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Translational Initiation Factor Expression and Ribosomal Protein Gene Expression Are Repressed Coordinately but by Different Mechanisms in Murine Lymphosarcoma Cells Treated with Glucocorticoids

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P1798 murine lymphosarcoma cells cease to proliferate upon exposure to 10^{-7} M dexamethasone and exhibit a dramatic inhibition of rRNA and ribosomal protein synthesis (O. Meyuhas, E. Thompson, Jr., and R. P. Perry, Mol. Cell Biol. 7:2691–2699, 1987). These workers demonstrated that ribosomal protein synthesis is regulated primarily at the level of translation, since dexamethasone did not alter mRNA levels but shifted the mRNAs from active polysomes into inactive messenger ribonucleoproteins. We have examined the effects of dexamethasone on the biosynthesis of initiation factor proteins in the same cell line. The relative protein synthesis rates of eIF-4A and eIF-2α were inhibited by about 70% by the hormone, a reduction comparable to that for ribosomal proteins. The mRNA levels of eIF-4A, eIF-4D, and eIF-2α also were reduced by 60 to 70%, indicating that synthesis rates are proportional to mRNA concentrations. Analysis of polysome profiles showed that the average number of ribosomes per initiation factor polysome was only slightly reduced by dexamethasone, and little or no mRNA was present in messenger ribonucleoproteins. The results indicate that initiation factor gene expression is coordinately regulated with ribosomal protein synthesis but is controlled primarily by modulating mRNA levels rather than mRNA efficiency.

Biosynthesis of the translational apparatus, namely, ribosomes and their associated soluble factors, involves the coordinate accumulation of a large number of proteins. The expression of ribosomal protein genes in bacteria is coordinated in large part at the level of translation, where autogenous regulation of the translation of operon mRNA occurs by feedback inhibition with one of the ribosomal protein products (30). In mammalian cells, ribosomal protein synthesis appears not to be regulated autogenously (6, 18, 20, 40); instead, protein turnover plays a key role in their balanced accumulation (1, 18, 21). Much less is known about the regulation of gene expression for the mammalian soluble factors. Studies of the elongation factor eEF-1α show that its synthesis is strongly reduced in Friend erythroleukemia cells at stationary phase and is stimulated when cells are treated with fresh serum-containing medium. Regulation involves changes in mRNA translation efficiency (polysome size) and mRNA levels, the latter by affecting mRNA stability (34, 35).

In earlier reports, we evaluated the levels and synthesis rates of a number of initiation factors in HeLa cells deprived of or stimulated by serum (9, 10). Of those factors examined, most (eIF-2, eIF-3, eIF-4B, and eIF-4Fp220) were approximately equimolar with ribosomes in these cells, whereas two (eIF-4A and eIF-4D) were three- to fourfold higher in concentration and at least one (eIF-4E) was about threefold lower. The relative synthesis and turnover rates of both ribosomal proteins and initiation factors are not altered in these cells by growth rate changes caused by serum; the translational components follow total protein synthesis and degradation rates (10). We are interested in determining whether initiation factor synthesis is coordinately regulated with that of ribosomal components and therefore sought a system in which the relative synthesis rates of ribosomal components change appreciably. Such a system is the murine lymphosarcoma cell when growth is arrested by treatment with glucocorticoids. Dexamethasone (DEX) causes a cessation of cell division and an approximately threefold decrease in the relative rates of synthesis of ribosomal proteins (25). The inhibition occurs at the level of translation. Ribosomal protein mRNA levels are not altered by treatment with DEX, but mRNA in active polysomes is shifted into nonactive messenger ribonucleoprotein (mRNP) particles. We have used the same cell line and treatment with DEX to examine the expression of initiation factor genes. Such studies are made possible by the prior cloning of human cDNAs encoding eIF-2α (12), eIF-4A (28), and eIF-4D (37). We found that coordination of initiation factor and ribosomal protein synthesis occurred but that the level of gene expression regulated differed between the two groups of proteins.

