Translational Control of Gene Expression in a Normal Fibroblast. Characterization of a Subclass of mRNAs with Unusual Kinetic Properties†

William E. Walden and Robert E. Thach*
Department of Biology, Washington University, St. Louis, Missouri 63130
Received July 19, 1985; Revised Manuscript Received November 6, 1985

ABSTRACT: The translation of a small number of mRNAs in mouse SC-1 fibroblasts can be stimulated by cycloheximide, under conditions where the synthesis of most proteins is inhibited. These mRNAs are ordinarily present in small polyribosomes or messenger ribonucleoprotein particles, although the addition of cycloheximide drives them into large (≥5) polysomes. These mRNAs cannot be translated in vitro unless they are extracted with phenol. With such treatment, however, they are translated with normal competitive efficiencies. In iron-poor media, the mRNA for ferritin exhibits several of the distinctive kinetic properties of this class of mRNAs. With iron supplementation, however, ferritin translation appears normal. These observations are consistent with the existence of translational induction/repression systems in eukaryotes. Several types of evidence suggest that repressors may act by interfering with the interaction between mRNAs and limiting translational initiation components.

The translation rates for reovirus mRNAs are thought to be determined in part by how well they compete with other mRNAs for binding to a limiting, message-discriminatory initiation factor (Walden et al., 1981; Ray et al., 1983). Under ordinary conditions of cell culture, reovirus mRNAs compete poorly for this discriminatory factor and therefore are translated at a relatively low rate. As a result, they are found in polysomes of abnormally small size. However, in the presence of low concentrations of cycloheximide, the translation of these mRNAs is substantially increased, whereas that of most host proteins is inhibited. It has been shown that this unusual response to cycloheximide is in fact a logical consequence of mRNA competition and is to be expected of any message which competes poorly for discriminatory factor (Godefroy-Colburn & Thach, 1981; Ray et al., 1985).

In view of these results, it was of interest to determine whether poorly competing mRNAs might also exist in uninfected cells. If so, then it would suggest a wider role for message competition in regulating the synthesis of cellular proteins. In a preliminary investigation of this possibility, we have reported the existence of several such mRNAs in normal mouse fibroblasts (Walden & Thach, 1982). These mRNAs were found predominantly in small polysomes or messenger ribonucleoprotein (mRNP)[1] particles, and their translation rates were stimulated by cycloheximide. We describe here the identification of several more mRNAs of this class. These mRNAs cannot be translated in vitro unless they are first treated with phenol. This suggests that their low translational efficiency is due to the presence of proteins with which they are associated. The latter result suggests that these mRNAs may be identical with those recently described by other workers (Jenkins et al., 1978; Liautard & Egly, 1980; Bergmann et al., 1982; Akhayat et al., 1983).

It has been suggested that the substances which inhibit the translation of this class of mRNAs might be specific repressors (Preobrazhensky & Spirin, 1978; Bag & Sells, 1981; Bergmann et al., 1982; Bag, 1983). If this idea is correct, then derepression by a specific inducer substance should restore these mRNAs to a normal kinetic behavior. In particular, the stimulation of translation caused by cycloheximide should be eliminated by induction. We have found that this set of predictions holds true for ferritin synthesis, a process which is thought to be regulated by a translational repressor (Chu & Fineberg, 1969; Zahringer et al., 1976; Shull & Theil, 1982). Moreover, we show that the peculiar behavior of typical members of this class of mRNAs can be explained by an effect of repressors on mRNA competition. This behavior is quantitatively consistent with a previously described kinetic model (Godefroy-Colburn & Thach, 1981; Ray et al., 1985).

Materials and Methods

Cell Culture. SC-1 cells were grown as monolayers in McCoy 5A medium supplemented with 10% FBS. All experiments were performed at cell densities from 1×10^4 to 3×10^4 cells/cm^2.

In Vivo Protein Synthesis Analysis. SC-1 cell monolayers in 35-mm cluster dishes were preincubated at 37 °C for 15 min in methionine-free MEM-E supplemented with 5% FBS, 10 μM methionine, and cycloheximide as indicated under Results. The monolayers were washed once and labeled for 30 min at 37 °C in 0.5 mL of this medium containing 50 μCi of [^{35}S]methionine (Amersham; 600–1400 Ci/mmol). Pulsed cells were washed once with 2 mL of warm McCoy 5A medium containing 5% FBS, and the label was chased with an additional 2 mL of this medium for 15 min at 37 °C. The cells were washed twice with 2-mL aliquots of phosphate-buffered saline and lysed in immune buffer (phosphate-buffered saline containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA). Aliquots containing equal amounts of protein were diluted, made 1× in gel sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol), heated for 5 min in a boiling water bath, and analyzed by SDS–polyacrylamide gel electrophoresis as described below. Protein concentrations were determined by the method of Lowry et al. (1951).

For the analysis of ferritin synthesis, the growth medium on monolayers was replaced with complete MEM-E containing

† This work was supported by a grant from the National Science Foundation (PCM-8211567) and by a fellowship from the Monsanto Co.

[1] Abbreviations: SDS, sodium dodecyl sulfate; eIF, eukaryotic initiation factor; MEM-E, Earle's minimal essential medium; mRNP, messenger ribonucleoprotein; FBS, fetal bovine serum; BSA, bovine serum albumin; dsRNA, double-stranded RNA; NP-40, Nonidet P-40; Cl_3CCOOH, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/86/0425-2033$01.50/0

5% FBS, and the cells were incubated for 2 h at 37 °C. Induction of ferritin synthesis was achieved by supplementing this medium with 100 μM ferric ammonium citrate. Labeling of cells was as described above except that 100 μM ferric ammonium citrate was added to preincubation and pulse-label media where appropriate.

Ferritin Purification and Antisera Production. Ferritin was purified from mouse liver essentially as described by Linder and Munro (1972). The method involved heat extraction, pH and ammonium sulfate fractionation, and gel filtration through Ultrogel ACA34 (LKB). The purity of the preparation was determined by SDS–polyacrylamide gel electrophoresis.

Antisera to mouse liver ferritin were raised in rabbits by injecting the protein as a mixture with Freund's adjuvant according to standard procedures.

Immune Precipitation. Immune precipitations were performed according to the method of Cladaras and Kaplan (1984) with minor modifications. Briefly, aliquots of cell lysates, containing an equal amount of protein, were diluted to 0.5 mL with immune buffer, heated in a boiling water bath for 3 min, and mixed with 0.5 mL of immune buffer containing 2% BSA. For precipitation of in vitro products, 12.5-μL samples of translation mixes were combined with an equal volume of 10% SDS and boiled for 3 min before diluting to 0.5 mL with immune buffer containing 1% BSA. Forty microliters of IgG sorb (rehydrated in immune buffer; The Enzyme Center, Cambridge, MA) was added, and the mixtures were allowed to incubate at room temperature for 1 h. The IgG sorb was pelleted away from the mixtures by centrifugation in an Eppendorf microfuge for 3 min. Normal rabbit serum (4 μL) was added, and the mixtures were incubated at room temperature for at least 45 min. IgG sorb (40 μL) was then added and incubation continued for 30 min. The mixes were centrifuged, and the supernatants were saved. Rabbit anti-mouse ferritin serum (4 μL) was added to the supernatants, and specific immune complexes were allowed to form overnight at 4 °C. IgG sorb (40 μL) was added, and incubation was continued for 30 min at room temperature. The pellets (containing the specific immune complexes) were collected by centrifugation and washed 4 times with immune buffer. The pellets were resuspended in 50 μL of gel sample buffer, heated for 5 min in a boiling water bath, and repelleted by centrifugation. Antigen released into the supernatants was analyzed by SDS–polyacrylamide gel electrophoresis and fluorography as described below.

