

## Physical Change in Cytoplasmic Messenger Ribonucleoproteins in Cells Treated with Inhibitors of mRNA Transcription

GIDEON DREYFUSS,\* STEPHEN A. ADAM, AND YANG DO CHOI

*Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60201*

Received 3 October 1983/Accepted 16 December 1983

Exposure of intact cells to UV light brings about cross-linking of polyadenylated mRNA to a set of cytoplasmic proteins which are in direct contact with the mRNA *in vivo*. Substantial amounts of an additional protein of molecular weight 38,000 (38K) become cross-linked to the mRNA when cells are treated with inhibitors of mRNA synthesis (actinomycin D, camptothecin, and 5,6-dichloro-1- $\beta$ -D-ribofuranosyl benzimidazole) or after infection with vesicular stomatitis virus. Cordycepin, which inhibits polyadenylation but not mRNA synthesis, has no such effect. Inhibitors of protein synthesis and of rRNA synthesis are also without effect on 38K cross-linking to mRNA. The onset of the effect of inhibitors of mRNA synthesis on the UV cross-linkable interaction between mRNA and 38K is rapid and reaches a maximal level in less than 60 min, and it is completely and rapidly reversible. In cells treated with actinomycin D, the amount of 38K which becomes cross-linked to mRNA is proportional to the extent of inhibition of mRNA synthesis. The association of 38K with mRNA during transcriptional arrest does not require protein synthesis because simultaneous treatment with the protein synthesis inhibitor emetine does not interfere with it. The effectors which promote the interaction of 38K with mRNA do not affect the proteins which are in contact with polyadenylated heterogeneous nuclear RNA and do not markedly affect protein synthesis in the cell. The 38K protein can be isolated with the polyribosomal polyadenylated fraction from which it was purified, and monoclonal antibodies against it were prepared. Immunofluorescence microscopy shows mostly cytoplasmic and some nuclear staining. These observations demonstrate that commonly used inhibitors of transcription affect the physical state of messenger ribonucleoproteins *in vivo*.

The complexes of proteins with heterogeneous nuclear RNA (hnRNA) (heterogeneous nuclear ribonucleoproteins [hnRNPs]) and with mRNA (messenger ribonucleoproteins [mRNPs]) are considered to be the functional assemblies which are involved in the synthesis and function of these polynucleotides in the cell (for a review, see reference 33). The proteins which form these complexes are, therefore, of key interest and are the focus of much research. The major difficulties in identifying RNPs by conventional isolation techniques stem from the fact that the criteria of copurification of certain proteins with polynucleotides are, in general, not sufficiently stringent to ascertain whether these are genuine RNP components *in vivo*. This is due to the fact that nonspecific interactions between RNA and proteins are likely to occur *in vitro* (2, 14, 38). UV cross-linking of RNA to protein in intact cells overcomes these difficulties and allows the identification of proteins which are in direct contact with hnRNA and mRNA *in vivo* (10, 34, 35, 50, 51, 53).

The *in vivo* UV cross-linking approach relies on the fact that UV light of sufficient intensity generates photoreactive species of RNA which react virtually indiscriminately with molecules, including proteins, which are in direct contact with it. Cross-linked complexes of polyadenylated [poly(A)<sup>+</sup>] mRNA were isolated under protein-denaturing conditions to ensure that only proteins covalently linked to mRNA were purified with it, eliminating nonspecific associations of proteins with mRNA (51, 53). The cytoplasmic poly(A)<sup>+</sup> material selected by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography was digested with RNase, and the released proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (51, 53; G. Dreyfuss, Y. D. Choi, and S. A. Adam,

submitted for publication). Several nonribosomal proteins become cross-linked to the cytoplasmic poly(A)<sup>+</sup> mRNA in HeLa cells, including polypeptides of apparent molecular weights of 135,000 (135K), 93K, 72K, 68K, 53K, 50K, 43K, and 36K. Selective nuclease digestion indicated that the 72K polypeptide is the major protein which is preferentially associated with the polyadenylate segment *in vivo*. The major UV cross-linked mRNPs, particularly the 72K polyadenylate-binding protein and the 50K and 53K proteins, may be similar to proteins previously shown to be associated with mRNA *in vitro* (1, 3, 5, 16, 17, 20, 21, 24, 26, 30, 31, 44, 46, 52). The overall patterns of mRNPs of human, mouse, and lizard species were found to be very similar. The great similarity in the proteins which make up mRNPs in different vertebrate species and the apparent sequence-discriminating association of at least one of them with a portion of the mRNA suggested that mRNPs in vertebrates may be composed of a small and universal subset of proteins that form a relatively conserved structural and functional unit (Dreyfuss et al., submitted for publication).

