^{-4}).

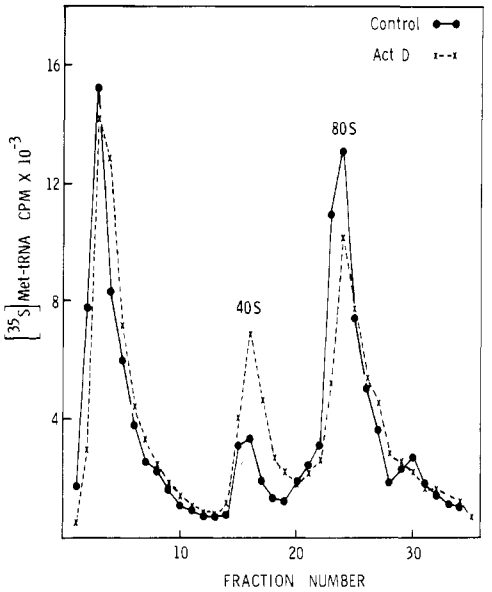

FIGURE 4: Binding of [^{35}S]methionine-tRNA$_f$ to ribosomal particles in the presence of 100 μM sparsomycin and an excess of added reticulocyte polysomal poly(A)+ RNA (globin RNA). The actinomycin D lysates were prepared from CHO cells treated with 1 μg/mL actinomycin D for 10 h. The [^{35}S]Met-tRNA$_f$ was prepared with total mouse liver tRNA and *E. coli* aminoacyl-tRNA synthetase which recognizes only the initiation tRNA^{Met}, not the internal tRNA^{Met} (Stanley, 1979). The protein synthesis systems were preincubated for 2 min at 30 °C with sparsomycin and then with 15 μg/200 μL reticulocyte poly(A)+ RNA and [^{35}S]Met-tRNA$_f$ (5.5 × 10^6 cpm/mL, ~1.3 × 10^6 cpm/pmol) for 20 min. The reactions were then quenched and analyzed as described in Figure 3, except that Millipore filtration in the presence of 5 mM $MgCl_2$ was used instead of CTAB precipitation. (●) Control lysate (ratio 80S/40S cpm = 4.1); (×) actinomycin D lysate (ratio 80S/40S cpm = 1.8).

[^{35}S]methionine into Met-tRNA$_f$ bound to the 40S ribosomal subunit can be distinguished from methionine incorporated into polypeptides by analyzing its susceptibility to hydrolysis by trichloroacetic acid; both forms are precipitated by cold trichloroacetic acid, but only Met-tRNA is hydrolyzed by trichloroacetic acid at 90 °C for 5–10 min. To minimize the incorporation of [^{35}S]methionine into polypeptides, these experiments were carried out in the presence of sparsomycin, an inhibitor of elongation (Goldberg et al., 1973).

Figure 3 shows the results of this type of experiment using lysates from cells treated with MPB. The results for lysates of cells pretreated with actinomycin D were the same (data not shown). In the presence of sparsomycin and no exogenously added mRNA, there was a predominant peak of radioactivity located over the more rapidly sedimenting form of the 40S ribosomal subunit. As determined by its trichloroacetic acid solubility characteristics, this radioactivity represents [^{35}S]Met-tRNA$_f$ bound to the 40S. Therefore, this peak represents the 40S initiation complex. It is also clear that both the control lysates and the MPB lysates have comparable levels. These results imply that the treated lysate has no deficiency in the level of tRNA^{Met}, in the level or rate of charging by the methionine aminoacyl synthesis, in the activity or quantity of eIF-2, and in the rate/level of 40S initiation complex formation.

A second independent assay of 40S initiation complex formation is to use precharged [^{35}S]Met-tRNA$_f$, instead of [^{35}S]methionine. This assay does not depend on the level and degree of charging of the tRNA^{Met} but only on the formation of the [eIF-2·GTP·Met-tRNA$_f$] ternary complex and its binding to the 40S ribosomal subunit. Even in this assay, there were comparable amounts of the 40S initiation complex formed in control lysates and in lysates prepared from cells exposed to 1 μg/mL actinomycin D for up to 10 h (see next section).