Polysome Preparation. Polysomes were prepared essentially as described by Walden et al. (1981). Cell cultures ($\sim 1 \times 10^8$ cells per gradient) were refed 30 min prior to harvest with fresh growth medium (where indicated, low levels of cycloheximide were added at this point). At harvest, the growth medium was removed, and the cells were rapidly cooled by adding ice-cold polysome buffer [100 mM sucrose, 100 mM KCl, 5 mM $Mg(OAc)_2$, 20 mM Tris-HCl, pH 7.4, and 100 μg/mL cycloheximide] and submerging the culture vessel in an ice/salt bath (−10 °C). All subsequent manipulations were done at 4 °C. Cells were collected by scraping with a rubber policeman and pelleted by centrifugation at 800*g* for 5 min. Pelleted cells were washed once with polysome buffer. The cells were pelleted again, and the packed cell volume was measured. An equal volume of polysome buffer supplemented with 1 mM dithiothreitol was added, and the suspension was transferred to a Dounce homogenizer. NP-40 was added to a final concentration of 1%. The cells were disrupted with 7 strokes of a tight-fitting pestle. Sodium deoxycholate was added to a final concentration of 1%, and nuclei were removed by centrifugation at 3000*g* for 5 min. The postnuclear supernatant was layered onto a 15–50% (w/v) sucrose gradient in 100 mM KCl, 5 mM $Mg(OAc)_2$, and 20 mM Tris-HCl, pH 7.4. The polysomes were resolved by centrifugation in a Beckman SW41 rotor at 36 000 rpm for 110 min at 4 °C. Gradient fractions (1.0 mL) were collected and monitored for absorbance at 260 nm with a Gilford recording spectrophotometer. After each fraction was diluted with an equal volume of TMK buffer [100 mM KCl, 3 mM $Mg(OAc)_2$, and 20 mM Tris-HCl, pH 7.4], polysomes and RNPs were collected by centrifugation through a 30% sucrose cushion (also in TMK) at 40 000 rpm for 54 h (at 4 °C) in a Beckman type 65 rotor. Pellets were gently rinsed once with TMK buffer, resuspended into 100 μL of TMK buffer, and stored at −70 °C. RNA was prepared from gradient fractions by phenol/chloroform extraction and ethanol precipitation.

In Vitro Translations. Translations in vitro were performed by using the fractionated translation system described by Brendler et al. (1981a) or a nuclease-treated wheat germ lysate (Bethesda Research Laboratories). Unless otherwise noted, [^{35}S]methionine was used as label. The protocol for in vitro translation competition assays has been described elsewhere (Brendler et al., 1981a,b). The purifications of rabbit hemoglobin mRNA and reovirus M_3 dsRNA have also been described (Brendler et al., 1981a). Polysomal RNA was used in the fractionated system at 0.67 μg per assay (this amount of polysomal RNA is about 60% of saturation). Reovirus M_3 dsRNA was added at 0.007 μg per assay. Prior to translation, reovirus M_3 dsRNA was denatured by heating at 67 °C for 15 min in 90% dimethyl sulfoxide, rapidly cooled, and diluted into sterile water at 0 °C (Brendler et al., 1981a).

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed essentially as described by Walden et al. (1981). Analysis of total protein synthesis was performed on 22-cm-long slab gels (1.2 mm thick) consisting of a 4% acrylamide stacking gel (1 cm) and a 7.5–15% linear acrylamide gradient separating gel. Electrophoresis was performed at 160 V (constant voltage), and the run was terminated after the tracking dye had exited the gel (about 14 h). Autoradiography was performed as described previously (Walden et al., 1981).

Analysis of immunoprecipitates was performed on 10% acrylamide slab gels (9 cm long with a 4% acrylamide stacking gel). Electrophoresis was carried out at constant current (30 mA) and proceeded until the tracking dye had reached the bottom of the gel. Fluorography of the gel was performed by using the APEX method (Jen & Thach, 1982).

Mathematical Modeling. All procedures and parametric values were essentially as previously described for reovirus-infected cells (Ray et al., 1985), except that viral mRNAs were omitted and the mRNA for P52 was assigned the following properties: a normally initiating component had a concentration of 0.009 nM and a dissociation constant (K_f) of 0.001 nM; a poorly initiating component had a concentration of 0.006 nM and a dissociation constant of 0.1 nM. Also, the concentrations of total discriminatory factor (f_t), total other limiting initiation factor (e_t), and total host mRNA ($\sum m_t^i$) were increased by 50% to take into account a slightly faster cell growth rate, following the logic previously described (Colburn & Thach, 1981).

Results

Stimulation of Synthesis of Individual Polypeptides by Cycloheximide. The question of whether mRNAs which compete poorly for initiation components exist in rapidly growing fibroblasts was investigated by pulse labeling cells in

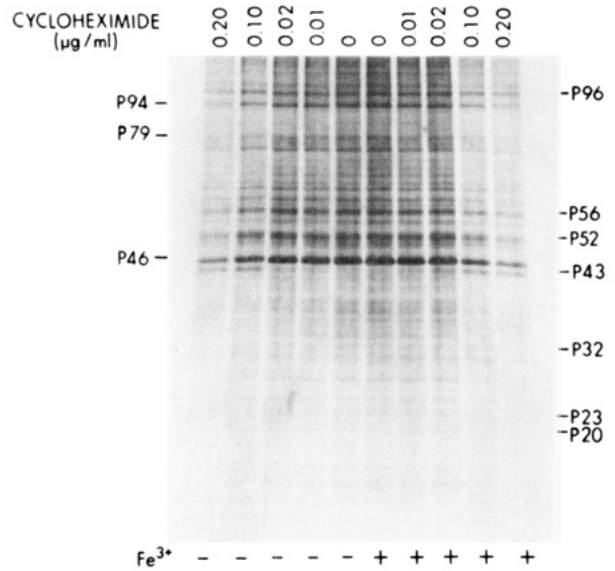

FIGURE 1: Effect of cycloheximide on protein synthesis rates in growing SC-1 cells. Cells which had been incubated in media lacking (–) or containing (+) additional ferric ammonium citrate (100 μM) were treated with cycloheximide at the concentrations indicated and labeled with [^{35}S]methionine. Labeled polypeptides were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography as described under Materials and Methods. Proteins whose synthesis is resistant to cycloheximide inhibition are identified on the right side of the figure. Representatives of those whose synthesis is inhibited normally are indicated on the left side of the figure.

