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Cytochalasin Releases mRNA from the Cytoskeletal Framework and Inhibits Protein Synthesis

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Cytochalasin D was shown to be a reversible inhibitor of protein synthesis in HeLa cells. The inhibition was detectable at drug levels typically used to perturb cell structure and increased in a dose-dependent manner. The drug also released mRNA from the cytoskeletal framework in direct proportion to the inhibition of protein synthesis. The released mRNA was unaltered in its translatability as measured in vitro but was no longer translated in the cytochalasin-treated HeLa cells. The residual protein synthesis occurred on polyribosomes that were reduced in amount but displayed a normal sedimentation distribution. The results support the hypothesis that mRNA binding to the cytoskeletal framework is necessary although not sufficient for translation. Analysis of the cytoskeletal framework, which binds the polyribosomes, revealed no alterations in composition or amount of protein as a result of treatment with cytochalasin D. Electron microscopy with embedment-free sections shows the framework in great detail. The micrographs revealed the profound reorganization effected by the drug but did not indicate substantial disaggregation of the cytoskeletal elements.

Cellular architecture appears to play a role in eucaryotic protein synthesis. Considerable evidence indicates that eucaryotic polyribosomes are bound to the structural networks of the cell. Biochemical evidence for this association is largely based on detergent extraction, which removes the soluble phase from the cell structure. In these studies, polyribosomes remain bound to the detergent-isolated cell structures (2, 9, 13, 16, 21, 22, 44). There are mRNA molecules free in the cytoplasm, but these are not translated (9).

Morphological studies show that the spatial distributions of polyribosomes and mRNA are not random. Polyribosomes in 3T3 cells are preferentially localized in perinuclear regions (13). A discrete distribution of actin mRNA in fusing myoblasts has been described (41) as well as a differential localization for histone and actin mRNA in the *Xenopus* oocyte (17). Such topographical concentrations are difficult to envision without an underlying organizing structure to which the polyribosomes and mRNA can bind. Those studies with both detergent extraction and morphological criteria found that the amount and the spatial distribution of polyribosomes were similar before and after the extraction (13). It is, therefore, unlikely that the observed polyribosome binding to skeletal structures is due to extraction artifacts. Other components of the protein synthesis apparatus, including initiation factors (16) and an aminoacyl-tRNA synthetase complex (29), also appear structure bound. Furthermore, immunofluorescence experiments indicate the colocalization of a protein homologous to the cap-binding protein with elements of the cytoskeleton (CSK) (50; reviewed in reference 31).

Some experiments have suggested that the association of polyribosomes with cell structure may be essential for translation in most eucaryotic cells. For example, newly formed vesicular stomatitis virus mRNA functions while bound to the cytoskeletal framework (CSKF) and ceases translation when released to the soluble phase (9). Maternally inherited mRNAs in the oocyte begin to function only after binding to the newly forming CSKF (30).

This report examines the association of polyribosomes with the CSKF by using cytochalasin D (CD) to disaggregate polyribosomes and release mRNA. These data show that when mRNA is released from the CSKF it ceases translation. Most important, the mRNA remaining on the CSKF functions at a normal rate. In contrast, the reticulocyte, with its presumably soluble protein synthetic system, shows no sensitivity to the drug.

MATERIALS AND METHODS

Materials. All chemicals and drugs, unless otherwise stated, were purchased from Sigma Chemical Co., St. Louis, Mo. CD was a stock solution of 5 mg/ml in dimethyl sulfoxide (DMSO). Actinomycin D was used from a stock solution of 2.0 mg/ml in 70% ethanol. Carrier DMSO was used in all controls, and the amount of DMSO added to each sample was adjusted to 1.5% of the total volume. Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass. Rabbit reticulocytes were prepared from anemic rabbits (Green Hectares, Madison, Wis.) by standard procedures and shipped on wet ice for use within 16 h of their isolation.

HeLa cells. HeLa S3 cells maintained in suspension culture were grown in spinner bottles at 37°C in Eagle minimum essential medium supplemented with 7% horse serum (Irvine Industries, Santa Ana, Calif.). Cells were maintained at 4×10^5 cells per ml. HeLa cells of the same strain were maintained in monolayers in a humidified atmosphere containing 5% CO_2 in Dulbecco modified Eagle minimum essential medium supplemented with 10% horse serum. Cells in monolayers (American Type Culture Collection CCL 2.2) were passaged twice weekly at 1:5 to 1:40 dilutions and typically used 32 to 48 h after plating. Preformulated, powdered medium was purchased from GIBCO Laboratories, Grand Island, N.Y.

Cell labeling. Suspension-grown HeLa cells were pelleted at $650 \times g$, washed in phosphate-buffered saline (PBS), and suspended in the appropriate medium, as indicated in the text and figure legends. The protein was pulse-labeled at 37°C with 25 to 100 μCi of L-[^{35}S]methionine (8.3 mCi/ml; 1064 Ci/mmol) per ml for the amounts of time indicated. Protein prepared for gel electrophoresis was typically la-

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beled for 30 min with the higher concentration of radiolabel in methionine-free medium. Unpublished results indicate that a 30-min pulse has been found to be sufficient to label cell protein to steady-state levels in the subcellular fractions. Rates of protein synthesis were determined with a 3-min pulse terminated by the rapid addition of 1,000-fold excess of unlabeled methionine. Time-critical experiments with cells in monolayers were carried out in a 37°C warm room. The medium in such experiments was further buffered by the addition of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES).

Cell fractionation. Cells were washed with PBS (4°C) as above. Pellets of suspension-grown cells were suspended in CSK buffer [100 mM NaCl, 300 mM sucrose, 10 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.8), 3 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 1.2 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100] and incubated on wet ice for 3 min. Cells in monolayers were extracted in culture plates floating on an ice bath. The CSKFs from suspension-grown cells were pelleted after centrifugation at 650 × *g* for 5 min and the supernatant (SOL fraction) was removed. The SOL fraction from monolayer cells was removed directly from the culture plate. The resulting nuclei-containing frameworks were suspended in RSB (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 7.4]) with 1% deoxycholate and 0.5% Tween 40, either by resuspending the pellet directly or removing the CSKFs from the culture plate with a rubber policeman in detergent-containing RSB. The nuclei and attendant intermediate filaments were removed by homogenizing with a Teflon-coated pestle in a glass Potter-Elvjhen homogenizer and pelleting at 1,000 × *g* for 5 min; the CSK fraction was obtained as the supernatant. For analysis by gel electrophoresis, the nuclei-containing pellet was suspended in sodium dodecyl sulfate (SDS) buffer containing 8.0 M urea (to solubilize intermediate filament proteins) and sonicated to yield the nuclear (NUC) fraction.

RNA isolation and in vitro translation. Cells were fractionated as described above except for the addition of 20 mM vanadyl adenosine to all buffers and the use of sterile technique throughout the procedures. The cell fractions were made up to 150 mM NaCl, 10 mM EDTA, 100 mM Tris hydrochloride (pH 7.6), and 1% SDS before adding proteinase K to 200 μg/ml. The samples were digested at 30°C for 30 min and extracted with two cycles of phenol-chloroform extraction essentially as described by Penman (33). poly(A)$^+$ RNA was purified by selection over oligo(dT)-cellulose (type III) obtained from Collaborative Research, Inc., Lexington, Mass. In some experiments, poly(A) was measured by hybridization to [^{3}H]poly(U) (2 to 10 Ci/mmol of UMP) as described by Milcarek et al. (26). Briefly, replicate RNA samples were hybridized to a molar excess of [^{3}H]poly(U) in 2× SSC (300 mM NaCl plus 30 mM sodium citrate [pH 7.4]). Single-stranded RNA was digested with pancreatic RNase A, and the trichloroacetic acid-precipitable radioactivity was determined and compared with a standard series with purified poly(A). Cellular mRNA was assumed to contain 4% poly(A) by mass. Purified poly(A)$^+$ RNA was translated in a reticulocyte-derived lysate obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., as described by the supplier. Incorporation of radioactive amino acid into protein was linear with the addition of poly(A)$^+$ RNA up to 0.4 μg/30-μl reaction.