Formation of the 80S Initiation Complex Is Affected by Pretreatment of Cells with Transcription Inhibitors. In the presence of added mRNA, precharged [^{35}S]Met-tRNA$_f$ can be used to measure the rate and extent of 80S complex formation in an unfractionated cell-free system. To trap the 80S initiation complex and to prevent loss of the ^{35}S radioactivity due to cleavage of the N-terminal methionine that often accompanies translation, sparsomycin can be used to block elongation. The formation of the 80S complex requires at least three major steps catalyzed by different initiation factors: binding of the 40S complex to the mRNA, movement of the 40S subunit along the mRNA to the initiation AUG codon, and joining of the 60S ribosomal subunit to the 40S complex. According to Kozak (1981), the binding of the mRNA appears to involve the 5′-capped end, with the first AUG codon encountered by the 40S complex being used most often as the initiation codon. Figure 4 shows the results of this type of experiment with added reticulocyte poly(A)+ "globin mRNA" added at a level determined by other experiments (see Figure 5) to be in excess. In this particular experiment the labeling time was 20 min since after this time there was no further incorporation of the [^{35}S]Met-tRNA$_f$ into either the 40S or the 80S complex. There are several observations that can be made. In both lysates there is significant and comparable binding of the [^{35}S]Met-tRNA$_f$ to the 40S and 80S ribosomal initiation complexes considered together. This implies that both lysates have the ability to form initiation complexes. However, the second major observation is that in the actinomycin D lysate the proportion of [^{35}S]Met-tRNA$_f$ found in 80S complexes compared to the total ribosomally bound radioactivity is much less than it is in the corresponding control lysates; the 80S/40S ratio of 1.8 is about 44% of the corresponding ratio in the control lysate. The actinomycin D lysate in this experiment was prepared from cell treated for 10 h of 1 μg/mL; our previous work for L-cells (1973) and unpublished for CHO cells[2] had shown that at this dose and exposure