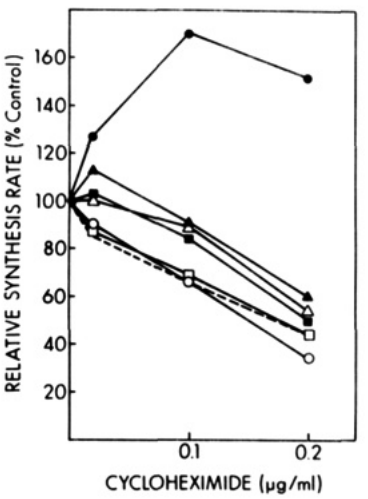

FIGURE 2: Quantitation of the effect of cycloheximide on in vivo protein synthesis. The synthesis of individual proteins in Figure 1 was quantitated by densitometry, and total protein synthesis was quantitated by Cl_3CCOOH precipitation of aliquots from each sample prior to SDS–polyacrylamide gel electrophoresis. Data are shown for proteins P43 (●), P52 (▲), P56 (■), P96 (△), P94 (○), and P79 (□) and for total protein synthesis (---).

the presence of low doses of cycloheximide. This technique has been shown to distinguish between mRNAs on the basis of their competitive efficiencies: the translation of mRNAs which are weak competitors is actually stimulated by cycloheximide, whereas the translation of messages which are strong competitors is inhibited (Walden et al., 1981). To this end, rapidly growing SC-1 cells were treated briefly with varying doses of drug and pulse labeled with [^{35}S]methionine. After a brief chase with cold methionine, the cells were lysed, and the products were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography (see Materials and Methods). A typical result is shown in Figure 1. Labeling of the great majority of proteins is reduced monotonically by cycloheximide addition (for example, note proteins P46, P79, and P94 in Figure 1); in contrast, the labeling of a few (for example, P20, P23, P32, P43, P52, P56, and P96) either is resistant to or is actually stimulated by cycloheximide. Of particular interest is the effect of cycloheximide on the synthesis of the protein identified as P43. Treatment of cells with 0.1 μg/mL cycloheximide increases the synthesis of this protein from that of being barely detectable to being one of the major protein bands in the one-dimensional gel pattern. A quantitation of these results is shown in Figure 2. These data show that the synthesis of P43 is stimulated by cycloheximide treatment by at least 70%. (If the contributions of minor proteins which partially overlap with P43 were taken into account, the degree of stimulation by cycloheximide would be much greater.) In addition, the synthesis of P52 and P56 is also shown to be stimulated, while the synthesis of P96 is more resistant to cycloheximide treatment than that of the majority of other proteins.

Similar experiments have been performed with inhibitors of initiation, such as harringtonine or medium hypertonicity. These agents did not stimulate the synthesis of any detectable protein band (data not shown). In particular, they did not affect differentially the synthesis of any of the bands that are stimulated by cycloheximide. This observation has important implications regarding the mechanism responsible for the cycloheximide effect, as is described under Discussion.

These results confirm and extend the more intensive analysis of P52 synthesis previously reported (P52 was previously called "C"; Walden & Thach, 1982). They are very similar to those obtained in reovirus-infected SC-1 cells where the synthesis of viral proteins is enhanced by cycloheximide (Walden et al., 1981).

Analysis of mRNA Distribution. Since the average number of ribosomes on a mRNA is determined by the length of its coding region and the rates of ribosome attachment and elongation, any mRNA which is specifically regulated in one or more of its initiation steps is likely to have an abnormal number of ribosomes associated with it. Since those messages whose translation is stimulated by cycloheximide are thought to be poor initiators, they should therefore be found sedimenting in unusually small polysomes. To determine if this is the case, the sedimentation profile of specific polysomes in SC-1 cells was determined. This was done by subjecting cytoplasmic extracts to centrifugation through sucrose gradients, extracting the RNA from each fraction, and translating this RNA in an in vitro translation system. Under the conditions employed, the amount of polypeptide synthesized was directly proportional to the level of mRNA in each fraction (Brendler et al., 1981a). The products of the in vitro translation were then analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. The results of an analysis of this type are shown in Figure 3.

On inspection of Figure 3A, it is evident from the pattern of proteins synthesized that polysome size is proportional to polypeptide size for most of the mRNAs in SC-1 cells. Similar results have been observed by Lodish and Porter (1980). This implies that most mRNAs initiate translation at nearly the same rate. However, there are mRNAs which do not sediment in polysomes in proportion to the size of their coding region. These tend to be mRNAs which sediment in smaller than average polysomes, in monosomes, and in free mRNP particles (this group of particles will be referred to hereafter as "free mRNP"). Most prominent is the mRNA encoding P52. Others (notably the mRNAs encoding P96, P74, P56, P43, P37, P34, and P32) can be seen more clearly in Figure 3B, which is a longer exposure of the gel shown in Figure 3A.

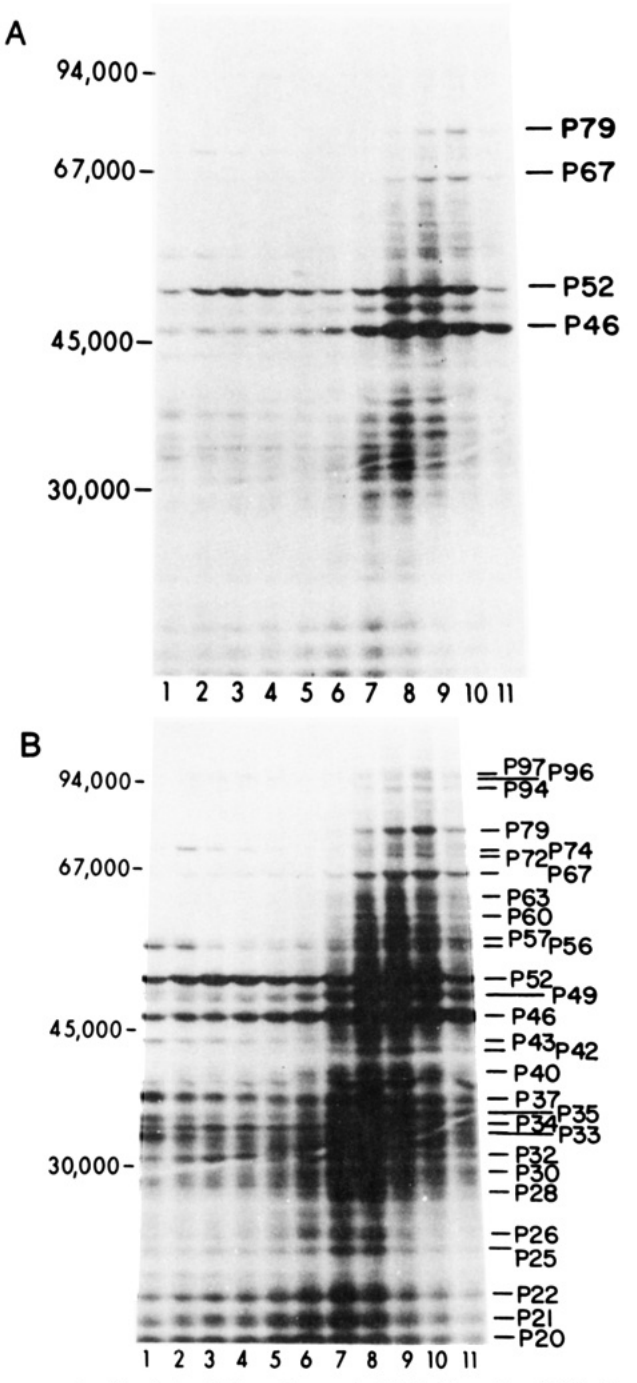

FIGURE 3: Analysis of the polysomal distribution of mRNAs by sedimentation in a sucrose gradient. (A) Polysomes were prepared from SC-1 cells and fractionated by sucrose gradient sedimentation as described under Materials and Methods. A representative portion of the RNA extracted from each gradient fraction was translated (at subsaturating levels) in the fractionated cell-free system prepared from Krebs ascites cells. Translation products were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. The numbers on the left indicate the position of migration of molecular weight markers. Lanes 1 and 2 show the products resulting from translating mRNA sedimenting at 20–50 S and 50–80 S, respectively. Lanes 3–11 show the products resulting from translating mRNA which sediments in polysomes of 1–2 (lane 3), 2–3 (lane 4), 3–4 (lane 5), 4–6 (lane 6), 6–8 (lane 7), 8–11 (lane 8), 11–14 (lane 9), 15–18 (lane 10), and >18 (lane 11) ribosomes per mRNA. (B) Longer exposure of the gel shown in panel A.