Polyribosome analysis. rRNA was labeled overnight with [5,6-^{3}H]uridine (37 Ci/mmol) at a concentration of 0.1 to 0.5 μCi/ml. After extraction with the buffers described above containing 20 mM vanadyl adenosine, portions of each fraction representing equivalent numbers of cells were applied to a 15 to 40% linear sucrose gradient in HSB (0.5 M NaCl, 50 mM $MgCl_2$, 10 mM Tris hydrochloride [pH 7.4]). The gradients were centrifuged for 90 min at 40,000 rpm in a Beckman SW41 rotor at 4°C. Fractions were collected from the bottom of the gradient, and the radioactivity was determined by scintillation counting in Hydrofluor (National Diagnostics, Sommerville, N.J).

Electron microscopy. HeLa cells, grown in suspension or on Formvar-coated gold grids, were fractionated as described above. The resulting structures were fixed in 2% glutaraldehyde in CSK buffer for 30 min at 4°C. The fixed structures were washed three times in 0.1 M sodium cacodylate (pH 7.2) (5 min per wash, 4°C), briefly postfixed with 1% OsO_4 in 0.1 M sodium cacodylate for 3 min at 4°C, and subsequently washed in 0.1 M sodium cacodylate. The structures were dehydrated through a series of increasing ethanol concentrations, ending with three changes of 100% ethanol. Preparations for scanning electron microscopy (model DS 130; International Scientific Instruments, Inc.) were fixed in PBS, processed as above, dried through the critical point, and sputter coated with gold-palladium, and viewed. Extracted whole mounts were viewed directly after critical point drying. Specimens for embedding in diethylene glycol distearate (DGD) were processed as described (7) by immersing in *n*-butyl alcohol (*n*-BA)-ethanol (2:1) and then in *n*-BA-ethanol (1:2), followed by four changes in 100% *n*-BA for 15 min each. The structures were transferred to DGD (Polysciences Inc., Warrington, Pa.) through a series of *n*-BA-DGD mixtures of 2:1 then 1:2 for 10 min each at 60°C, followed by three changes of 100% DGD for 1 h each. Embedded structures were allowed to solidify, and DGD blocks were cut with glass knives at an angle of 10° on an MT-2B Porter-Blum ultramicrotome. Sections were placed on Parlodion- and carbon-coated grids. DGD was removed by immersing the grids in 100% *n*-BA at 23°C (three washes, each for 1 h). The grids were returned to 100% ethanol through a graded series of ethanol-*n*-BA mixtures and dried through the CO_2 critical point. Sections were examined in a JEOL EM 100 B electron microscope at 80 kV.

Electrophoresis. Two-dimensional gel electrophoresis was performed essentially by the method of O'Farrell (32) with equilibrium isoelectric focusing gels containing 2% of a 4:1 mixture of pH 3.5 to 10 and pH 5 to 7 Ampholines (LKB, Sweden). The second dimension was separated with 10% polyacrylamide gels containing SDS as described by Laemmli (18). Isoelectric points were determined with parallel runs of a calibration standard (pI range 4.7 to 10.6) obtained from BDH Chemicals Ltd., Poole, England. One-dimensional gel electrophoresis was performed by the method of Laemmli (18). Radioactive proteins were visualized by the method of Bonner and Laskey (4) by using Kodak X-Omat AR film preflashed to obtain a linear response (19).

RESULTS

Effect of CD on cell morphology. The fungal metabolite CD is well known for its profound effects on cell morphology. The perturbations of structure are manifest at concentrations as low as 0.5 to 2 μg/ml (27, 28, 38). Higher concentrations of CD have little additional effect on external gross morphology but, as shown here, do further alter the organization of the internal CSKF. Figure 1 shows scanning electron micrographs of HeLa cells exposed to low (2 μg/ml) and high (32

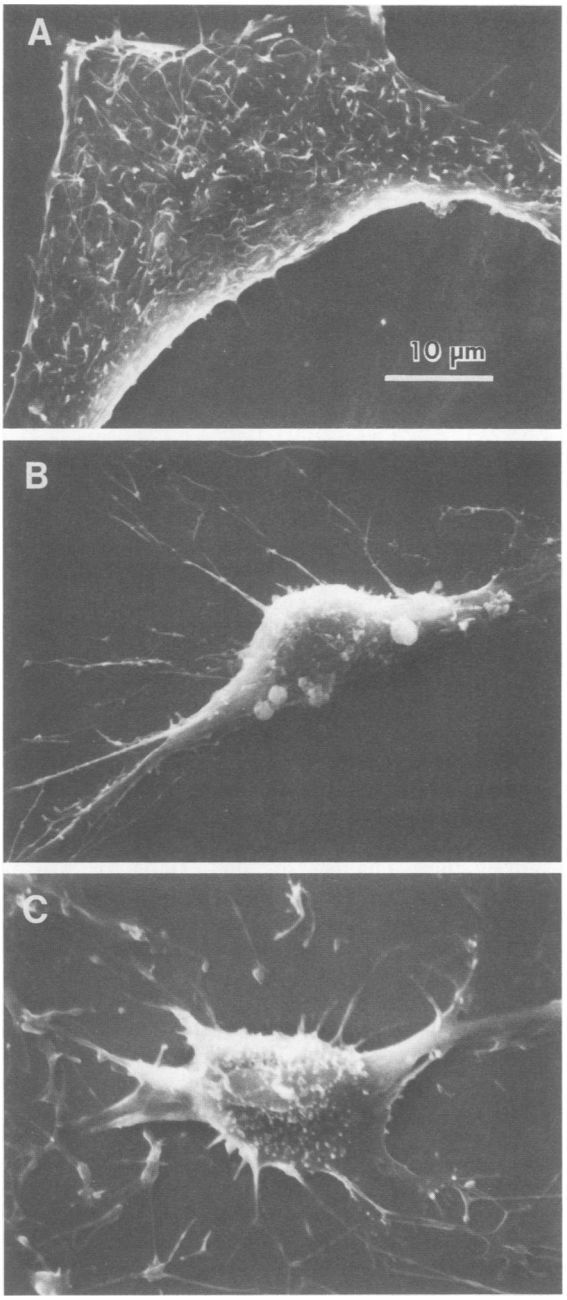

FIG. 1. Scanning electron micrographs of HeLa cells in monolayers. Cells were grown on glass cover slips and exposed to 1.5% DMSO (A), 2 μg of CD per ml (B), or 32 μg of CD per ml (C) for 30 min prior to gluteraldehyde fixation and processing as described in Materials and Methods. The bar in Fig. 1A indicates 10 μm.

μg/ml) concentrations of CD for 30 min. In both treatments, the cell bodies retracted, leaving fine, arborized processes attached to the substrate. The alterations in morphology were almost complete at a drug concentration of 2 μg/ml; a higher concentration resulted in only minor additional effects.

Effect of CD on filaments of the CSKF. The altered external morphology of CD-treated cells reflects the reorgnization of the internal filamentous CSKF (28, 38). Some changes in the fibers of the CSK, such as the dispersal of actin stress cables, are visible at the light microscopic level (46). More insight into the effects of the drug requires electron microscopy. However, much of the filamentous structure of the CSKF is masked in conventional embedded-section electron micrographs (Fig. 2A). Embedment-free electron microscopy is more suited to imaging three-dimensional fiber networks (7, 8). Fiber networks can be visualized in great detail in unembedded whole mounts of the CSKF provided the cells are relatively flat (Fig. 2B). The CD-treated cells are no longer well spread, and their CSKF is concentrated near the nucleus. The thickness of these cells precludes effective whole-mount electron microscopy (Fig. 2C). However, the recently developed techniques of embedment-free sections allow the organization of the CSKF from CD-treated cells to be examined (7).

The CSKF of suspension-grown HeLa cells was prepared and processed for resinless-section electron microscopy. Control cells were compared with cells treated with either 2 or 32 μg of CD per ml for 30 min. The cells were extracted with Triton X-100 in CSK buffer as described in Materials and Methods. The CKSF was fixed, dehydrated in alcohol, and infiltrated with the removable embedding compound DGD. Sections were cut and the DGD was removed as described in Materials and Methods. The self-supporting sections were dried through the critical point and viewed directly in the electron microscope.

Figure 2D shows a resinless-section image of the control HeLa CSKF. Although actin is a prominent cytoskeletal protein, the 6-nm microfilaments, marked as MF, constituted only a modest portion of the structure. As noted in other reports, there are many structural proteins composing the Triton-extracted CSKF (5, 12, 39). This complexity of composition is consistent with the many different structural components visualized here. CD renders the filament networks topographically heterogeneous so that regions rich in filaments alternate with empty areas. Dense foci, with many short filaments, were prominent. These foci were often adjacent to filament-depleted regions. Many fibers coalesced into loose bundles that typically followed the curvature of the nuclear lamina.