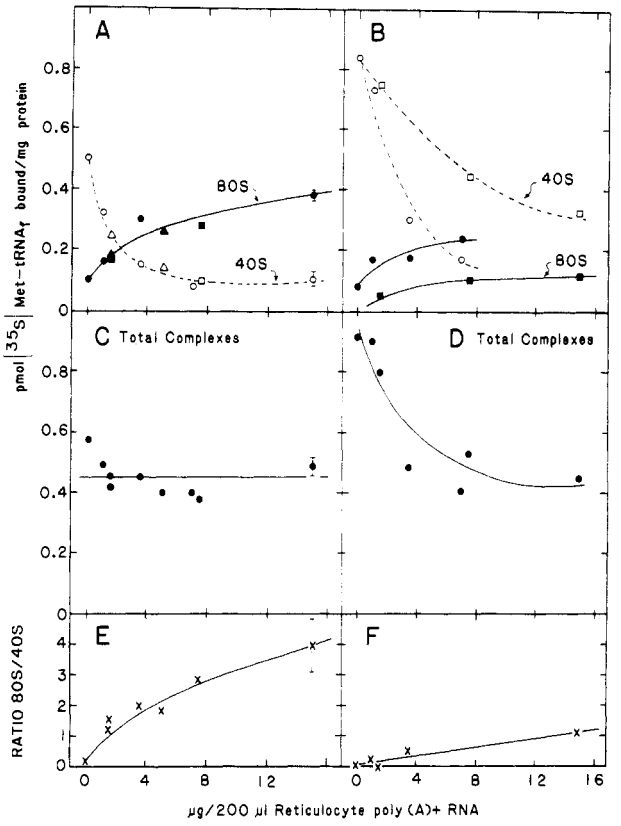

FIGURE 5: Effect of reticulocyte globin mRNA concentration on the distribution of [^{35}S]Met-tRNA$_f$ in 40S and 80S initiation complexes after 10 min of incubation in sparsomycin-treated lysates. The reactions were incubated and analyzed as described in Figure 4. A number of different matched pairs of lysates were used, but the incubation time was always 10 min, which is before the incorporation of the Met-tRNA$_f$ into the 80S complexes levels off. Thus, the results reflect the rate of conversion of the 40S complex to the 80S complex. The actinomycin D lysates were prepared from CHO cells treated for 7–10 h at 1 µg/mL. The results of the different experiments and lysates (four for control; three for actinomycin D) are normalized by plotting picomoles of [^{35}S]Met-tRNA$_f$ bound per milligram lysate protein. (A) The distribution of 40S (open symbols; each lysate indicated with a different symbol) and 80S (closed symbols) radioactivity in control lysates. (B) The distribution of 40S and 80S radioactivity in actinomycin D lysates. (C) Total binding in ribosomal particles in control lysates. (D) Total binding in ribosomal particles in actinomycin D lysates. (E) The 80S/40S ratio in the control lysates (±SEM). (F) The 80S/40S ratio in the actinomycin D lysates (±SEM).

time, the level of protein synthesis and polysomes in intact cells is reduced to 30–40% of the control level. Therefore, these experiments suggest that either the rate or efficiency of the formation of the final 80S complex in the lysates prepared from treated cells is deficient compared to that in control lysates.

One way of analyzing this in more detail is to determine the dependence on the mRNA concentration of the efficienty of conversion of the 40S complex to the 80S complex in the presence of sparsomycin. This is important because considering only the absolute level of ^{35}S incorporation into either the 40S or the 80S complex at only one time point (or mRNA concentration) can be initially somewhat misleading if one is attempting to analyze the efficiency or rate of 80S complex formation. An incubation time of 10 min was chosen since this time is before the incorporation of [^{35}S]Met-tRNA$_f$ into the 80S complex had reached its maximum level and before the translational ability of the lysate had begun to decrease from its initial rate. Since several different lysates and [^{35}S]Met-tRNA$_f$ preparations were used in different experiments, the results were normalized by calculating the picomoles of bound ^{35}S radioactivity per quantity of lysate protein. Because of the presence of sparsomycin to block elongation, the endogenous mRNA is sequestered in polysomes, and so the effective concentration of the endogenous mRNA capable of binding 40S complexes is low compared to the concentration of the added free mRNA which thus dominates the reaction. The results, seen in Figure 5, show that the extent of 80S formation from binding of mRNA and the 60S ribosomal subunit is a function of the level of available mRNA. In the control lysate, the conversion is essentially quantitative with the ratio of ^{35}S radioactivity in the 80S complex to that in the 40S complex reaching a maximum of about 4 (Figure 5C,E). The striking results with the actinomycin D lysates are that the conversion is not quantitative and that their 80S/40S ratio is significantly lower at each mRNA concentration than is the control lysate's ratio (Figure 5D,F). The conversion is not quantitative, and the ratio artificially rises because there is a [mRNA) dependent loss in the level of [^{35}S]Met-tRNA found in the 40S initiation complex without a concomitant increase in 80S radioactivity (Figure 5B,D vs. Figure 5A,C). The lysates from actinomycin-treated cells were somewhat more variable in their response than were control lysates; while the overall level of binding and the general trend were the same in all actinomycin lysates and significantly different from that in control lysates, the exact sensitivity of the 40S complex and the extent of 80S formation varied by up to 2-fold. In addition, other experiments[2] using ^{125}I-labeled mRNA as an independent probe of 80S formation have also indicated that 80S formation is slower or less efficient in actinomycin D lysates. Thus, these experiments suggest that the mRNA binding affinity is lower in the actinomycin D lysates or that the efficiency of 40S movement and/or 60S subunit joining is less in these lysates.