MATERIALS AND METHODS

Cell culture. P1798.S20 cells were grown in suspension as described by Cavanaugh and Thompson (4). Briefly, cells were maintained in RPMI 1640 (J. R. Scientific) containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2), 20 μM 2-mercaptoethanol, and 5% (vol/vol) fetal bovine serum (HyClone). No antibiotics were added. Cells were grown in suspension in an incubator with 95% air and 5% CO_2 at 37°C. Under these conditions, the log-phase population doubling time is about 14 h. Cell density was maintained within 0.1×10^6 to 1.0×10^6 cells per ml. Cells were treated with 10^{-7} M DEX (tissue culture grade, dissolved in 70% ethanol; Sigma Chemical Co.) for 24 h and are referred to as treated cells. After treatment with DEX, cells were centrifuged at $500 \times g$ (clinical centrifuge, position 4) for 5 min; cell pellets were washed three times

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with RPMI 1640, resuspended in fresh medium, and allowed to grow for an additional 24 h. Such cultures are referred to as rescued cells.

Polysome fractionation. Cells (5×10^7 to 1×10^8) growing for 23 h in 100 ml of medium containing DEX were diluted with 100 ml of fresh medium containing DEX and harvested 1 h later. Control cells (5×10^7 to 1×10^8) growing for 23 h in 100 ml of DEX-free medium were diluted with 100 ml of fresh medium and harvested 1 h later. Cell cultures were poured over frozen crushed phosphate-buffered saline, and cells were pelleted and washed three times with phosphate-buffered saline. Cells were suspended in 1 ml of lysis buffer (10 mM HEPES [pH 6.8], 5 mM $MgCl_2$, 100 mM KCl, 10 μg of cycloheximide per ml, 1 mM dithiothreitol, 0.5% Nonidet P-40) and lysed in a Dounce homogenizer with seven strokes. The lysate was centrifuged for 10 min at 8,000 rpm in a Sorvall SS-34 rotor, and the supernatant was layered onto a 15 to 45% (wt/wt) sucrose (ultrapure; ICN Pharmaceuticals Inc.) gradient in lysis buffer (no detergents) and centrifuged in a Beckman SW41 rotor for 70 min at 35,000 rpm. Gradients were fractionated by displacement from the bottom by using an ISCO model 185 gradient fractionator and scanned at A_{260} with a UA-5 monitor; 12 fractions (1.1 ml each) were collected.

Plasmids and probes. The following plasmids were used as sources of specific DNA probes. For the eIF-2α probe, a 1.4-kilobase (kb) *Tha*I-*Hin*dIII fragment was derived from pUC8-2α (constructed and provided by M. Humbelin), a plasmid containing a complete human eIF-2α cDNA inserted into the *Hin*cII-*Hin*dIII site of pUC8. For the eIF-4A probe, a 0.44-kb *Eco*RI fragment was derived from p8.30 (a generous gift from P. Nielsen, Max Planck Institute for Immunobiology, Freiburg, Federal Republic of Germany), a plasmid containing the 0.44-kb mouse eIF-4A (28) cDNA inserted into the *Eco*RI site of pUC8. For the eIF-4D probe, a 0.56-kb *Eco*RI-*Pst*I fragment was derived from pBS-4D (constructed and provided by Z. Smit-McBride), a plasmid containing the 0.56-kb human eIF-4D cDNA (37) inserted into the *Eco*RI-*Pst*I site of the Blue Script plasmid (Stratagene). For the actin probe, a 1.15-kb *Pst*I fragment was derived from pActin (26), a plasmid containing the 1.15-kb mouse actin cDNA inserted into the *Pst*I site of pUC9. For the ribosomal protein L32 probe, a 1.6-kb *Sac*I fragment was derived from p4A/Sac 1.6 (8), a plasmid containing the 1.6-kb processed gene of mouse L32 inserted into the *Sac*I site of pUC12. Cleaved fragments were purified by agarose gel electrophoresis and labeled with [α-^{32}P]ATP by the Multiprime labeling system (Amersham Corp.) to a specific activity of ca. 10^9 cpm/μg of DNA.