Assuming that ribosome elongation rates are roughly equivalent on most mRNAs (Lodish, 1976), it follows that initiation on these mRNAs is slower than on the average cellular mRNA. (The possibility that these mRNAs might be in free mRNP because of an abnormally high elongation rate can be ruled out, as will be described below.) It is also apparent that the majority of these mRNAs which sediment as free mRNP encode proteins whose synthesis is stimulated when cells are treated with a low dose of cycloheximide [compare Figures 1 and 3B; see also Walden & Thach (1982)]. Thus, the results of polysome size analysis support the notion that initiation on the cycloheximide-stimulatable mRNAs is unusually slow.

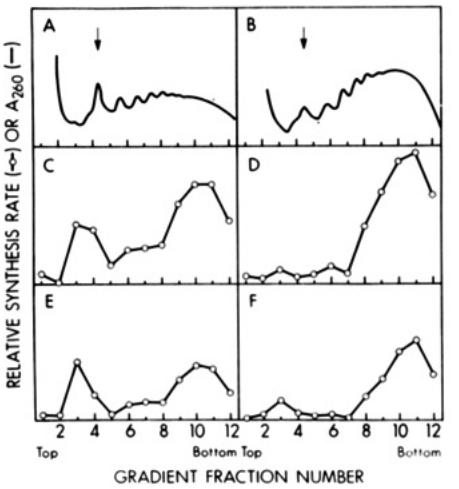

FIGURE 4: Effect of cycloheximide on polysome distributions. Polysomes were prepared and analyzed as described for Figure 3 and under Materials and Methods. The relative synthesis of specific proteins was determined by densitometry. (A, C, and E) Polysome profiles from cells incubated in normal media. (B, D, and F) Polysome profiles from cells incubated for 30 min prior to harvest in media containing 0.2 μg/mL cycloheximide. Data are shown for total polysomes (A_{260} profile) (panels A and B), P52 mRNA (panels C and D), and P56 mRNA (panels E and F). The arrow indicates the position of 80S monosomes.

It is interesting that a major portion of several mRNAs which sediment as free mRNP also sediments in normal sized polysomes. This has been noted by others for other systems as well (Geoghegan et al., 1979; Lee & Engelhardt, 1979; Croall & Morrison, 1980; Lodish & Porter, 1980; Yenofsky et al., 1982). Particularly obvious examples of this phenomenon are the messages encoding P52 and P56 (Figure 3). This curious bimodal distribution suggests that these as well as a number of other proteins are encoded by mRNAs that are heterogeneous with regard to translational efficiency; one component is very weak in initiation, while the other is normal. [That the polypeptides encoded by both message components are the same has been confirmed for several species by peptide mapping procedures (Walden & Thach, 1982; unpublished results).]

Effects of Cycloheximide on Polysome Profiles. The results thus far presented show a correlation between a mRNAs ribosome density and the ability of cycloheximide to stimulate its translation (i.e., those mRNAs whose translation is stimulated are also found sedimenting as free mRNP). Therefore, it is expected that cycloheximide treatment should drive these same mRNAs into much larger polysomes. To determine if this does happen, polysomes from SC-1 cells which had been incubated in the presence or absence of 0.2 μg/mL cycloheximide for 30 min were prepared and analyzed as described under Materials and Methods. Figure 4 shows the results obtained for the mRNAs encoding P52 and P56. The distribution of P52 mRNA (panel C) and P56 mRNA (panel E) is bimodal in the absence of cycloheximide, confirming the pattern shown in Figure 3. The two peaks of mRNA occur at 0–1 and 8–14 ribosomes per message, respectively. Inclusion of 0.2 μg/mL cycloheximide in the cell culture medium for 30 min prior to harvest causes a shift of free 80S monosomes

into polysomes (compare panel A to panel B), while inhibiting the overall rate of protein synthesis by 40–60% (cf. Figure 2). This treatment drives most of the weakly initiating component of both P52 mRNA and P56 mRNA into very large polysomes (Figure 4; panels D and F, respectively). The average number of ribosomes per weakly initiating mRNA increases from less than one to at least five. In contrast, the distribution of P43 mRNA is monomodal in the absence of cycloheximide (Figure 3B), suggesting the existence of a single species of mRNA. Inclusion of cycloheximide in the cell growth medium drives approximately 70% of this P43 mRNA into large polysomes (data not shown). Similar results have been obtained for the majority of mRNAs which sediment in free mRNP particles in SC-1 cells [see Figure 6 of Walden & Thach (1982); unpublished results].

In control experiments, it was established that the cycloheximide-induced shift in mRNA profiles is complete in 30 min for most mRNAs; a few require 45 min for the new steady-state distribution to be reached. This rules out any likelihood that the induced changes are dependent upon transcriptional events.

Two important conclusions are suggested by these experiments. First, it is clear that most of the mRNAs ordinarily found in free mRNP are in fact translatable in vivo but are translated at a low rate in the absence of cycloheximide. Second, these mRNAs load a disproportionate number of ribosomes when cycloheximide is added: whereas the average message increases its number of ribosomes by approximately 30%, ribosome loading on the weak mRNAs increases by more than 5-fold (Figure 4). This is consistent with the observation (Figures 1 and 2) that cycloheximide addition must actually increase the initiation rate on these mRNAs.

Analysis of mRNP Activity in Vitro. A number of investigators have noted that mRNA isolated as free mRNP can be translated in vitro only after treatment with high salt or phenol to remove associated protein (Civelli et al., 1976, 1980; Jenkins et al., 1978; Liautard & Egly, 1980; Bergmann et al., 1982). To see if mRNAs found in free mRNP in SC-1 cells exhibit the same characteristics, polysomes and RNP isolated from gradient fractions either were used directly for translation in vitro or were phenol extracted, and the remaining RNA was ethanol precipitated before translation. To ensure that only initiation events were recorded, labeled formyl[^{35}S]-methionyl-tRNA was employed. The products of both sets of translation experiments were then analyzed by SDS–polyacrylamide gel electrophoresis. The results of an analysis of this type are shown in Figure 5. The mRNAs which sediment in large polysomes are translated efficiently in vitro both before and after phenol extraction (compare lanes 6–8 with lanes 14–16). In contrast, the mRNAs sedimenting as free mRNP particles are translated very inefficiently or not at all in vitro prior to phenol extraction. However, once extracted they translate quite well (compare lanes 1–3 with lanes 9–11 in Figure 5). To determine whether the suppression of translation of mRNAs in mRNP particles is due to the presence of a general, trans-acting inhibitor, reoviral M_3 or globin mRNAs were included in translation reactions containing free mRNP. Both of these control mRNAs were translated normally, indicating that the inhibitors present in the mRNP fraction act only in a cis, and not a trans, mode (data not shown). These results are similar to those reported by others (Civelli, 1976; Jenkins et al., 1978; Liautard & Egly, 1980; Civelli et al., 1980; Bergmann et al., 1982). They also suggest that phenol-extractable substances may play a role in limiting the translation of free mRNPs in vivo.