The level of mRNA binding and of movement of the 40S complex along this mRNA can be examined by using the inhibitor edeine during the cell-free incubation. As has been shown by Kozak & Shatkin (1978) and Kozak (1979), edeine alters the 40S complex such that it does not stop at the initiating AUG codon—thus, the 60S subunit cannot bind—but continues to move down the mRNA allowing additional 40S complexes to bind to the same mRNA. The result is that with increasing time the mRNA molecules can have one, two, three, or even more 40S complexes bound to each mRNA, with the precise number bound being dependent on the rate of binding and movement and the availability of 40S complexes. Figure 6 shows the result of such an experiment with exogenously added mRNA in excess of the amount of endogenous mRNA in the lysate. The concentration of edeine used in this experiment (5 µM) completely inhibits initiation as well as protein synthesis in both lysates as assayed by leucine incorporation and sensitivity to pactamycin.[2] While the overall level of ^{35}S-labeled 40S initiation complexes is comparable between the control and treated lysates, the ratio of multiple to single 40S complexes is significantly lower in the lysates prepared from actinomycin D treated cells (0.79 vs. 1.52). If exogenous mRNA is not added, there are essentially no complexes in the >40S region. This implies that these >40S complexes seen in Figure 6 are not nonspecific dimers of the 40S complex but represent more than one 40S complex bound to the same mRNA molecule. In addition, treatment of both the 40S and the >40S complexes with hot trichloroacetic acid resulted in solubilization of greater than 85% of the [^{35}S]methionine radioactivity.[2] This is consistent with the ^{35}S radioactivity being present in [^{35}S]Met-tRNA and not in a synthesized

[2] Unpublished observations.

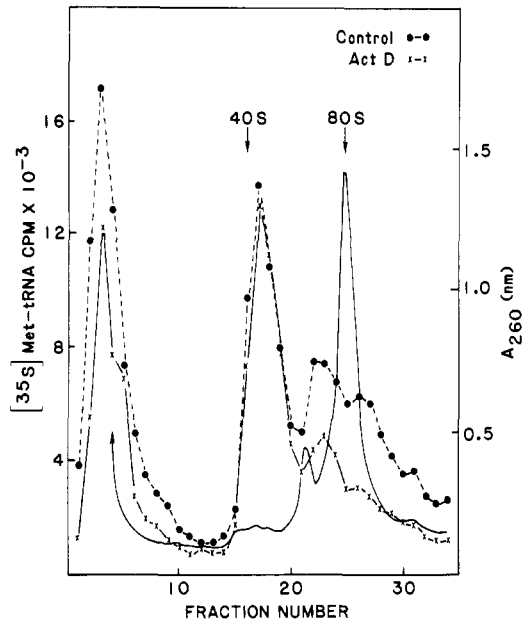

FIGURE 6: Binding of [^{35}S]Met-tRNA$_f$ to ribosomal particles in the presence of 5 μM edeine and added reticulocyte poly(A)+ RNA. The reaction is basically the same as in Figure 4 except that incorporation was for 10 min, and the actinomycin D lysates were prepared from CHO cells treated for 7 at 1 μg/mL. The radioactivity in particles ≥40S sequentially represent one, two, three, etc., 40S initiation complexes bound to the same mRNA molecule; edeine prevents the binding of the 60S subunit to form the 80S initiation complex. In these gradients, we are unable to distinguish between free 40S initiation complexes and 40S complexes bound to mRNA which would sediment slightly faster (in reticulocyte lysates the difference is 48S vs. 43S (Safer et al., 1978) so the "40S" peak represents both free and bound 40S complexes. (—) A_{260} (nm); (●) control (ratio >40S/40S cpm = 1.52); (×) actinomycin D (ratio >40S/40S = 0.79).

polypeptide. Therefore, one reason the actinomycin D lysates are less efficient in forming the 80S initiation complex could be that in these lysates mRNA binding and/or 40S movement is less efficient.