The differences observed between the effects of low (2 μg/ml) and high (32 μg/ml) concentrations of CD were ones of degree of filament reorganization. The micrographs suggest an increased number of free filament ends at the high concentration of CD. Such an increase in apparent ends is in accord with the postulated action of CD in fracturing actin filaments (14, 25, 40) or capping filament ends (23, 24). The high drug concentration resulted in more foci and a diminution of filamentous structures, possibly through increased lateral associations of the filaments of the CSKF or perhaps as the result of fiber scissions. The micrographs did not suggest a massive loss of cytoskeletal material. This is corroborated by direct measurements of the protein content in the structural fractions described below.

Protein content and composition of the CSKF from CD-treated cells. Treatment of cells with CD had almost no effect on the measured protein content and composition of the CSKF. Suspension-grown HeLa cells were prelabeled with [^{35}S]methionine, treated with CD, and fractionated as described in Materials and Methods. The SOL components were released with nonionic detergent extraction. The resulting nuclei-containing CSKFs were further extracted with the mixed detergent, Tween 40 and deoxycholate, in low-ionic-strength buffer (RSB). This second extraction removed most of the CSKF proteins from the nucleus. The fraction solubilized by the mixed detergent was the CSK, and it

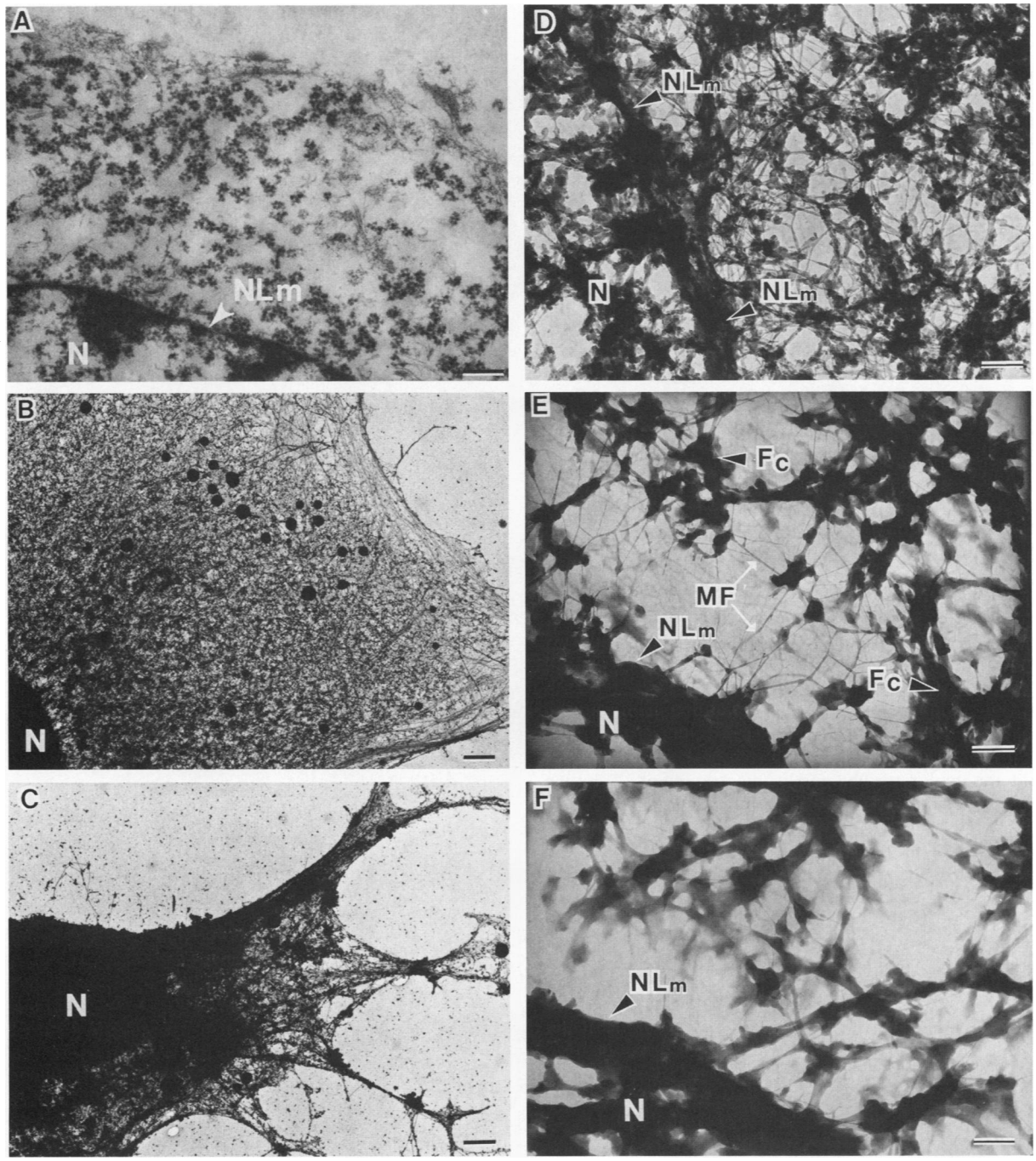

FIG. 2. Transmission electron micrographs of HeLa cells and CSKFs. N, Nucleus; NLm, nuclear lamina; MF, presumptive microfilaments; Fc, CD-induced foci. (A) Untreated suspension-grown HeLa cells were extracted with Triton X-100, fixed, and processed for Epon embedding as described in Materials and Methods. The bar is 0.2 μm. (B) Whole-mount view of control monolayer HeLa CSKF. The Bar is 5 μm. (C) Whole-mount view of monolayer HeLa CSKF from cells treated with 2 μg of CD per ml for 30 min. The bar is 5 μm. (D) Resinless section of suspension-grown control HeLa CSKF. The bar is 0.2 μm. (E) Resinless section of the CSKF from HeLa cells exposed to 2 μg of CD per ml for 30 min. The bar is 0.2 μm. (F) Resinless section of the CSKF from cells exposed to 32 μg of CD for 30 min. The bar is 0.2 μm.

included most of the proteins of the CSKF, including the filamentous actin and essentially all of the cell polyribosomes (22). The separated NUC fraction includes the chromatin-associated proteins, heterogeneous nuclear ribonucleoprotein, and the nuclear matrix (8, 15). In accord with other reports, the intermediate filaments are retained with the isolated nucleus (5, 8, 11, 42, 49).

Despite the pathological appearance of CD-treated cells, the data in Table 1 show that there is no loss of total [^{35}S]methionine-labeled protein. Therefore, processes leading to significant reduction in cell mass, such as cytoplasmic extrusion and enucleation, were not significant during the course of these experiments. The data in Table 1 further show that exposure to CD had no gross effect on the

TABLE 1. Distribution of [^{35}S]methionine-labeled proteins after a 30-min exposure to CD

Subcellular fraction	Acid-precipitable radioactivity (cpm)/1,000 cells with CD concn (μg/ml) of:		
	0	2	64
SOL	1,325	1,343	1,302
CSK	314	320	296
NUC	146	149	152
Total	1,785	1,822	1,750

composition of the CSK fraction. Electropherograms of the [^{35}S]methionine-labeled proteins in the SOL and CSK fractions are shown in Fig. 3. The pattern of labeled CSK proteins obtained from cells exposed to 2 μg of CD per ml (lane 5) was indistinguishable from that of the control (lane 4). The CSK proteins obtained from cells treated with 64 μg of CD per ml (lane 6) were also similar to the control, although two differences were apparent at the high drug concentration. There was an apparent increase in the amount of CSK proteins migrating in the region of actin, at 42 to 44 kilodaltons, and a small reduction in proteins migrating in the lower-molecular-weight range between 18 and 27 kilodaltons. Proteins migrating in the lower-molecular-weight range include the ribosomal proteins (20). Their decrease may reflect the loss of polyribosomes from the CSKF at the higher concentration of CD (described below).

The CSK proteins were further analyzed by two-dimensional gel electrophoresis. Figure 4 shows the complex pattern of CSK proteins from control and drug-treated cells. Actin and alpha-actinin were tentatively identified by their apparent molecular weight and isoelectric point. Tubulins were largely absent since the microtubules were depolymerized by the cold extraction. The intermediate filament proteins were not present in the CSK fraction since they remain with the nucleus (5, 8, 11, 42, 49). The pattern of CSK proteins isolated from cells exposed to CD was the same as the control pattern at both low (Fig. 4B) and high (Fig. 4C) levels of CD. The diminution of putative ribosomal proteins at high drug levels, noted in the one-dimensional gel, was not seen here because the basic ribosomal proteins do not focus in these equilibrium gels (20).