Preparation and blot analysis of RNA. Total RNA from P1798.S20 cells was isolated by the guanidinium-cesium chloride method (23). Washed cells were lysed into 6 M guanidinium isothiocyanate–5 mM sodium citrate–0.1 M 2-mercaptoethanol–0.5% Sarkosyl (CIBA-GEIGY Corp.) and then brought to 0.4-g/ml cesium chloride (high purity; CABOT). RNA was pelleted through a 1.2-ml cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.5) by centrifugation in a Beckman SW60 rotor at 41,000 rpm for 15 h at 20°C. The RNA pellet was dissolved in 10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–1% sodium dodecyl sulfate (SDS) and extracted once with a 4:1 mixture of chloroform and 1-butanol. The aqueous phase was transferred to a fresh tube, and the organic phase was reextracted with an equal volume of 10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–1% SDS. The aqueous phases were combined, and the RNA was precipitated with ethanol.

For Northern (RNA) blots, total RNA was subjected to electrophoresis (100 V for 5 h) in a 1% agarose gel containing 2.2 M formaldehyde. The RNA was then transferred to nitrocellulose sheets (Schleicher & Schuell, Inc.) as described by Maniatis et al. (23). For slot blot analysis, RNA was incubated with 7% formaldehyde in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C for 10 min and then diluted with 10× SSC and applied to nitrocellulose with a Schleicher & Schuell slot blot apparatus. The Northern and slot blots were baked under vacuum for 2 h at 80°C and prehybridized for 2 to 3 h at 42°C with 50% formamide–2.5× Denhardt reagent, 100 μg of denatured salmon sperm DNA per ml–0.1% SDS–5× SSC. Hybridization was in the same buffer, using ^{32}P-labeled probes (ca. 10^7 cpm). The blots were incubated overnight or for 40 h at 42°C, washed twice for 15 min each time in 1× SSC–0.1% SDS at room temperature and then washed twice for 15 min each time in 0.1× SSC–0.1% SDS at 42°C. The blots while still damp were wrapped in Saran Wrap and exposed to Kodak X-Omat AR film at −70°C with an intensifying screen (Dupont Cronex Lightning-Plus).

Cell labeling and protein extraction. P1798 cells (1 to 5 ml at 10^5 cells per ml) were centrifuged, and the supernatant was carefully removed. The cell pellet together with 0.1 ml of conditioned medium was suspended in 1 ml of methionine-free minimal essential medium (GIBCO Laboratories) containing 5% fetal bovine serum, 5 mM HEPES (pH 6.8), and [^{35}S]methionine (330 μCi/ml, 1,160 Ci/mmol; Amersham). For treated cells, the medium also contained 10^{-7} M DEX. The culture was incubated at 37°C for 30 min with shaking. The cells were then harvested by three washes with 5 ml of phosphate-buffered saline and lysed with lysis buffer (9.8 M urea, 2% pH 3.5 to 10 LKB Ampholines, 1% 2-mercaptoethanol). A sample of lysate was taken out and precipitated with 10% trichloroacetic acid. The precipitate was collected on a glass fiber filter and washed with cold 10% trichloroacetic acid, and the radioactivity on the dried filter was counted in toluene scintillation mixture, using an LKB 1212 Rackbeta liquid scintillation counter. The incorporation was linear for 1 h. Two-dimensional isoelectic focusing–SDS-polyacrylamide gel electrophoresis and immunoblot identification of factor protein spots were done as previously described (9, 24). Lysates of pulse-labeled control and treated cells were analyzed by two-dimensional gel electrophoresis and autoradiography. The relative rate of synthesis is defined as the fraction of radioactivity incorporated into specific initiation factor proteins compared with the rate for total proteins. By applying the same amount of total radioactivity in each gel, the relative synthesis rates were directly compared by visualizing the intensities of factor protein spots on the autoradiogram. Quantitation was done by determining the exposure time required for equalizing the intensities of the same factor spot on the two pictures.