Discussion

The inhibition of protein synthesis in intact mammalian cells by transcriptional inhibitors that has been described by several laboratories (Singer & Penman, 1972; Craig, 1973, 1979; Goldstein & Penman, 1973; Cooper & Braverman, 1977, 1980; unpublished data) can also be detected in lysates prepared from treated cells. This finding makes possible a biochemical analysis of this inhibition. Using such lysates, we have found that the alteration of translational activity is not due to a direct effect of the various inhibitors of RNA synthesis on the translational apparatus but is more indirect, resulting from the response of intact cells to these inhibitors. The inhibition of protein synthesis does not appear to be due to the presence of an inhibitor but may be caused by a loss or alteration of some factor necessary for translation. The step in protein synthesis that is indirectly altered by these inhibitors is probably after the binding of the [eIF-2·GTP·Met-tRNA$_f$] complex to the 40S ribosomal subunit and prior to the formation of the 80S initiation complex.

One simple explanation of these effects could be that an unstable RNA is required for translational initiation in nucleated mammalian cells. Inhibition of the synthesis of such an RNA would therefore cause a progressive inhibition or protein synthesis. We cannot yet rule out this possibility, but no such RNA with the necessary characteristics has been described in normal cells. The only RNA that has been reported to be involved in protein synthesis which might share some similarities to this hypothetical unstable RNA is adenovirus VAI RNA, since in its absence the translation of late viral mRNA is less efficient (Thimmappaya et al., 1982). However, the mechanism of its action is still unclear.

While the mixing experiments suggest that there is no dominant soluble inhibitor of translational initiation that is induced in the treated cells, it is clear that this possibility has not been completely ruled out. For example, if there had been an inhibitor, its activity could have been lost during the lysate preparation, its activity counteracted by something in the control lysate, its concentration in the mixed lysates too low to be effective, or the time course of the assay too short for the inhibitor's effect to become manifest. It is clear, nevertheless, that if an inhibitor is present in the treated cells or inducible in the lysates from treated cells, this putative inhibitor is significantly different, at least quantitatively, from the inhibitor induced by hemin starvation in reticulocyte lysates or induced by interferon treatment of nucleated cells followed by virus infection or exposure to double-stranded RNA (Jagus et al., 1981). The most likely explanation of our results is that some component required for translation is missing or less active in the lysates from treated cells. This is supported by our preliminary results that one can prepare a fraction from reticulocyte ribosomal salt wash that stimulates translation in treated lysates but not in control lysates. Which factor(s) is (are) deficient or impaired in their function is still unclear, as is the detailed mechanism of this effect.

We have, however, made progress in identifying which steps in the initiation of protein synthesis that may be affected by these inhibitors. The observation that lysates prepared from treated cells have undiminished levels of the 40S initiation complex has several implications: (1) The level of the initiator Met-tRNA$_f$ and its charging is not inhibited and thus not rate limiting. This would rule out Cooper's suggestion for how actinomycin D affects translation (Cooper & Braverman, 1977). (2) The formation of the ternary (GTP·eIF2·Met-tRNA$_f$) complex is also unaffected which implies that eIF-2 must be functioning normally. (3) The availability of competent "native" 40S subunits must also not be rate limiting which suggests that the antiassociation initiation factor eIF-3 is probably functioning normally.

The fact that the formation of the 40S initiation complex is not altered by transcription inhibitors clearly distinguishes this mode of translational control from that described in other systems. For example, translation is inhibited in rabbit reticulocytes or lysates starved for iron or hemin, exposed to low levels of double-stranded RNA, or treated with compounds oxidizing their intracellular glutathione. In all of these cases, at least one specific soluble protein kinase becomes activated and phosphorylates the α-subunit of eIF-2 which alters the catalytic recycling of this initiation factor and ultimately results in an inhibition of the eIF-2-dependent formation of the 40S complex (Jagus et al., 1981; Clemens et al., 1982). In addition, these three inhibitory systems can cause their effects in cell-free reticulocytes lysates and do not require intact cells. Because rabbit reticulocytes lack a functional nucleus, this might not seem surprising, but this type of translational control using a soluble kinase to inactivate eIF-2 is seen in other nucleated mammalian cells. In these cases, however, treatment of the intact cell is often necessary. For example, after several hours of exposure of the cells to interferon, lysates from these cells become sensitive to double-strand RNA and exhibit the eIF-2 phosphorylation (Jagus et al., 1981). A decreased level of the 40S complex formation is seen in lysates prepared from cells starved for particular amino acids (Pain et al., 1980).