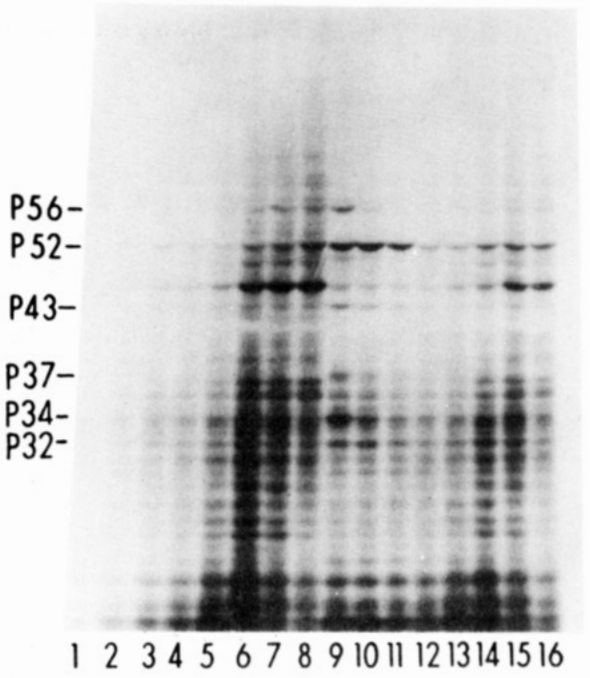

FIGURE 5: Analysis of mRNP activity in vitro. Polysomal and free RNPs were collected from sucrose gradient fractions by centrifugation as described under Materials and Methods. RNA was extracted from half with phenol/chloroform and ethanol precipitated. A representative portion of the RNP and the RNA was translated in the fractionated cell-free system by using formyl[^{35}S]methionyl-tRNA as label. Translation products were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. Lanes 1–8, translation products of unextracted RNP; lanes 9–16, translation products of extracted RNA. Translation results are shown for mRNAs sedimenting at 20–50 S (lanes 1 and 9) and 50–80 S (lanes 2 and 10) or in polysomes of 1–2 (lanes 3 and 11), 2–3 (lanes 4 and 12), 3–5 (lanes 5 and 13), 5–7 (lanes 6 and 14), 7–9 (lanes 7 and 15), and 9–11 (lanes 8 and 16) ribosomes per mRNA.

Analysis of Translation Efficiencies in Vitro. We next sought to determine whether the mRNAs in free mRNP are translated as efficiently as their polysomal counterparts after phenol extraction. For this purpose, the method of Brendler et al. (1981a) was employed to measure mRNA competitive ability. Briefly, a subsaturating amount of either nonpolysomal or polysomal RNA (the "test" message) was translated in the presence of increasing amounts of rabbit globin mRNA (the "competing" message). It has been shown that under these conditions the translation of each test message is affected in a manner characteristic of its affinity for a component referred to as the "message discriminatory factor", which has been recently identified as eIF-4F and/or eIF-4A (Ray et al., 1983; Sarkar et al., 1984). The mRNAs with the lowest affinities for discriminatory factor are the most rapidly inhibited by increasing concentrations of competing mRNA, while those with the greatest affinities are affected the least.

An analysis of this type was performed using mRNA isolated from free mRNP. A small amount of reovirus M_3 mRNA was included in these translation assays as a standard message. The results of these experiments indicated that the mRNAs encoding P52 and P56 from the free mRNP fractions compete as well as their counterparts isolated from large polysomes. This fact is expressed quantitatively through the use of "Q-prime plots" (Brendler et al., 1981a; Godefroy-Colburn & Thach, 1981), which are shown in Figure 6, where the data from several independent competition experiments have been combined. The slope of the lines drawn through each set of points is equal to the ratio of the dissociation constants of the mRNA–discriminatory factor complexes (K_f)

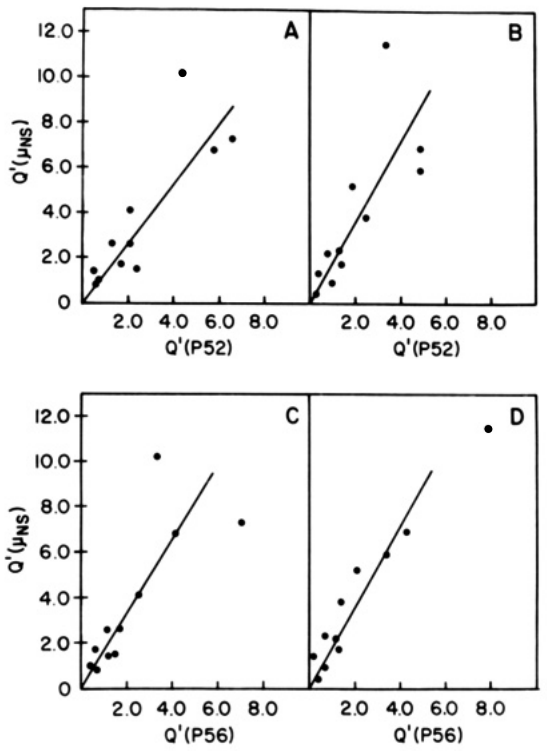

FIGURE 6: Analysis of in vitro competition data by Q' plots. The competition data from several experiments were analyzed by using Q' plots according to the theory of Godefroy-Colburn and Thach (1981). The quantity $Q' = Q/(Q_0 - Q)$ (where Q_0 is the synthesis of a particular protein in the absence of globin mRNA and Q is the synthesis in the presence of competing levels of globin mRNA) was calculated for protein P52, protein P56, and μ_{ns}. The Q' values for μ_{ns} were plotted against those for either P52 or P56. The slopes of the resulting plot are an indication of the affinity of a particular mRNA for the discriminatory factor relative to that of reovirus M_3 RNA. The data shown are for RNA extracted from mRNP particles and small polysomes (panels A and C) or from large polysomes (panels B and D).

for the test message and the reovirus M_3 standard. It is evident in Figure 6 that the mRNAs from large polysomes and from mRNP particles produce lines with essentially the same slopes; hence, their dissociation constants for binding to discriminatory factor must be the same. (In contrast, if the free mRNP particle mRNAs had retained after phenol treatment the defect which causes their poor translation in vivo, the slopes of their Q-prime plots, and hence their relative dissociation constants, would have been at least 5-fold higher.) The absolute values of the constants for both types of P52 and P56 mRNA are similar to that for reovirus M_3 mRNA, which is a relatively strong competitor (Brendler et al., 1981b). Thus, it is clear that the mRNAs which are derived from the free mRNP fraction by phenol extraction are capable of initiating normally and are indistinguishable in this assay from their counterparts derived from large polysomes. Taken together with the results described above, these data suggest that phenol extraction has removed an inhibitory substance which is responsible for the inefficient translation on these mRNAs in vivo.