RESULTS

Inhibition of translation by DEX treatment. Murine P1798 lymphosarcoma cells grow rapidly in suspension culture and exhibit a doubling time of 12 to 14 h. When such cells were chilled and harvested rapidly and the distribution of ribosomes in polysomes was analyzed by sucrose density gradient centrifugation, the resulting polysome profile (Fig. 1a) was typical for cells active in protein synthesis. About 80% of the ribosomes were in polysomes, and the polysome envelope included quite large polysomes. In contrast, when P1798 cells were treated with 10^{-7} M DEX for 24 h and

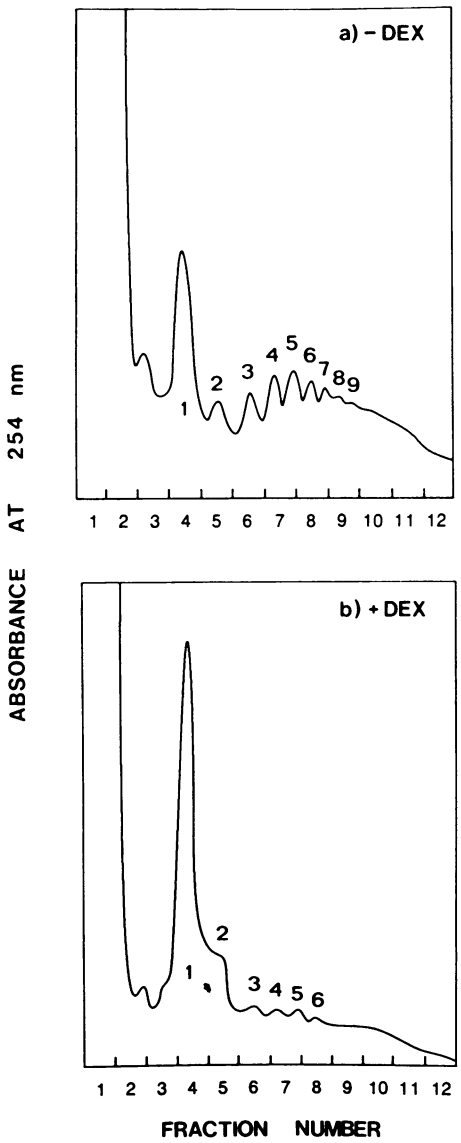

FIG. 1. Polysome profiles of DEX-treated and untreated cells. P1798 cells (ca. 6×10^7) were either not treated (a) or treated with 10^{-7} M DEX for 24 h (b). An equal number of treated and untreated cells (6×10^7) was then lysed and analyzed by sucrose gradient centrifugation (see Materials and Methods). Shown are typical scans of optical density at A_{260}. Because of substantially more ribosome biosynthesis during the 24-h incubations with untreated cells, the total optical density in the profile is greater for panel a than for panel b. Sedimentation is from left to right.

analyzed, the polysome profile (Fig. 1b) indicated a strong inhibition of initiation of protein synthesis. Only 30% of the ribosomes remained in polysomes, and the average polysome size was reduced. The ca. 2.5-fold reduction in active, translating ribosomes is roughly consistent with a 3.5-fold reduction in radiolabeled amino acids incorporated over a pulse of 30 min after 24 h of treatment with the hormone. These results on global translation rates are comparable to those reported by Meyuhas et al. (25), who also reported that the relative rate of ribosomal protein synthesis is reduced approximately an additional threefold over total protein synthesis rates. We examined the relative rates of synthesis of eIF-2α and eIF-4A by comparing their spot intensities in autoradiograms of pulse-labeled cell lysates fractionated by high-resolution isoelectric focusing–SDS-polyacrylamide gel electrophoresis (see Materials and Methods). When equal amounts of radioactivity were loaded on the gels, the eIF-2α and eIF-4A spots were about threefold reduced in intensity by DEX treatment (results not shown).