Release of polyribosomes from the CSKF by CD. Although CD did not change the protein composition of the CSK

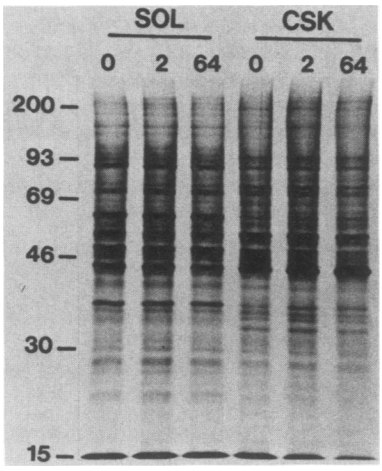

FIG. 3. Fluorogram of prelabeled SOL and CSK proteins of HeLa cells exposed to CD. Cells were prelabeled with [^{35}S]methionine, divided into three cultures, and exposed to CD at concentrations of 0, 2, and 64 μg/ml for 30 min. The SOL and CSK fractions were prepared as described in Materials and Methods, and equivalent amounts of radioactivity were loaded onto the one-dimensional polyacrylamide gel. The concentration of CD used is indicated directly above each lane. The approximate position of radioactive molecular weight markers are indicated (M_r, in thousands) at left. The actual radioactivity recovered for all fractions is presented in Table 1.

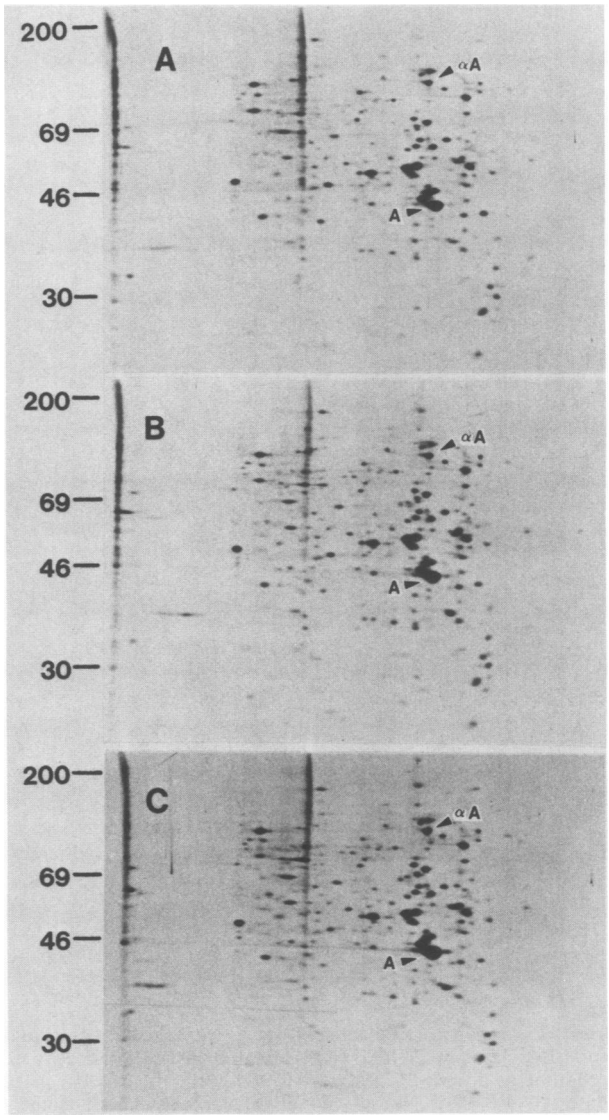

FIG. 4. Two-dimensional gel electropherograms of CSK proteins of HeLa cells exposed to CD. Equivalent amounts of the CSK proteins from the samples prepared for Fig. 3 were analyzed by the two-dimensional gels shown here. (A) Control cell proteins; (B) proteins from cells exposed to 2 μg of CD; (C) proteins from cells exposed to 64 μg of CD per ml. Actin (indicated by A) and alpha-actinin (αA) are indicated based on apparent molecular weight and isoelectric point (5). The positions of radioactive molecular weight markers are indicated (M_r, in thousands). Each gel was loaded with approximately 240,000 cpm, and the fluorogram was slightly overexposed to reveal minor proteins and any subtle changes in the pattern of proteins isolated in the CSK fraction.

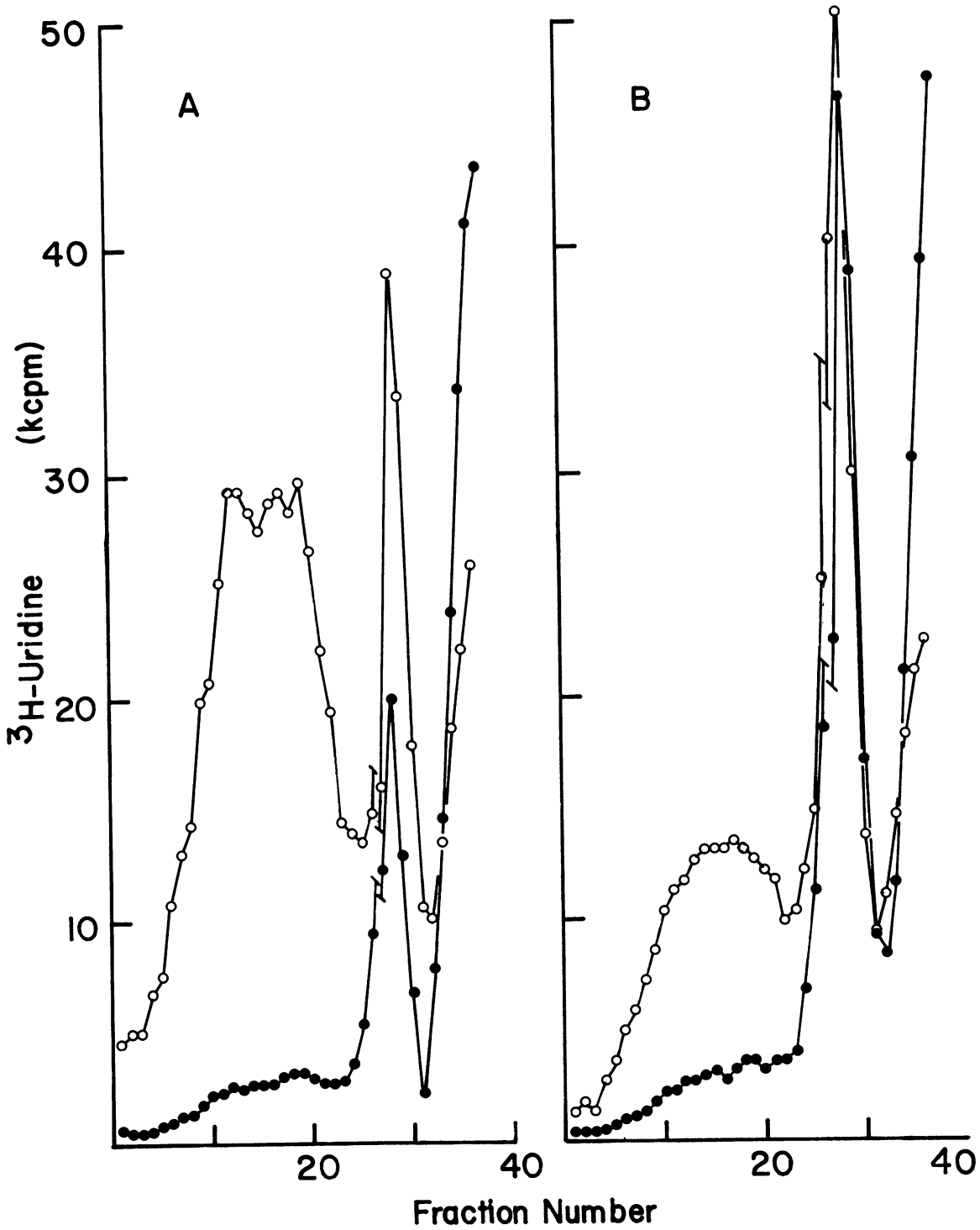

FIG. 5. Distribution of polyribosomes between the SOL and CSK fractions of control and CD-treated HeLa cells. Monolayer cultures of HeLa cells were labeled with [^{3}H]uridine overnight, exposed to DMSO (A) or 8 μg of CD per ml for 20 min (B), and fractionated as described in Materials and Methods. Material from equivalent numbers of cells was loaded onto each sucrose gradient. Symbols: ●, SOL fraction; ○, CSK fraction. The total gradient radioactivity recovered from control cells was 1.29×10^6 cpm, and that from CD-treated cells was 1.23×10^6. Values plotted for fractions above number 26 are scaled down by a factor of 0.6.