The initiation step that does appear to be affected in lysates isolated from cells treated with transcription inhibitors is the formation of the 80S complex. This step involves the binding of the 40S initiation complex to the 5′ end of the mRNA, movement of the complex to the initiating AUG codon, and the joining of the 60S ribosomal subunit. Our data are not conclusive but indirect, since it involves analyzing the mRNA-dependent shift of [^{35}S]Met-tRNA from the 40S complex to the 80S complex. The main observation (Figure 5) was that in the presence of mRNA, most of the labeled 40S complexes in the actinomycin D lysates were not quantitatively or efficiently shifted into 80S complexes but disappeared. This was in contrast to the situation in the control lysates where the conversion was quantitative. Preliminary experiments (unpublished data) analyzing the mRNA binding ability of lysates prepared from actinomycin D treated cells have shown that while [^{125}I]mRNA is bound to 40S complexes, the appearance of the bound mRNA in the 80S complex is significantly slower than in control lysates. The inhibition is not complete but roughly corresponds to the extent of protein synthesis inhibition in treated intact cells. These experiments independently confirm the more indirect findings described in this paper using [^{35}S]Met-tRNA that the lesion induced by actinomycin D is after the formation of the 40S complex. However, further experiments analyzing the kinetics and concentration dependence of the mRNA binding will be needed to pinpoint the lesion.

Cooper & Braverman (1980) have also reported that in lymphocytes treated with pactamycin and cycloheximide, additional exposure to actinomycin D results in a decrease in the accumulation of 80S complexes. This would suggest that actinomycin D may interfere with some step before the formation of the 80S complex. This is consistent with our results. However, as discussed earlier, their proposal of $tRNA^{Met}$ as being one possible labile, sensitive, and rate-limiting RNA species is not supported by our experiments.

The decrease in 80S complex formation efficiency could be due to a number of possibilities including a lessened stability of the 40S complex, a decrease in 40S complex binding to mRNA, slower or less efficient movement of the 40S to the AUG codon, and a less efficient joining of the 60S subunit. In the absence of added mRNA, or in the presence of either edeine or the nonhydrozable GTP analogue which prevent 60S joining but not mRNA binding (Benne & Hershey, 1978), the level of 40S complexes in both the control and actinomycin D lysates did not decrease over time. Thus, it appears unlikely that the 40S complexes formed in the treated cell's lysate are inherently less stable than are the control's 40S complexes. The instability of the 40S complexes in the treated cell lysates occurs only in the presence of added mRNA; whether it is caused by a stimulated deacylase or is due to an induced disassembly it not yet clear. While the possible importance of the 60S joining reaction as the lesion site in 80S formation has not been directly studied, we believe that it is less likely to be involved, based on the results of the experiments using edeine. Kozak & Shatkin (1978) have shown that in the presence of edeine, the 40S complex binds to and moves down the mRNA but that the complex moves past the initiation AUG codon and so prevents the joining of the 60S subunit. This work was done with wheat germ extracts and several different mRNAs including globin mRNA which we have used as our exogenous RNA. While the edeine response in mammalian extracts has not been studied in as complete a manner as was done for wheat germ extracts in that paper, Kozak (1979; personal communication) has found that rabbit reticulocyte lysates exhibit qualitatively the same response to edeine as do wheat germ extracts. This finding together with our observations on the properties and the sizes of the >40S complexes found in the presence of different amounts of added mRNA in CHO extracts makes it reasonable to assume that the edeine response in our extracts is basically the same as that described in wheat germ. If the actinomycin D induced lesion were at the 60S joining step, one would expect to see no difference between the control and actinomycin D lysates exposed to edeine in terms of the distribution of the ^{35}S-labeled 40S complexes on the added mRNA. However, as was shown in Figure 6, the actinomycin D lysate had a significantly lower number of 40S complexes on each mRNA. This would suggest that the defect may be in the binding and/or movement of the 40S complex on the mRNA rather than in the 60S joining step.