Factor mRNA distributions in polysome profiles. To investigate the basis for the reduced rates of initiation factor synthesis, the distributions of the initiation factor mRNAs in polysomes were determined by sucrose density gradient centrifugation and Northern blotting. Untreated and 24-h hormone-treated cells were lysed in the presence of cycloheximide, and lysates were fractionated by centrifugation into 12 fractions as described in Materials and Methods (Fig. 1). RNA from each fraction was isolated and first analyzed by Northern blot hybridizations with initiation factor cDNA probes. For each probe, a single size class of mRNAs was observed (results not shown), consistent with recognition by the probes of only their cognate mRNAs. Then equal volumes of each gradient fraction were analyzed by slot blot hybridizations (Fig. 2). With untreated cells, the mRNAs for initiation factors were found predominantly in the polysome fractions (Fig. 2, fractions on the right side of the vertical line) (Table 1). With DEX-treated cells, the overall intensities of the mRNAs were reduced, but there was little change in the sizes of polysomes and negligible conversion of polysomal mRNA into mRNPs. The similar sizes of initiation factor polysomes indicated that active mRNAs were translated with approximately equal efficiencies in the two cell states (assuming that elongation rates are constant). The RNA polysome profiles for initiation factors were in striking contrast to those for ribosomal proteins (L32 in Fig. 2), which show a large increase in their distribution to the mRNP fractions (25).

Reduction of initiation factor mRNA levels by DEX treatment. The sum of the slot intensities of the 12 gradient fractions (Fig. 2) for each of the initiation factors from DEX-treated cells clearly was reduced. Since an equal number of cells was applied to these gradients and since nondividing cells generally have less RNA content (19), a firm conclusion about the cellular levels of factor mRNAs could not be reached on the basis of these Northern and slot blot analyses. To better measure possible changes in factor mRNA levels in DEX-treated cells, slot blot hybridizations to equal amounts of total cellular RNA were carried out with cDNA probes for eIF-2α, eIF-4A, and eIF-4D. The autoradiograms shown in Fig. 3 were quantitated by densitometric scanning; values are reported in Table 1. The relative level of each of the three initiation factor mRNAs dropped about threefold, whereas the relative levels for actin and ribosomal protein L32 remained constant. In contrast to ribosomal proteins (25), the initiation factor mRNA levels paralleled their relative protein synthesis rates. This finding suggests that initiation factor gene expression may be controlled not at the level of protein synthesis but rather at the level of transcription or posttranscriptional events.

DISCUSSION

We have found in this study that glucocorticoids specifically inhibit initiation factor gene expression in P1798 murine lymphosarcoma cells. Our results show that the inhibition of initiation factor gene expression occurs mainly by reducing the levels of the initiation factor mRNAs. The translation efficiency of initiation factor mRNA is not altered (assuming that elongation rates are constant), and the threefold de-