fraction, high levels of the drug did dissociate polyribosomes from the CSKF. To show this, HeLa cell RNA was labeled for 12 h with [^{3}H]uridine. The cells were then exposed to CD for 30 min, and the polyribosomes were prepared. Figure 5 shows the resulting polyribosome profiles and their partition between the SOL and CSK fractions. Figure 5A shows that greater than 97% of the polyribosomes are bound to the CSKF. This value is in agreement with previous results (9, 22). Polyribosomes from cells exposed to 8 μg of CD per ml are shown in Fig. 5B. This concentration of CD inhibits

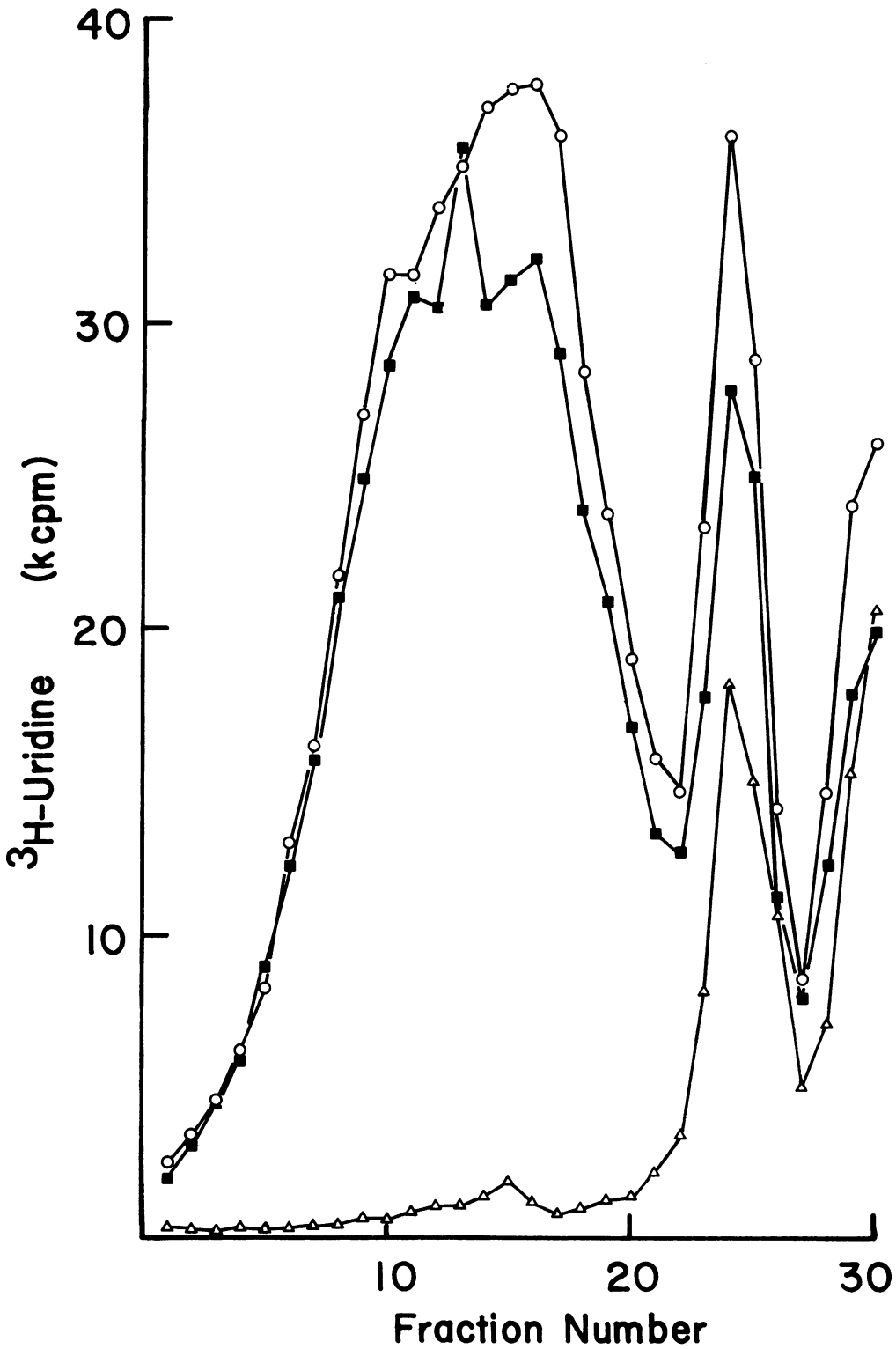

FIG. 6. Recovery of polyribosomes after disruption by high levels of CD. Suspension-grown HeLa cells were labeled overnight with [^{3}H]uridine and exposed to 64 μg of CD per ml for 20 min. Polyribosomes derived from the CSK fraction of untreated cells (○), cells exposed to the drug (△), and cells 20 min after removal of the drug (■) were analyzed by sucrose density sedimentation. (As in Fig. 5, no polyribosomes were found in the SOL fraction, and this fraction was not analyzed here.)

protein synthesis by approximately 50%. The amount of ribosomes in polyribosomes is also reduced by 50% at this drug concentration. Although reduced in amount, the size (or sedimentation distribution) of the remnant polyribosomes is unchanged by CD. Few, if any, other inhibitors of protein synthesis produce this type of polyribosome pattern (45).

The effect of CD on both protein synthesis and polyribosomes is rapidly and completely reversible. Figure 6 compares the polyribosome profiles of cells exposed to 64 μg of CD per ml with those obtained 20 min after the removal of the drug. The amount of polyribosomes in the recovering cells nearly returned to that in the control cells and was associated with the CSKF. The recovery of protein synthesis and the reappearance of polyribosomes occurred before the cells regained normal morphology (data not shown).

Inhibition of protein synthesis by CD. Figure 7 shows a typical time course of inhibition of protein synthesis by CD. Cells were exposed to 8 μg of CD per ml and pulse-labeled with [^{35}S]methionine at the indicated times. The inhibition of protein synthesis was complete by 15 min and remained constant over the time examined (60 min). The dose-dependent inhibition of protein synthesis by CD in subconfluent monolayer cultures is shown in Fig. 8. The cells were washed and maintained in media containing CD at the concentrations indicated in Fig. 8. After 20 min of exposure to the drug, the cells were pulse-labeled with [^{35}S]methionine for 3 min, and the pulse was terminated by the addition of excess unlabeled methionine. The data in Fig. 8 show that inhibition of protein synthesis became detectable at about 2 μg of CD per ml, with 50% inhibition occurring at 10 μg/ml. The inhibition curve is biphasic and can be modeled as the sum of two independent exponential functions. The apparent inhibition constants derived from a least-squares fit of the data are 1.4×10^{-5} M and 1×10^{-4} M. The inhibition of protein synthesis occurred at concentrations of CD almost an order of magnitude above those required to alter the morphology of the HeLa cell. These levels are, in turn, an order of magnitude greater than the concentration of CD shown to affect glucose transport in these cells (27).

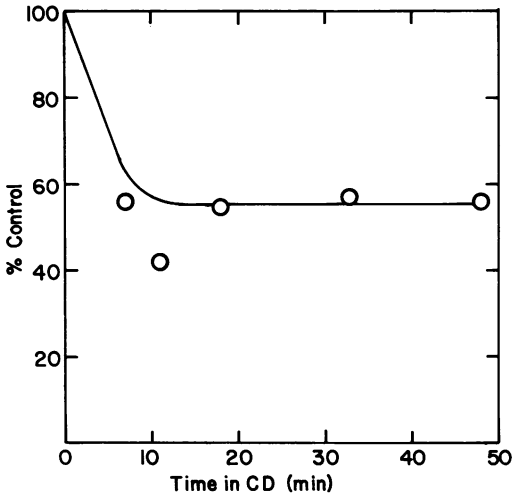

FIG. 7. Time course of the CD-induced inhibition of protein synthesis. Replicate monolayer HeLa cultures were washed, treated with 8 μg of CD per ml, and pulse-labeled as described in Materials and Methods. The time plotted indicates the endpoint of a 3-min pulse. The total acid-precipitable radioactive protein isolated at the end of the pulses indicated is expressed as a percentage of the untreated value.