Analysis of Ferritin mRNA Translation. The data presented thus far are consistent with the following model: (1) several mRNAs in mouse fibroblasts are translated inefficiently as a result of their inability to interact with a limiting initiation component; (2) this inability is primarily due to specific repressors of translation, which can be removed by phenol extraction and/or ethanol precipitation; and (3) once the repressor has been removed, the mRNA interacts normally with components of the translation machinery. The effects

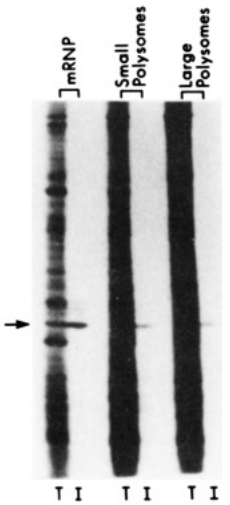

FIGURE 7: Polysomal distribution of ferritin mRNA. RNA isolated from free mRNP (20–80S particles), small polysomes (2–4 ribosomes per mRNA), or large polysomes (>5 ribosomes per mRNA) was translated in a wheat germ lysate as described under Materials and Methods. T, total translation products; I, products precipitated with antisera to mouse ferritin. The arrow shows the position of migration of mouse ferritin.

of repression on translational ability suggested by this model can be investigated by studying an mRNA for which a repressed and a derepressed state can be established. The synthesis of ferritin has been shown to be regulated at the level of translation by a process that apparently involves a translational repressor (Chu & Fineberg, 1969; Zahringer et al., 1976; Shull & Theil, 1982). The translational state of ferritin mRNA is known to be affected by iron, such that elevated levels of iron lead to a derepression of the ferritin mRNA and hence increased ferritin synthesis (Zahringer et al., 1976). Thus, ferritin mRNA translation should provide a good system for testing the model outlined above.

It was first necessary to determine the translational state of ferritin mRNA in growing SC-1 cells. It has been shown by others that, under conditions of limiting iron supply, the mRNA encoding ferritin is present both in polysomes and in free mRNP (Zahringer et al., 1976; Watanabe & Drysdale, 1981). By contrast, in the presence of excess iron, all the ferritin mRNA is driven into polysomes (Zahringer et al., 1976). To determine if a similar situation exists in SC-1 cells, mRNA was isolated from free mRNP (≤80S particles), small polysomes (two to four ribosomes per mRNA), or large polysomes (more than five ribosomes per mRNA). These were then translated in vitro, and the relative amount of ferritin mRNA sedimenting in each fraction was determined by immunoprecipitation of ferritin-specific products followed by SDS–polyacrylamide gel electrophoresis as described under Materials and Methods. The results, shown in Figure 7, reveal that the bulk of the mRNA encoding ferritin sediments in free mRNP, while only a minor portion sediments in small or large polysomes. This result indicates that the majority of ferritin mRNA is translated very inefficiently in SC-1 cells under normal conditions and suggests that ferritin synthesis should be markedly increased by the addition of iron to the culture medium. Confirming this prediction, we found that the synthesis of ferritin is induced severalfold upon incubation of SC-1 cells in the presence of elevated levels of iron (Figure 8). Thus, ferritin mRNA is apparently strongly repressed under normal conditions in SC-1 cells and is derepressed when the cells are exposed to elevated levels of iron.

As a test of the model outlined at the beginning of this section, we determined the effects of cycloheximide on ferritin

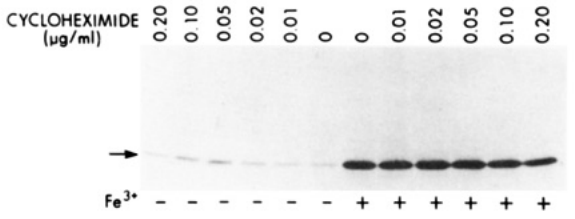

FIGURE 8: Effects of cycloheximide on ferritin synthesis in vivo. SC-1 cells were incubated for 2 h prior to cycloheximide treatment and pulse labeling in normal media (−) or in media containing an additional 100 μM ferric ammonium citrate (+). Ferritin was immunoprecipitated from cell lysates and analyzed by SDS–polyacrylamide gel electrophoresis as described under Materials and Methods. The arrow shows the position of migration of mouse ferritin.

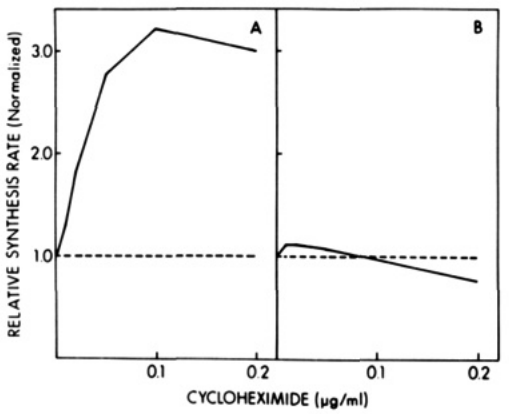

FIGURE 9: Comparison of the effects of cycloheximide on ferritin synthesis under conditions of low and high exogenous iron. The data shown in Figure 8 were quantitated by densitometry and normalized to the total protein synthesis rate at each cycloheximide concentration. The total protein synthesis rate was determined by Cl_3CCOOH precipitation. (A) Cells incubated in low-iron media. (B) Cells incubated in high-iron media. Data are shown for ferritin (—) and total protein (- - -) synthesis.

synthesis in normal cells and in cells incubated in the presence of elevated levels of iron. The model predicts that cycloheximide should cause a stimulation of ferritin synthesis under normal conditions, whereas in the presence of elevated iron this stimulation of ferritin synthesis by cycloheximide should be eliminated. To test this prediction, SC-1 cells were incubated in the presence or absence of exogenous iron (100 μM ferric ammonium citrate) and treated with increasing doses of cycloheximide. They were then pulse labeled with [^{35}S]-methionine, and the products were analyzed by SDS–polyacrylamide gel electrophoresis. The results of such an analysis are shown in Figure 8, and quantitation of the data is presented in Figure 9. Cycloheximide treatment in the absence of iron causes a marked increase in the synthesis of ferritin (left six lanes in Figure 8). In contrast, ferritin synthesis is not stimulated by cycloheximide when cells have been incubated in medium containing exogenous iron (right six lanes in Figure 8). The extent of these effects is more clearly evident in Figure 9. Here it is apparent that under normal culture conditions ferritin synthesis is stimulated by more than 3-fold relative to total protein synthesis by cycloheximide (panel A, Figure 9). In contrast, in the presence of elevated iron, cycloheximide has no stimulatory effect on ferritin synthesis but inhibits it at a rate equal to that of total protein synthesis. This reversal of the cycloheximide effect on ferritin synthesis by iron is by no means a general one, since the effects of cycloheximide on other proteins' synthesis is virtually identical under both culture conditions (most notably P43; see Figure 1). It should be noted that these data do not bear on the question of whether or not under derepressed conditions ferritin mRNA might be an unusually strong competitor, as recently suggested for bullfrog ferritin mRNA (Schaefer & Theil, 1981; Shull & Theil, 1982, 1983).

Table I: Polysome Size of P52 mRNA Components at Varying Cycloheximide Concentrations Predicted by Mathematical Modeling

inhibitor concn (μg/mL)	polysome size for		
	actin	P52 (normal)	P52 (repressed)
0	11.5	12.7	1.0
0.1	16.9	18.7	11.6
0.25	18.1	20.0	13.2

In control experiments, it was shown that both excess iron and cycloheximide treatment drive a significant proportion of ferritin mRNA into polysomes, as expected.