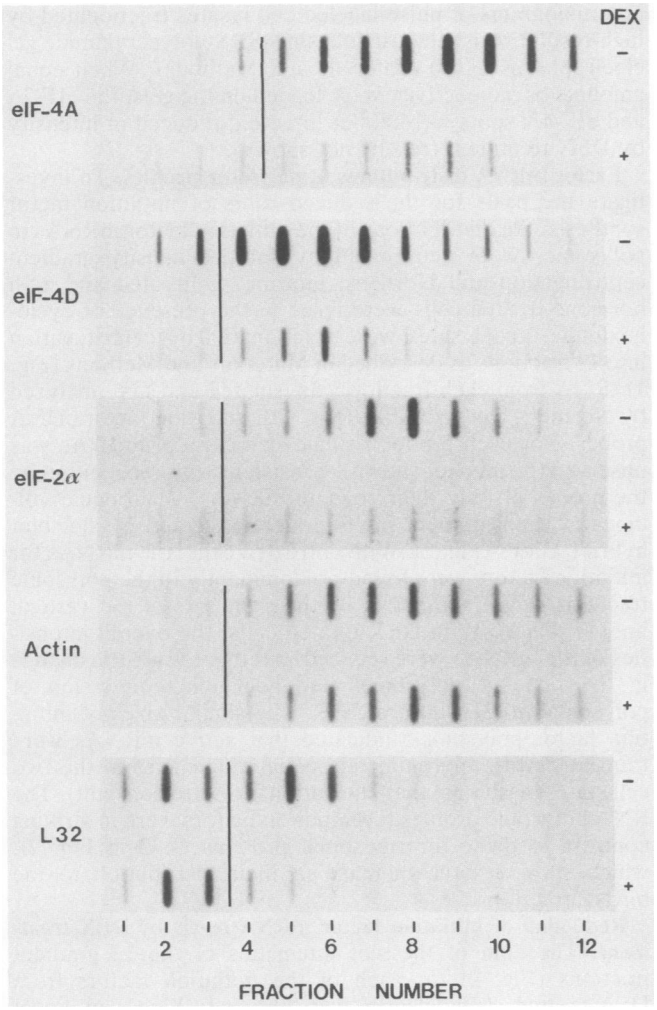

FIG. 2. Slot blot hybridization analyses of mRNAs in polysome gradients. DEX-treated (+) and untreated (−) cell lysates were analyzed by sucrose density gradient centrifugation as described in the legend to Fig. 1 and Materials and Methods. Each of the 12 gradient fractions was brought to 1% SDS, and protein was extracted three times with phenol-chloroform-isoamyl alcohol (24:24:1). RNA in the aqueous phase was precipitated with ethanol and dissolved in 200 μl of water, and 100 μl was applied to slots for hybridization as described in Materials and Methods. Shown are autoradiograms of the slot blots exposed for 1 day to Kodak X-Omat AR film. The vertical line separates the polysomal fractions (>80S, right) from subpolysomal fractions (<80S, left).

crease in the relative level of mRNA is consistent with the threefold decrease in the relative rate of factor protein synthesis. This inhibition of about threefold is comparable to that of ribosomal proteins (25). Thus, our results show that the biosynthesis of initiation factors and the biosynthesis of ribosomes are coordinately regulated in DEX-treated cells. Coordinate regulation also occurs in procaryotic cells growing at different rates (16).

In many cases, glucocorticoids such as DEX enhance gene transcription (41), but these hormones can regulate specific mRNAs by other mechanisms. For example, glucocorticoids enhance the stability and increase the length of the 3′ poly(A) tail of human growth hormone mRNA (7, 31) and stimulate transport of mRNA from nucleus to cytoplasm (14). Glucocorticoids may also negatively regulate gene

TABLE 1. Comparison of levels and polysomal association of various mRNAs in DEX-treated and untreated P1798 lymphosarcoma cells

Protein	Relative mRNA level of +DEX cells[a] (% −DEX)	% mRNA in polysomes[b]		No. of codons[c]	Avg no. of ribosomes/ mRNA[d]	No. of codons/ ribosome[e]
		−DEX	+DEX			
eIF-4A	27	96	80	406	9–10	43
eIF-4D	33	90	87	153	3–4	44
eIF-2α	35	96	85	315	8–10	35
Actin	100	92	94	374	8–10	42
L32	90	58	20	135	3–4	40

[a] Data derived from Fig. 3. +DEX, DEX-treated cells; −DEX, untreated cells.
[b] Data derived from Fig. 2.
[c] Derived from available amino acid sequences as follows: mouse eIF-4A (29), human eIF-4D (37), rat eIF-2α (12), rat actin (26), and mouse L32 (8).
[d] Derived from a comparison of the peak polysomal fractions in untreated cells (Fig. 2) and the corresponding polysomal profile (Fig. 1).
[e] Obtained by dividing the number of codons in an mRNA by the average number of ribosomes in the peak polysomal fractions.

expression. For example, they inhibit the transcription of several genes, including those encoding stromelysin (13), pro-opiomelanocortin (5, 17), and human glycoprotein hormone α subunit (2), through negative regulation mediated by 5′-flanking sequences. These hormones can also decrease the amount of specific mRNAs by posttranscriptional regulation, as shown by DEX inhibition of accumulation of interferon mRNA in human fibroblasts (15). This inhibition does not reflect changes at the level of transcription of the mRNA. Our study suggests that DEX may negatively regulate initiation factor mRNAs through transcriptional mechanisms. Since transcriptional inhibition by DEX is likely to be mediated by glucocorticoid-responsive elements (2), we have examined the sequences of a possibly functional eIF-4A retroposon (29) and the human eIF-2α gene (M. B. Hümbelin, B. Safer, J. A. Chiorini, J. W. Hershey, and R. B.