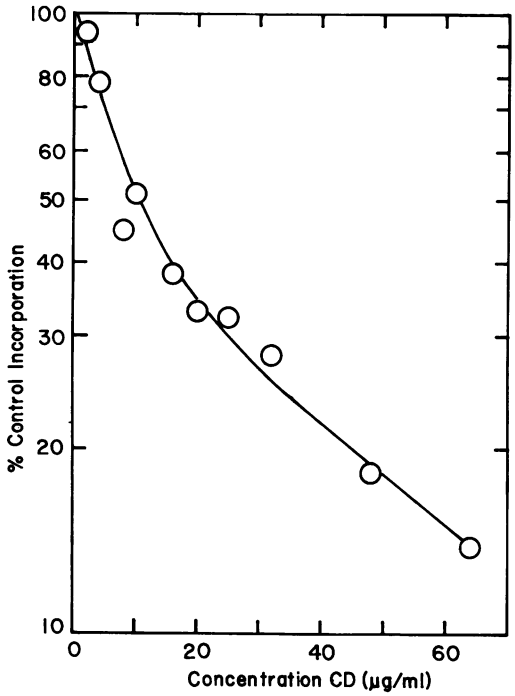

FIG. 8. Dose-dependent inhibition of protein synthesis by CD. Monolayer cultures of HeLa cells were exposed to concentrations of CD as indicated on the ordinate for 20 min, and the level of protein synthesis was determined by a pulse-label with [^{35}S]methionine. The total acid-precipitable radioactivity is plotted as a percentage of that by the untreated sample. The inhibition curve drawn through the data represents the best fit obtained by the method of least squares to a biphasic function comprising the sum of two independent exponential components. The exponential constants obtained in this manner (apparent K_d of inhibition) are 7 and 54 μg/ml, or 1.4×10^{-5} and 1×10^{-4} M.

Release of mRNA from the CSKF by CD. Previous results suggested that polyribosomes are linked to the CSKF by their mRNA (9, 22). Other experiments showed that mRNA not bound to the CSKF is also not translated. Taken together, these observations suggested that the binding of mRNA to the CSKF may be obligatory for its translation (9). The experiments presented here show that CD disaggregates a portion of the polyribosomes and removes a fraction of the mRNA from translation. The remaining polyribosomes evidently function normally. These phenomena could be explained if CD released a portion of mRNA from the CSKF and these ceased functioning while the mRNA remaining bound continued to be translated. This hypothesis is supported by the findings described below in which the release of mRNA from the CSKF is shown to closely parallel the inhibition of protein synthesis.

The release of poly(A)$^+$ RNA from the CSK to the SOL fraction by CD was measured. RNA was labeled with [^{3}H]uridine in the presence of either 5-fluorouridine or 0.04 μg of actinomycin D per ml to suppress the labeling of rRNA. Cells were exposed to several concentrations of CD for 30 min. RNA was extracted from the cell fractions and purified, and the poly(A) RNA was selected with oligo(dT)-cellulose as described in Materials and Methods. Alternatively, unlabeled RNA was isolated from cells exposed to CD, and the distribution of poly(A) among the cell fractions

TABLE 2. Distribution of poly(A)$^+$ RNA between the SOL and CSK fractions after a 30-min exposure to CD

Assay conditions[a]	% Total poly(A)$^+$ RNA CSK-associated with CD concn (μg/ml) of:			
	0	4	16	64
[^{3}H]Uridine				
Actinomycin	78.8	68.6	40.1	17.4
5-Fluoro-U	85.2	66.3	51.8	12.9
poly(U) hybridization	74.9	61.2	50.0	12.5

[a] [^{3}H]uridine was incorporated in the presence of either 0.04 μg of actinomycin D per ml or 5-fluorouridine (5-Fluoro-U). The assay by the poly(U) hybridization technique had no inhibitors.

was assayed by poly(U) hybridization. The poly(U) technique is less sensitive than radiolabeling but eliminates possible complications arising from the inhibitors of rRNA synthesis.

The results of both methods of measuring the location of poly(A)$^+$ RNA agree and are presented in Table 2. Approximately 75% of the mRNA was associated with the CSKF in control cells. The remaining 25% of poly(A)$^+$ RNA was in the SOL fraction and was not associated with polyribosomes. The data of Table 2 indicate that CD displaced mRNA from the CSKF in a dose-dependent manner. The amount of mRNA released closely paralleled the inhibition of protein synthesis. The data from several experiments are summarized in the bar graph in Fig. 9. At each dose, the proportion of poly(A) retained on the CSKF corresponded to the level of protein synthesis remaining. These data, together with the normal sedimentation profile of the remaining polyribosomes, imply that the CD-induced inhibition of protein synthesis may be the result of the release of mRNA from the CSKF.

A previous report noted that cytochalasin B preferentially dislodges polyribosomes translating a specific lens protein mRNA in lens explants (35). No obvious selectivity of mRNA release was found in CD-treated HeLa cells in these experiments. The pattern of newly synthesized proteins seen in the fluorogram of Fig. 10 is largely unchanged by the presence of CD. Cells were exposed to 4 or 32 μg of CD per ml, pulse-labeled in the presence of the drug, and fractionated to yield the SOL, CSK, and NUC fractions as described in Materials and Methods. Equivalent amounts of radioactivity were loaded onto each lane of the one-dimensional SDS gel. No marked selectivity in withdrawal of certain mRNA from translation in the presence of CD was apparent. The synthesis of three proteins in the SOL fraction having apparent M_rs of 37,000, 49,000, and 82,000, respectively, appears slightly more resistant to the CD-induced inhibition. This is not the case for the majority of proteins, and to the limit of resolution afforded by the one-dimensional gels, mRNA appears to be withdrawn from translation in a uniform manner. The difference from previous findings may simply reflect the undifferentiated state of HeLa cells, as the same investigators found that stably transformed lens fibroblasts no longer exhibited a preferential displacement of polyribosomes (34).

Translation of mRNA release by CD. The cessation of translation of mRNA upon release from the CSK could reflect an alteration in the RNA molecules themselves. There is at least one example of changes in the translatability of mRNA accompanying major changes in the rate of protein

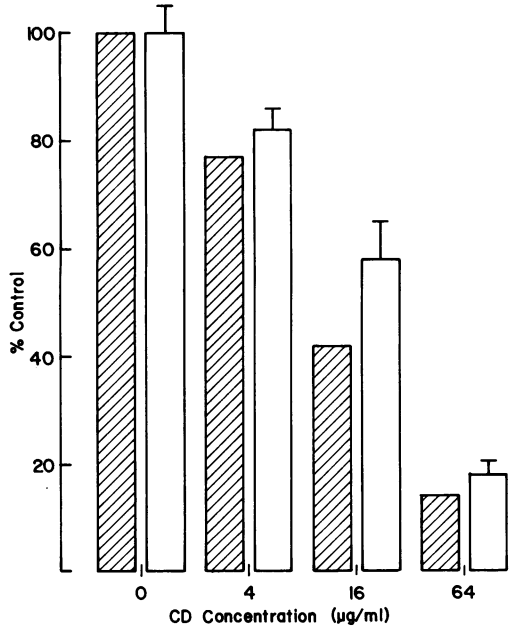

FIG. 9. Concomitant inhibition of protein synthesis and release of mRNA from the CSKF. The inhibition of protein synthesis represented at each concentration indicated is obtained from the best-fit curve of Fig. 8. The percentage of poly(A)$^+$ RNA retained is expressed as a percentage of the cytoplasmic poly(A) retained on the CSKF in untreated cells. The data are taken from a number of experiments and include the data of Table 2. The standard error of the mean for each group is indicated. For 0 μg/ml, n = 7; for 4 μg/ml, n = 4; for 16 μg/ml, n = 3; for 64 μg/ml, n = 5. ▨, Protein synthesis; □, CSK-bound mRNA.