Discussion

The preceding results define some peculiar properties of a minor class of mRNAs present in mouse fibroblasts. Their presence in free mRNP and their anomalous response to cycloheximide addition are especially striking. What makes these observations interesting is the fact that the mRNA for ferritin exhibits these same properties in iron-poor media but behaves normally in media supplemented with iron. Ferritin synthesis is thought to be regulated at the translational level by an iron-inducible repressor (Chu & Fineberg, 1969; Zahringer et al., 1976; Shull & Theil, 1982). This supports the suspicion that many or all members of this peculiar class of mRNAs may be translationally inducible/repressible (Preobrazhensky & Spirin, 1978; Bag & Sells, 1981; Bergmann et al., 1982; Bag, 1983). The fact that they all contain tightly bound, phenol-extractable substances that inhibit translation in vitro is consistent with this idea.

In view of this intriguing possibility, it is of interest to speculate upon the reasons for the peculiar kinetic behavior of these mRNAs. One simple explanation is that the bound repressor-like substances interfere with their ability to bind to the message-discriminatory factor previously described (Walden et al., 1981; Godefroy-Colburn & Thach, 1981; Ray et al., 1983; Sarkar et al., 1984). In this case, cycloheximide would stimulate their translation rates and drive them into large polysomes in the same way as it does reovirus mRNAs. In the presence of inducers, on the other hand, repressors would be absent, and the mRNAs would compete with normal or even supranormal (Shull & Theil, 1982, 1983) affinities for discriminatory factors. Thus, they would now be found in normal-size polysomes, and their translation would not be stimulated by cycloheximide.

That this simple concept can quantitatively account for the cycloheximide stimulation of one of these mRNAs (that encoding P52) has been shown previously (Walden & Thach, 1982). The kinetic algorithm previously described (Godefroy-Colburn & Thach, 1981; Ray et al., 1985) was also used to model the experimental data presented in this paper. We assumed for this purpose that there are two types of message encoding the same P52 protein, one repressed and the other not (Walden & Thach, 1982). This accounts for the stimulation by cycloheximide and the bimodal polysome size distribution. The latter results are shown in Table I and are to be compared to the experimental data in Figure 4. A number of variants of this model can also be used to fit the experimental data. In particular, one in which it is assumed that the bound repressor is in equilibrium with free repressor, and that bound repressor altogether excludes recognition by discriminatory initiation factor, produces results identical with those shown in Table I and in Walden and Thach (1982). The

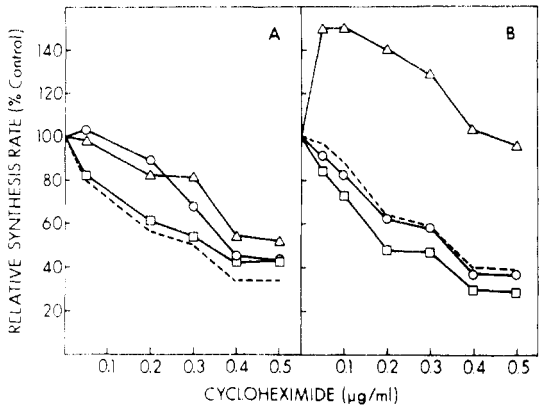

FIGURE 10: Effect of cycloheximide on P52 synthesis in reovirus-infected cells. The effect of cycloheximide on translation in reovirus-infected cells was determined as previously described [see Figures 2B and 3B of Walden et al. (1981)]. Data are shown for proteins P52 (○), protein P67 (□), reovirus σ^3 (△), and total protein synthesis (---). (A) 5-h postinfection; (B) 8-h postinfection.

data for P43 can also be modeled successfully by these methods, assuming that only one species of message exists (not shown).

Alternative explanations of our data have been considered. For example, if the postulated repressors possessed extremely short half-lives in vivo, then a slight general inhibition of translation could conceivably lead to a derepression of this subset of mRNAs. However, this idea can be ruled out by the observation that inhibitors of initiation, such as harringtonine and medium hypertonicity, do not produce the stimulation of translation seen with cycloheximide. Another reasonable idea is that the cycloheximide effect has nothing to do with mRNA competition or limiting initiation factors but rather is solely due to the nature of the interaction between repressor and mRNA. While attractive in its simplicity, this concept cannot explain one rather peculiar observation: the stimulation of P52 synthesis by cycloheximide vanishes in reovirus-infected cells. The competition/discrimination model, in contrast, actually predicts such an effect. This is due to the fact that the values of the dissociation constants used to model the data in Table I necessitate that the repressed mRNAs must be very much poorer competitors for discriminatory factor than reovirus mRNAs, which are one-third to half as efficient as average host mRNAs under normal conditions of cell culture (Ray et al., 1985). Because of this relationship among the dissociation constant values, the large numbers of reovirus mRNAs introduced into the infected cell act to buffer the concentration of free discriminatory factor, and thereby prevent its rising to a level that would stimulate translation of repressed mRNAs.[2] That this predicted effect is actually observed is shown in Figure 10B, where the effect of cycloheximide addition on the translation rates of mRNAs in reovirus-infected cells 8-h postinfection is depicted [data were obtained by quantitation of bands shown in Figure 3A of Walden et al. (1981)]. As previously reported, reovirus mRNA translation is markedly stimulated by cycloheximide; however, that of P52 mRNA is not. This suppressive effect on the P52 response to cycloheximide is evidently a function only of viral mRNA concentrations, since at a slightly earlier time, when these concentrations are much lower, the effect is not seen (Figure 10A). As expected, sucrose gradient analysis showed that the bimodal polysome distribution of P52 mRNA was not altered by cycloheximide in reovirus-infected cells (data not shown). These observations suggest that the peculiar kinetic behavior of P52 mRNA, at least, cannot adequately be explained in terms of repressors alone. It is evidently sensitive to the presence of other unrelated mRNAs in the same cell.

The possibility that the mRNAs in the free mRNP fraction initiate slowly because they lack good "consensus" sequences around the starting AUG's (Sargan et al., 1982; Kozak, 1984a,b) has also been considered. The strongest argument against this idea is that the three ferritin L-chain mRNAs that have been sequenced have very good consensus sequences surrounding the AUG's (Liebold et al., 1984; Dorner et al., 1985; Didsbury et al., 1986). It remains possible that other mRNAs in the free mRNP fraction may lack good consensus sequences. In this case, the stimulation of their translation by cycloheximide and their activation by phenol treatment would still require further explanation.

At this point, it should be emphasized that it seems highly unlikely that all repression/induction systems will work the same way. Rather, it seems more likely that a multiplicity of control mechanisms should have evolved, each peculiar to its unique context. Moreover, it also seems likely that some members of the class of mRNAs described here will owe their peculiar behavior to some phenomenon other than repression. In any case, these studies offer new insights into these questions and suggest new avenues for attacking the problem experimentally.

Registry No. Iron, 7439-89-6.

[2] To see why this is so, let us consider the theoretical effect on the free discriminatory factor concentration of adding cycloheximide to virus-infected cells. Before viral mRNA synthesis has begun, 0.1 μg/mL cycloheximide increases the free factor concentration from 1.2 to 16.8 nM, a 14-fold effect. Even when the viral mRNA concentration is relatively low, representing 5% of the total mRNA present, the increase is from 1.0 to 12.9 nM. However, when the viral mRNA concentration has risen to 28% of the total mRNA, it binds much of the free factor, so the increase induced by cycloheximide is from 0.4 to 1.1 nM, which is only a 2.7-fold effect.

REFERENCES

Akhayat, O., Vincent, A., Goldenberg, S., Person, A., & Scherrer, K. (1983) *FEBS Lett. 162*, 25–32.

Bag, J. (1983) *Eur. J. Biochem. 135*, 187–196.