These conclusions were reinforced by experiments with vesicular stomatitis virus (VSV)-infected cells which demonstrated that viral mRNAs also become associated with the same proteins which are found associated with the entire population of poly(A)<sup>+</sup> mRNA in the cell (Dreyfuss et al., submitted for publication). These experiments exploited the selective insensitivity of the RNA transcriptase of the virus to actinomycin D so that VSV mRNAs could be specifically labeled and thereby tag only the proteins which are in contact with these mRNAs after UV cross-linking in intact cells. It was found that one prominent protein of 38K which was not normally found cross-linked to mRNA in uninfected cells became cross-linked to VSV mRNAs in actinomycin D-treated infected cells. It also became apparent that treatment of cells with actinomycin D without VSV infection and also

\* Corresponding author.

VSV infection by itself could bring about the same effect. Since both actinomycin D and VSV infection are known to inhibit nuclear DNA-dependent RNA transcription, it raised the possibility that the increased cross-linking of the 38K protein with mRNA occurs as a result of arrest of transcription. This report presents studies which examine the relationship between inhibitors of nuclear transcription and the mRNPs in the cytoplasm.

#### MATERIALS AND METHODS

**Cell culture and labeling.** HeLa cells were grown in monolayer culture in Dulbecco modified Eagle medium containing 10% fetal calf serum at 37°C in 5% CO<sub>2</sub> atmosphere. Cultures were supplemented with penicillin-streptomycin, and used at subconfluent densities. Cells were labeled for 4 h with [<sup>35</sup>S]methionine at 10 μCi/ml in methionine-free medium containing 2% undialyzed fetal calf serum in the presence of actinomycin D (0.04 μg/ml) and ethidium bromide (1.5 μg/ml) added 30 min before addition of the label to suppress ribosomal (9, 40, 54) and mitochondrial (28, 58) RNA and protein synthesis, respectively. Cell culture materials were from GIBCO Laboratories, and radiochemical reagents were obtained from New England Nuclear Corp. Infections with the Indiana strain, Glasgow serotype of VSV (from Harvey Lodish, Massachusetts Institute of Technology) were at a multiplicity of 10 PFU per cell as previously described (7, 32).

**UV irradiation.** The culture medium was removed, and cell monolayers were washed twice with Ca<sup>2+</sup> and Mg<sup>2+</sup> containing phosphate-buffered saline (PBS). Irradiation was carried out in PBS at room temperature with a 15-W germicidal lamp (Sylvania G15T8) placed 4.5 cm away from the cell monolayer for 3 min. Under these conditions, the UV dose as measured by a light intensity meter was approximately  $6.5 \times 10^3$  ergs/mm<sup>2</sup>.

**Cell fractionation and oligo(dT)-cellulose chromatography.** After UV irradiation, the PBS was removed, and the cells were allowed to swell for 5 min in ice-cold 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl 1.5 mM MgCl<sub>2</sub> containing 0.5% aprotinin (Sigma Chemical Co.) and 1 μg each of pepstatin A and leupeptin per ml. Triton X-100 was added to a final concentration of 0.5% followed by 0.5% deoxycholate and 1% Tween 40, and the cells were homogenized by four passages through a 25-gauge needle. The nuclei were removed by low-speed centrifugation, and the cytoplasmic fraction was adjusted to 1 mM EDTA, 1% mercaptoethanol, and 0.5% SDS. After heating at 90°C for 5 min, rapid chilling, and addition of LiCl to 0.5 M, the cytoplasmic extract was incubated for 15 min with oligo(dT)-cellulose (Type 3; Collaborative Research) with constant agitation. The oligo(dT)-cellulose was then packed in a column and washed with >10 column volumes of binding buffer (10 mM Tris-hydrochloride, pH 7.4, 500 mM LiCl, 1 mM EDTA, 0.5% SDS). The performance of the column was monitored by liquid scintillation counting. The eluted poly(A)<sup>+</sup> material was reheated to 65°C for 5 min and oligo(dT)-cellulose chromatography was repeated. When a poly(A)<sup>+</sup> hnRNA fraction from UV irradiated cells was prepared, procedures were similar except that the nuclear fraction was used after DNase I digestion (50 μg/ml for 15 min at 37°C), the buffers contained 10 mM vanadyladenosine, and heating of samples before the first oligo(dT)-cellulose chromatography was only to 65°C. The poly(A)<sup>+</sup> material was precipitated overnight at –20°C with 3 volumes of ethanol.