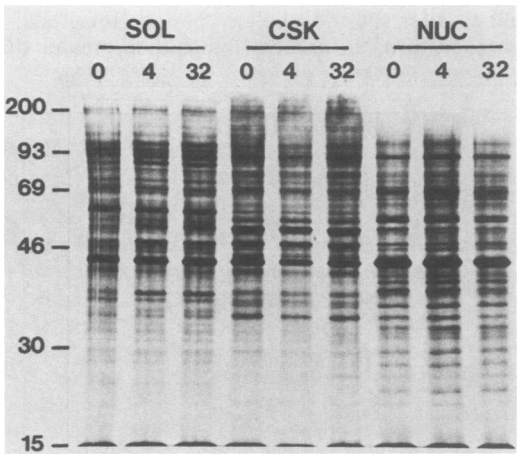

FIG. 10. Fluorogram of HeLa proteins synthesized in the presence of CD. Cells were exposed to 0, 4, or 32 μg of CD per ml for 15 min, [^{35}S]methionine was added, and the cells were labeled for an additional 15 min. The cells were harvested and fractionated as described in Materials and Methods to yield the three fractions indicated. Equivalent amounts of radioactivity from each fraction were analyzed in each lane of the one-dimensional polyacrylamide gel. The concentration of CD used is indicated directly above each lane. The position of radioactive molecular weight markers is indicated (M_r, in thousands) at the left. Material from cells treated with 2 μg/ml was derived from approximately 1.23× the number of cells as the control; that treated with 32 μg/ml was from 2.6× the number of cells.

synthesis. Anchorage-dependent fibroblasts, cultured in suspension, convert their mRNA to a form untranslatable in vitro (10). The mRNA released from the CSKF by CD was tested for possible alterations in translatability. RNA was prepared from the SOL and CSK fraction of cells exposed to 50 μg of CD per ml for 30 min. Poly(A)$^+$ RNA was purified by oligo(dT) selection, and the amount of poly(A) present in each fraction was assayed by the poly(U) hybridization technique. Equivalent, nonsaturating amounts of poly(A)$^+$ mRNA were added to a reticulocyte system. Table 3 shows the stimulation of [^{35}S]methionine incorporation into acid-precipitable material. The results are slightly complicated by the prior existence of mRNA of low relative translatability in the soluble phase (SOL fraction) which mixes with the mRNA released from the CSKF. When suitable corrections are made, the mRNA released into the soluble phase by CD shows an unchanged translatability.

The observation of mRNA of low translatability in the SOL fraction in control cells confirms a previous observation (9) that there are in all cells mRNA molecules in the SOL fraction that do not translate efficiently. These poorly translated molecules closely resembled the active mRNA on the CSKF in gross properties and were revealed only when the separation into CSK and SOL mRNA was made. The CSK mRNA was translated greater than fourfold more efficiently than an equivalent amount of SOL mRNA. In this experiment, the translational efficiency of the CSK-bound, poly(A)$^+$ RNA was comparable to that of globin mRNA. Treatment with CD released fully translatable CSK-bound mRNA into the pool of poorly translatable SOL mRNA, forming a mixture that was less active than the pure CSK message but much more active (threefold) than SOL mRNA alone.

The data in Table 3 also show that CD has no direct effect on cell-free protein synthesis. The addition of CD directly to the reticulocyte-derived in vitro translation system showed that CD was not a direct inhibitor of translation. The total acid-precipitable material obtained after translation of globin mRNA was unaffected by the presence of 50 μg of CD per ml in the translation mixture. Similar results were obtained with HeLa mRNA (data not shown). This result suggests that CD does not act specifically at the level of the ribosome-mRNA interaction.

Insensitivity of the reticulocyte to inhibition by CD. The results suggest that CD inhibits protein synthesis by disrupting the association between mRNA and the CSKF. It was of interest to examine what effect the drug would have on protein synthesis in a cell which has no apparent CSKF (36). The effect of CD on protein synthesis in intact rabbit reticulocytes was determined. Reticulocytes were prepared from anemic rabbits and used within 16 h of isolation, at which time more than 99% of the cells excluded trypan blue. The reticulocytes were washed in PBS and diluted in serum-free minimum essential medium with 1/10 the normal amount of methionine. CD was added in DMSO carrier as indicated in the legend to Fig. 11. The cells were pulse-labeled with [^{35}S]methionine after a 30-min exposure to the drug. Suspension-cultured HeLa cells were treated in a similar manner. Figure 11 shows the acid-precipitable radioactivity expressed as a percentage of the untreated culture in the two cell types. HeLa cells showed the expected inhibition of protein synthesis. In contrast, the rabbit reticulocytes showed no detectable inhibition of protein synthesis over the range of concentrations of CD tested.

TABLE 3. In vitro translation of poly(A)$^+$ RNA isolated from CD-treated cells

Poly(A)$^+$ RNA source	Acid-precipitable [^{35}S]methionine (cpm) incorporated above background/ng of added poly(A)$^+$ RNA for subcellular fraction indicated		
	SOL	CSK	Globin
Control	714	3,303	
CD (50 μg/ml)	1,845	1,058	
Globin[a]			3,659
Globin + CD[b]			3,602

[a] Incorporation was directed by an equivalent amount of purified globin mRNA.

[b] Incorporation was directed by an equivalent amount of globin mRNA in the presence of 50 μg of CD per ml in the reticulocyte translation mixture.

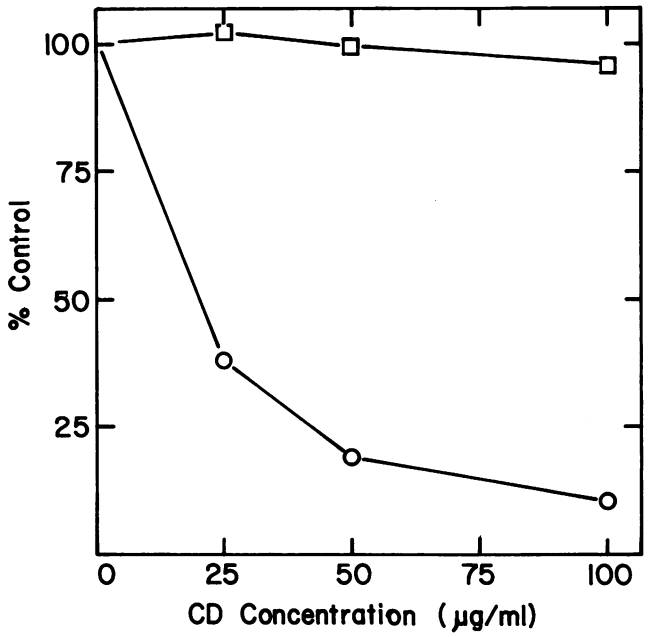

FIG. 11. Failure of CD to affect protein synthesis in intact reticulocytes. Cells were washed with PBS and suspended at 5×10^7 reticulocytes or 1×10^6 HeLa cells per ml in medium containing 1/10 the normal amount of methionine. CD in DMSO carrier was added to the concentrations indicated, adjusting the amount of DMSO to 2% of culture volume in all cases. The cultures were incubated for 30 min, with 150 μCi of [^{35}S]methionine per ml being added during the final 10 min of the treatment. The cells were extensively washed in PBS and then lysed in buffer containing SDS. Portions were decolorized by H_2O_2 and NaOH, and the acid-precipitable radiolabeled protein was determined. The results are expressed as a percentage of the untreated sample, where the control level of incorporation represented 2.08 cpm per HeLa cell and 0.23 cpm per reticulocyte. Symbols: ○, HeLa protein synthesis; □, reticulocyte protein synthesis.

DISCUSSION

These experiments explore the functional significance of the association of polyribosomes with the CSKF. The release of mRNA from the CSKF by CD is used to probe this interaction. At sufficiently high concentrations, CD inhibits protein synthesis in an unusual manner. CD reduces the amount of polyribosomes in proportion to the degree of inhibition. The remaining polyribosomes, which retain an association with the CSKF, are unaltered in their sedimentation distribution. This pattern of inhibition could not result from simple lesions in initiation or elongation which lead to altered polyribosome distributions (45). The results are

consistent with a model in which, in the presence of CD, a portion of the mRNA molecules is withdrawn from active translation. In parallel with this withdrawal from translation, the mRNA molecules are released from the CSKF to the soluble phase. The mRNAs released from the CSKF cease translation, while the mRNAs remaining bound to the CSKF translate normally. Since a number of investigators have shown that removing the ribosomes from the mRNA by a variety of means does not release mRNA from the CSKF (3, 9, 16, 44), the release of these molecules is probably prior to and not a consequence of its cessation of translation. A likely interpretation of the results is that freeing mRNA from the CSKF removes it from translation.

It was necessary to first clarify the action of high concentrations of CD on cytoskeletal organization. The drug exerts morphological effects at concentrations lower than those required to inhibit protein synthesis. The scanning electron micrographs in Fig. 1 show that 2 μg of CD per ml profoundly alters cell morphology. This drug concentration has only a minor effect on protein synthesis (Fig. 6). Higher concentrations of CD have only modest additional effects on external gross morphology but further alter the arrangement of the interior filament networks.