Bag, J., & Sells, B. H. (1981) *Mol. Cell. Biochem. 40*, 129–141.

Bergmann, I. E., Cereghini, S., Geoghegan, T., & Brawerman, G. (1982) *J. Mol. Biol. 156*, 567–582.

Brendler, T. G., Godefroy-Colburn, T., Carlill, R. D., & Thach, R. E. (1981a) *J. Biol. Chem. 256*, 11747–11754.

Brendler, T. G., Godefroy-Colburn, T., Yu, S., & Thach, R. E. (1981b) *J. Biol. Chem. 256*, 11755–11761.

Chu, L. L. H., & Fineberg, R. A. (1969) *J. Biol. Chem. 244*, 3847–3854.

Civelli, O., Vincent, A., Buri, J.-F., & Scherrer, K. (1976) *FEBS Lett. 72*, 71–76.

Civelli, O., Vincent, A., Maundrell, K., Buri, J.-F., & Scherrer, K. (1980) *Eur. J. Biochem. 107*, 577–585.

Cladaras, M. H., & Kaplan, A. (1984) *J. Biol. Chem. 259*, 14165–14169.

Croall, D. E., & Morrison, M. R. (1980) *J. Mol. Biol. 140*, 549–564.

Didsbury, J. R., Theil, E. C., Kaufman, R. E., & Dickey, L. F. (1986) *J. Biol. Chem. 261*, 949–955.

Dorner, M. H., Salfeld, J., Will, H., Liebold, E. A., Vass, J. K., & Munro, H. N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3139–3143.

Geoghegan, T., Cereghini, S., & Brawerman, G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5587–5591.

Godefroy-Colburn, T., & Thach, R. E. (1981) *J. Biol. Chem. 256*, 11762–11773.
Jen, G., & Thach, R. E. (1982) *J. Virol. 43*, 250–261.
Jenkins, N. A., Kaumeyer, J. F., Young, E. M., & Raff, R. A. (1978) *Dev. Biol. 63*, 279–298.
Kozak, M. (1984a) *Nucleic Acids Res. 12*, 857–872.
Kozak, M. (1984b) *Nature (London) 308*, 241–246.
Lee, G. T.-Y., & Engelhardt, D. L. (1979) *J. Mol. Biol. 129*, 221–233.
Liautard, J. P., & Egly, J. M. (1980) *Nucleic Acids Res. 8*, 1793–1804.
Liebold, E. A., Aziz, N., Brown, A. J. P., & Munro, H. N. (1984) *J. Biol. Chem. 259*, 4327–4334.
Linder, M. C., & Munro, H. N. (1972) *Anal. Biochem. 48*, 266–278.
Lodish, H. F. (1976) *Annu. Rev. Biochem. 45*, 39–72.
Lodish, H. F., & Porter, M. (1980) *J. Virol. 36*, 719–733.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Preobrazhensky, A. A., & Spirin, A. S. (1978) *Prog. Nucleic Acid Res. Mol. Biol. 21*, 1–38.
Ray, A., Walden, W. E., Brendler, T., Zenger, V. E., & Thach, R. E. (1985) *Biochemistry 24*, 7525–7532.
Ray, B. K., Brendler, T. G., Adya, S., Daniels-McQueen, S., Miller, J. K., Hershey, J. W. B., Grifo, J. A., Merrick, W. C., & Thach, R. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 663–667.
Sargan, D. R., Gregory, S. P., & Butterworth, P. H. W. (1982) *FEBS Lett. 147*, 133–136.
Sarkar, G., Edery, I., Gallo, R., & Sonenberg, N. (1984) *Biochim. Biophys. Acta 783*, 122–129.
Schaefer, F. V., & Theil, E. C. (1981) *J. Biol. Chem. 256*, 1711–1715.
Shull, G. E., & Theil, E. C. (1982) *J. Biol. Chem. 257*, 14187–14191.
Shull, G. E., & Theil, E. C. (1983) *J. Biol. Chem. 258*, 7921–7923.
Walden, W. E., & Thach, R. E. (1982) in *Interaction of Translational and Transcriptional Controls in the Regulation of Gene Expression* (Grunberg-Manago, M., & Safer, B., Eds.) pp 399–416, Elsevier Biomedical, New York.
Walden, W. E., Godefroy-Colburn, T., & Thach, R. E. (1981) *J. Biol. Chem. 256*, 11739–11746.
Watanabe, N., & Drysdale, J. (1981) *Biochem. Biophys. Res. Commun. 103*, 207–212.
Yenofsky, R., Bergmann, I., & Brawermann, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5876–5880.
Zahringer, J., Baliga, B. S., & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 857–861.

Chicken U2 and U1 RNA Genes Are Found in Very Different Genomic Environments but Have Similar Promoter Structures†

Gina M. Korf‡ and William E. Stumph*
Department of Chemistry and Molecular Biology Institute, San Diego State University, San Diego, California 92182
Received September 10, 1985; Revised Manuscript Received December 2, 1985

ABSTRACT: We have cloned and analyzed a gene that codes for chicken U2 small nuclear RNA (snRNA). In the haploid chicken genome, there are approximately 35–40 copies of the U2 RNA gene arranged in tandemly repeated units 5.35 kilobase pairs in length. This U2 gene organization contrasts with that of chicken U1 RNA genes, which are found in heterogeneous genomic environments. Although U snRNA genes are transcribed by RNA polymerase II, they lack the usual TATA and CAAT homologies found in the 5′ control regions of most RNA polymerase II transcription units. Nevertheless, a comparison of chicken U2 and U1 RNA gene 5′-flanking DNA sequences reveals two upstream blocks of homology which are also evolutionarily conserved in U2 and U1 RNA genes of other vertebrate species. The first block of conserved sequence is centered around position −55 relative to the RNA cap site, and the other is located near position −200. Interestingly, stretches of sequence with the potential to form Z DNA are located either within or immediately adjacent to both of these two conserved upstream sequence elements, suggesting a possible role for Z DNA in U1/U2 gene expression. Moreover, the chicken U2 and U1 gene promoter regions also contain specific short sequences (i.e., the hexamer GGGCGG and the octamer ATGCAAAT) that have been shown to be required for the expression of a number of mRNA-encoding genes. These findings suggest that the transcription of snRNA genes is controlled by a complex set of factors, some shared with other RNA polymerase II transcription units and others which may be unique to the snRNA genes.

The small nuclear RNAs (snRNAs)[1] of the U family represent a special class of RNA polymerase II transcripts (Eliceiri, 1980; Roop et al., 1981; Murphy et al., 1982; Mattaj & Zeller, 1983) which are metabolically stable and are not polyadenylated. With the exception of U6 RNA, they also contain an unusual 2,2,7-trimethylguanosine cap structure instead of the 7-methylguanosine cap found on mRNA molecules [for a review, see Busch et al. (1982)]. The snRNAs U1–U6, as well as the recently discovered U7–U10 snRNAs (Strub et al., 1984; Reddy et al., 1985), exist in vivo as integral

† This work was supported by grants to W.E.S. from the National Science Foundation (PCM-8310683) and the National Institutes of Health (GM-33512) and in part by the California Metabolic Research Foundation.
* Correspondence should be addressed to this author.
‡ G.M.K. is a predoctoral student in the Department of Biology, San Diego State University.

[1] Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; bp, base pair(s); kbp, kilobase pair(s).

0006-2960/86/0425-2041$01.50/0