There are two examples of translational control which may have some resemblance to the experimental system analyzed in this paper and provide some insight as to the lesion site. These are the shut-off of host cell protein synthesis induced by viral infection—especially by picornaviruses such as poliovirus (Trachsel et al., 1980; Tahara et al., 1981)—and the inhibition of protein synthesis that occurs when cells go through mitosis (Tarnowka & Baglioni, 1979). In the polio-infected cells the host mRNA with its 5′-methyl cap is less able to bind to the 40S complex compared to the uncapped viral RNA which becomes the predominant translated mRNA. This new preference for viral mRNA appears to be due to an alteration in the activity (or amount) of the initiation factor(s) responsible for mRNA cap recognition and mRNA binding (Trachsel et al., 1980; Tahara et al., 1981). Recent work has shown that in lysates from polio-infected cells there is a deficiency in the newly described initiation factor eIF-4F (also known as cBP-II) required for mRNA binding and that this is correlated with the proteolysis of the 220 000-dalton polypeptide making up this factor (Etchison et al., 1982; Grifo et al., 1983). Whether or not mRNA binding in mitotic cells is the lesion site in that system is not clear, because while lysates from mitotic cells can bind mRNA (Tarnowka & Baglioni, 1979), the efficiency or rate of binding was not definitely established. It was clear in these experiments, however, that 40S initiation complex formation was not significantly affected (Tarnowka & Baglioni, 1979). It is perhaps not surprising that our results with inhibitors of RNA synthesis share similarities with the mitotic cell system and picornavirus infection, since in both of these cases RNA synthesis is inhibited. In some of the virally infected systems that have been described, simultaneous actinomycin D treatment speeds up the extent of host translation shut-off; in others where the turnoff is fairly rapid, there is less of an effect (Leibowitz & Penman, 1971; Jen et al., 1980). However, because picornavirus infection can inhibit host cell translation faster than can just actinomycin D treatment, it seems likely that the inhibition of host RNA synthesis is not the entire explanation of the viral effect on protein synthesis; other effects on the initiation factors required for mRNA binding or mRNA competition are probably more important (Trachsel et al., 1980; Jen et al., 1980; Tahara et al., 1981; Ray et al., 1983).

In summary, the experiments described in this paper show that the lesion in translation induced in intact cells by inhibitors of RNA synthesis is maintained in lysates prepared from treated cells and so can be analyzed biochemically. The transcription inhibitors do not act directly on the translational apparatus, nor do they induce an inhibitor of protein synthesis. Instead there is a decline in the level or activity of some

factor—as yet, unknown—required for protein synthesis. To localize the affected initiation step more precisely, the efficiency of the various subreactions of the initiation process were analyzed in cell-free lysates isolated from control and treated cells. The results show clearly that there is no inhibition of any of the steps leading up to the formation of the 40S initiation complex but that there appears to be some alteration in the efficiency of 80S complex formation which might involve mRNA binding and movement. This type of translational regulation may be important in the metabolism of a cell, since it would allow coordination between nuclear transcription and cytoplasmic protein synthesis that would be independent of the stabilities of the various mRNAs present in the polysomes. Further experiments will be necessary to determine which factor(s) is (are) involved and to determine whether or not this regulatory mechanism is also involved in situations in which cytoplasmic translation is altered by events such as mitosis or viral infection which are known to affect RNA synthesis.

Acknowledgments

We thank Virginia Clements for her preparation of the cell-free lysates and skillful assistance in some of the experiments. Dr. Sharon Krag of the School of Public Health and Hygiene at Johns Hopkins University generously provided our initial culture of the CHO cells. We are grateful to Dr. John Douros of the National Cancer Institute for his gift of sparsomycin, Dr. Gary Neil of Upjohn Company for pactamycin, and Dr. Paul England of Johns Hopkins University for his helpful comments on the manuscript.

Registry No. Actinomycin D, 50-76-0; MPB, 13083-37-9.

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