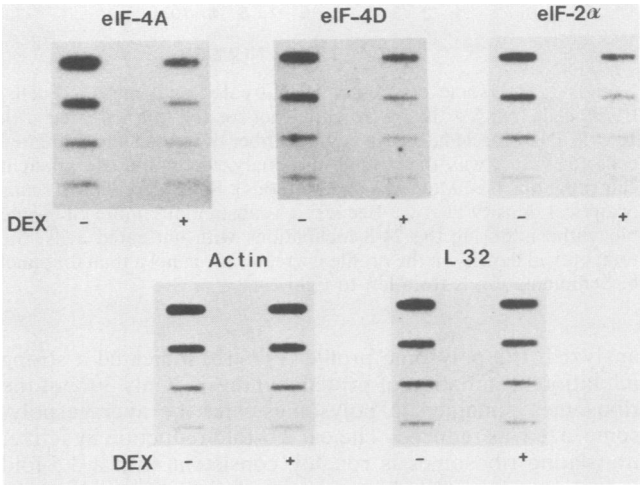

FIG. 3. Slot blot hybridization analyses of the effects of DEX on the levels of factor mRNAs in P1798 cells. Total RNA was prepared from DEX-treated (+) and untreated (−) cells and was applied to nitrocellulose by twofold serial dilutions, starting from 5 μg, as described in Materials and Methods. The filters were hybridized to ^{32}P-labeled cDNA probes for eIF-2α, eIF-4A, eIF-4D, actin, and L32. Shown are autoradiograms of the slot blots exposed for 1 day to Kodak X-Omat AR film.

Cohen, Gene, in press) but have been unable to find any such elements. Another possible explanation for the effect of DEX on initiation factor mRNA levels is an increased turnover of these mRNAs. Such a mechanism accounts for the decrease in type I procollagen mRNA in DEX-treated fibroblasts (33, 38). It has been suggested that DEX may activate some sequence-specific nucleases that degrade mRNA with AUUUA sequence motifs in their 3′ untranslated regions (15, 36). However, the 3′ untranslated regions in the eIF-4D (incomplete), eIF-4A, and eIF-2α cDNAs reveal no such sequence repeats. Regulation at the level of RNA splicing or nuclear transport also is possible (14, 39). Further experimentation is required to identify which steps in the transcription, processing, and turnover of initiation factor mRNAs are controlled by glucocorticoids in lymphosarcoma cells.

It is striking that initiation factor mRNA and ribosomal protein mRNA are two very different classes of mRNA with respect to translational efficiency. Initiation factor mRNAs are translated efficiently, and little of these mRNAs is detected in the mRNP fractions of sucrose gradients. The ribosome loading densities (number of codons per ribosome) of eIF-4A and eIF-4D mRNAs (Table 1) are similar to those of actin and L32 (42 and 40, respectively). The average message in HeLa cells, which occurs in polysomes containing seven to eight ribosomes (3, 11) and encodes a 37,000-dalton protein (27), has a comparable ribosome loading density of 43 codons per ribosome. eIF-2α mRNA is more efficient than the average, since it has a ribosome loading density of 35 (Table 1), which is close to that of globin mRNA in reticulocytes (ca. 32). This result confirms our previous findings in HeLa cells, where eIF-2α mRNA is also translated efficiently (12). The efficiency of eIF-2α mRNA is not obviously explained by its 5′ leader sequences, which have extensive stable secondary structures (Hümbelin et al., in press). Such structures are thought to decrease the translation efficiency of mRNAs (22, 32).

It appears that translational regulation is not directly involved in the control of initiation factor protein synthesis. This is in contrast to the mechanism for the inhibition of ribosomal protein synthesis, in which the level of ribosomal protein mRNA is not changed. Instead, the translation of ribosomal protein mRNA is inhibited, since DEX treatment leads to a shift in the distribution of mRNA from active polysomes into inactive mRNPs (25). However, we cannot exclude the possibility that a similar translational mechanism leads to the decrease in the amount of initiation factor mRNAs. If translation of a portion of the mRNAs were inhibited, resulting in their conversion into mRNP particles, and if such mRNPs were rapidly degraded, the polysomal profiles reported in Fig. 2 would result. Therefore, a mechanism of shifting mRNA from polysomes to mRNPs, which accounts for the decrease in ribosomal protein synthesis, could also be involved in the decrease of initiation factor mRNA levels.

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