The effect of CD on cell structure is best seen by direct observation of the interior fibers. Figure 2A shows that the conventional embedded-section electron micrographs give only a partial picture of the CSKF fiber networks. An effective method of imaging the CSKF is to use the techniques of embedment-free electron microscopy. The resinless sections (Fig. 2D and E) afford more detailed views of the CSKF from CD-treated cells. Changes in the extent and nature of interfilament associations are apparent. Fibers become clustered into dense patches or loosely connected bundles alternating with empty regions. The micrographs of Fig. 2E and F show some of the differences between low and high drug concentrations. The frequency of dense patches is greater at the high level of CD. Presumptive microfilaments appear shortened and clustered, especially at the high drug concentration. These observations are consistent with other reports of the effects of lower doses of CD (28, 38, 46). We find that even high levels of CD do not diminish or disaggregate the cytoskeletal networks but do effect profound rearrangements of the cytoplasmic architecture.

The failure of CD to disassemble the CSKF is more clearly shown by direct measurements of its protein content and composition. These experiments used [^{35}S]methionine-labeled cells and the same detergent extraction procedure used for electron microscopy. The data in Table 1 and Fig. 3 and 4 compare the protein content and composition of the normal and CD-treated CSKFs. Neither total protein content nor the amounts of individual proteins are altered by drug concentrations much higher than those normally used to perturb cell organization. Even the amount of nonextractable actin, the presumptive target of the drug in the intact cell, does not decrese in the presence of CD.

CD is an effective but unusual inhibitor of protein synthesis. The inhibition becomes noticeable at concentrations just above those commonly used to alter cell morphology. The polyribosome profiles in Fig. 5 show the unusual mode of protein synthesis inhibition. The polyribosomes in CD-treated cells are reduced in amount but exhibit a normal sedimentation distribution. The reduction of polyribosome mass closely parallels the reduction in protein synthesis (Fig. 8). This implies that the remnant polyribosomes, which remain associated with the CSKF, are functioning at near normal rates. Few, if any, inhibitors have this effect on the protein synthesis machinery. Inhibitors of initiation decrease the average polyribosome size, while inhibitors of elongation reduce the rate of protein synthesis without a concomitant reduction in polyribosome mass (45). In both cases the number of active mRNA molecules does not change. Furthermore, the gel patterns of proteins synthesized in the presence of CD (Fig. 10) appear free of the stuttering or smearing that characterize premature peptide termination. In the presence of CD, a portion of mRNA molecules is completely removed from translation while the remainder continues to function normally. The cessation of mRNA function accompanies, and may be the result of release from the CSKF.

The mRNA molecules displaced from the CSKF into the SOL compartment by the action of CD are fully active in a cell-free translation system (Table 3). However, the partition of mRNA into CSK-bound and SOL fractions reveals a previously unseen heterogeneity in translatability. The mRNA normally found free in the soluble phase is much less translatable in vitro, on a molar basis, than the CSK-bound RNA. Studies of these soluble mRNAs show them to be full sized, with sequences that cross-hybridize extensively with those of active messages (unpublished observations; S. Farmer, personal communication). Nevertheless, these mRNA molecules from the SOL fraction have an as yet undetermined lesion and do not stimulate protein synthesis in either the reticulocyte- or wheat germ-derived cell-free translation systems. The presence of these pseudomessages is masked in total cell extracts but becomes readily apparent when they are separated from the active mRNA bound to the CSKF. The presence of the inactive mRNA affects the results shown in Table 3. The mRNA released from the CSKF in the presence of cytochalasin mixes with the inactive poly(A)$^+$ RNA of the SOL fraction, and the composite translates with an intermediate level of efficiency. The mRNA from the CSK fraction, derived largely from bound polyribosomes, translates more effectively than mRNA from the SOL fraction. This difference is not due to extraction artifacts since, once shifted to the SOL fraction by CD, such active mRNA remains fully translatable.

Seemingly intact but inactive mRNA has been reported previously. Anchorage-dependent fibroblasts, when cultured in suspension, remove their mRNA from translation and store it in a quasistable form (10). These molecules are almost untranslatable when tested in vitro. The inactive mRNA from the suspended cells is converted to an active form when the cells are allowed to reattach. Whether the lesion in mRNA from the suspended cells is the same as that in the mRNA-like molecules from the SOL fraction is not known and warrants further investigation.

The release of mRNA from the CSKF is not characteristic of agents or conditions that inhibit protein synthesis. Inhibitors of elongation, such as emetine and cycloheximide, as well as disaggregators of polyribosome structure, such as sodium fluoride, pactamycin, verucarin, and heat shock, fail to change the association of cytoplasmic poly(A)$^+$ RNA with the CSKF (9, 16, 22, 45). The effect of virus infection on mRNA binding is more complex. Infection with vesicular stomatitis virus leads to the inhibition of host protein synthesis, with the host mRNA remaining on the CSKF (3). In contrast, two viruses that produce a substantial cytopathic effect, poliovirus and adenovirus, displace host mRNA from the CSKF and inhibit host protein synthesis (21, 44). It is, however, not likely that the release of mRNA from the CSKF is the primary means by which host protein synthesis is inhibited (43).

CD does not appear to directly affect the components of the protein synthesis system. It has no effect when added to an initiating reticulocyte in vitro system. This suggests that the drug does not act at the level of initiation and elongation and is in agreement with the normal polyribosome sedimentation distribution obtained in the presence of the drug. The reticulocyte seems to have little cytostructure, and its polyribosomes are distributed uniformly throughout the cytoplasm (36). It seems likely that there is no cytoskeletal role in vivo for the highly specialized protein synthesis of the reticulocyte. The data in Fig. 9 are consistent with this hypothesis. Levels of CD that strongly inhibit HeLa cells have no effect on protein synthesis in the intact reticulocyte. The results are consistent with the proposal that in cells with a cytoskeletal-associated protein synthesis system, mRNA binding to the CSKF is necessary although clearly not sufficient for translation.

The nature and purpose of the association of protein synthesis with structural elements of the cell remain unknown. A review discussing this subject has recently been published (31). There may be several reasons for the association of mRNA with the CSKF. The seemingly obligatory binding of mRNA for translation may reflect a need for positioning the molecule near appropriate initiation (16) or other regulatory factors, including the cap-binding protein (50) and high-molecular-weight aminoacyl-tRNA synthetase complexes (29). The binding of mRNA may also have a topographical significance. Such localization may be important for the placement of protein products at the cellular level (6, 12) and in the segregation of maternal mRNA in the early embryo (6, 30).

The results presented here do not address the mechanism by which CD releases mRNA from the CSKF. In particular, it is not known how this release relates to the alteration of actin filaments. Other investigators have noted related effects of cytochalasin on the protein synthetic apparatus. Howe and Hershey (16) found the eucaryotic initiation factors to quantitatively associate with the CSKF in HeLa cells. In this same study, CB released these factors as well as mRNA to the soluble phase (16). Cytochalasin has been reported to release polyribosomes from cultured lens cells and cytoskeletal remnants of these cells (34, 35). Finally, polyribosomes of a rat liver preparation were found to associate with a Triton X-100–insoluble matrix in a manner dependent on the integrity of filamentous actin (1).

Little is known of the nature of mRNA binding to the cytoarchitecture. mRNA, in the form of ribonucleoprotein, does not require the presence of ribosomes for binding to the CSKF (3, 9, 16, 44). Specific mRNAs lacking a 5′ cap or 3′ poly(A)$^+$ tract were found to bind the CSKF (3), suggesting that binding must include internal portions of the mRNA, possibly through the mRNA-associated proteins. Because colchicine has little effect on protein synthesis and because the microtubules are depolymerized in the extraction protocol without releasing polyribosomes, the associations studied here most likely do not involve the microtubule system (21). Morphological studies indicate that the state of intermediate filament organization is probably irrelevant to protein synthesis (44, 47, 48). The experiments reported here and discussed previously do indicate that actin fibers are somehow involved in the functioning of the protein synthesis system. However, the data do not actually show that mRNA is bound directly to actin fibers. In view of the complex interactions possible with the actin filament system (37), no specific conclusions concerning its role in protein synthesis are possible. The finding that the presumptive actin filaments are shortened but not reduced in amount when CD releases mRNA might suggest that the actin fibers themselves are not the polyribosome binding site.

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