**RNase digestion.** The poly(A)<sup>+</sup> material was pelleted by centrifugation at 12,500 × g and suspended in 50 μl of 10 mM

Tris-hydrochloride, pH 7.4, containing 1 mM CaCl<sub>2</sub>, and digestion with RNase was carried out with 25 μg of pancreatic RNase A (Worthington Diagnostics) and 400 U of micrococcal nuclease (P-L Biochemicals) per ml for 60 min at 37°C. To inhibit possible traces of protease, the pancreatic RNase was preboiled, and aprotinin (0.5%), pepstatin A (1 μg/ml), and leupeptin (1 μg/ml) (Sigma) were included in the digestion mixture. After the RNase digestion, the proteins were precipitated by addition of 3 volumes of ethanol at –20°C for at least 2 h.

**Gel electrophoresis.** Protein samples were electrophoresed on an SDS-containing discontinuous polyacrylamide gel electrophoresis system. The separating gel was prepared from a stock of 33.5% acrylamide and 0.3% *N,N'*-bisacrylamide to a final concentration of 12.5% acrylamide. The separating gel buffer contained 0.38 M Tris-hydrochloride, pH 9.1. The stacking gel was prepared from a stock of 30% acrylamide and 0.44% *N,N'*-bisacrylamide to a final acrylamide concentration of 4% in 0.125 M Tris-hydrochloride, pH 6.8. Both gels contained 0.1% SDS and were polymerized with ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine. The electrode tank buffer was 25 mM Tris–192 mM glycine containing 0.1% SDS. Samples were prepared by boiling for 3 min in a 0.125 M Tris-hydrochloride, pH 6.8, buffer containing 1% SDS, 5% mercaptoethanol, 10% glycerol, and bromophenol blue. After electrophoresis <sup>35</sup>S-labeled material, the gels were stained with Coomassie blue and impregnated with PPO (2,5-diphenyloxazole), and fluorography was performed with preflashed X-ray films (27).

**Preparation and fractionation of polyribosomal poly(A)<sup>+</sup> mRNPs.** Partial purification of 38K was carried out from the polyribosomal fraction in which it was found to be substantially enriched. Polyribosomes were prepared from HeLa cells pretreated for 15 min with cycloheximide (25 μg/ml). Cells were incubated on ice for 5 min in 10 mM Tris-hydrochloride buffer (pH 7.4)–10 mM NaCl–2.5 mM MgCl<sub>2</sub> containing 200 μg of cycloheximide per ml, the RNase inhibitor vanadyl-adenosine (10 mM), and the protease inhibitors aprotinin (0.5%), pepstatin A (1 μg/ml), and leupeptin (1 μg/ml). After cell lysis with 0.5% Triton X-100 and several passages through a 25-gauge needle, nuclei were deposited by low-speed spin, and the supernatant was overlaid on a 2-ml layer of 20% sucrose in 10 mM Tris-hydrochloride, pH 7.4, containing 2.5 mM MgCl<sub>2</sub> and 250 mM NaCl. Centrifugation in a Beckman SW50.1 rotor was at 50,000 rpm for 1 h at 4°C. The pellet and the sucrose layer were mixed with an equal volume of 10 mM Tris-hydrochloride, pH 7.4, containing 250 mM NaCl and 20 mM EDTA and applied to an oligo(dT)-cellulose column. The column was washed with the same buffer until no more protein was eluted and then eluted with 5 column volumes of 25% formamide in 10 mM Tris-hydrochloride, pH 7.4, followed by 5 column volumes of 50% formamide in the same buffer.

**Preparation of monoclonal antibodies to 38K.** The material which eluted from the oligo(dT)-cellulose column in the 50% formamide fraction was precipitated in the cold with 3 volumes of ethanol, and the pellet was dissolved in SDS-PAGE sample buffer and subjected to electrophoresis in the presence of SDS as described above. The gel was stained lightly with Coomassie blue, and the major band at molecular weight 38,000 (identified by peptide mapping as the 38K protein of interest) was excised. The protein was extracted from the crushed polyacrylamide into 1% SDS in 10 mM Tris-hydrochloride, pH 7.4, at room temperature for 2 h. The extracted protein was precipitated with 4 volumes of acetone, the pellet was dissolved in 0.5 ml of PBS and mixed

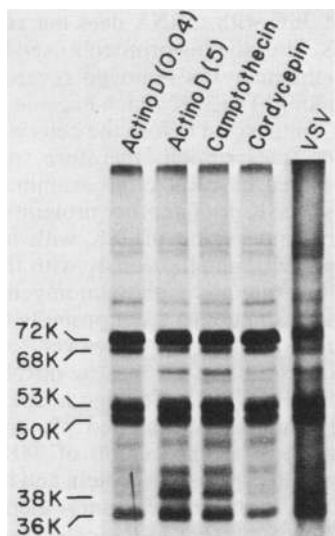


FIG. 1. Proteins UV cross-linked in vivo to poly(A)<sup>+</sup> mRNA after treatment with various inhibitors of transcription. Cells were labeled with [<sup>35</sup>S]methionine for 4 h as described in the text. The medium was replaced and the cells were exposed to actinomycin D (0.04 or 5 μg/ml), camptothecin (25 μg/ml), or cordycepin (50 μg/ml) for 2 h in complete medium before UV cross-linking. For mRNP complexes from VSV-infected cells, cells were labeled for 4 h with [<sup>35</sup>S]methionine and infected with VSV. UV cross-linking and isolation of complexes was performed at 3.5 h postinfection. UV cross-linking, poly(A)<sup>+</sup> mRNP isolation, RNase digestion, and electrophoresis were performed as described in the text.

with an equal volume of complete Freund adjuvant, and 0.5 ml corresponding to material from 20 10-cm tissue culture dishes was injected intraperitoneally per BALB/c mouse. A similar booster injection in incomplete Freund adjuvant was given on day 14, and the mice were hyperimmunized by an intravenous injection of the same antigen in PBS on day 28. Mice were sacrificed 3 or 4 days later, the spleens were removed, and the lymphocytes were fused with SP2/0 myeloma cells. Hybridoma culture, cloning, and screening procedures were essentially as previously described (13, 25). Culture supernatants were initially screened for specific antibody-producing colonies by β-galactosidase and immunoblotting by using the same material as the one injected as antigen. Positive colonies were expanded, recloned, and screened again with UV-cross-linked poly(A)<sup>+</sup> material from normal and actinomycin D-treated cells. Colonies which were positive only for the latter were selected. Ascites fluid for one of these, designated 5C2, was prepared by intraperitoneal inoculation into pristane-primed BALB/c mice.

**Immunoblotting and immunofluorescence.** Blotting of proteins from polyacrylamide gels onto nitrocellulose paper was carried out by electrotransfer at 0.5 A in 50 mM Tris-glycine (pH 9.1) containing 20% methanol at room temperature for 6 to 12 h. The nitrocellulose blot was treated by the method of Burnette (4) and exposed to monoclonal antibody ascites fluid and then to <sup>125</sup>I-labeled goat antimouse antiserum. Immunofluorescence was carried out on cells cultured on glass cover slips and fixed with 2% formaldehyde for 20 min at room temperature followed by 3 min in acetone at -20°C with a Zeiss Photomicroscope III equipped with a Planapo 63X 1.4 oil objective.

## RESULTS

**Inhibitors of mRNA transcription and the cross-linking of a 38K protein to mRNA in HeLa cells.** The proteins which

became cross-linked to poly(A)<sup>+</sup> mRNA as a consequence of exposure of intact cells to UV light in untreated cells and in cells treated with several different inhibitors of nuclear transcription were examined. The poly(A)<sup>+</sup> mRNP fraction, prepared from cells treated with actinomycin D at concentrations which inhibit RNA polymerase II transcription, contained a new 38K protein which was not cross-linked to poly(A)<sup>+</sup> mRNA in untreated cells. Other, less pronounced, changes in mRNPs were also seen. This effect was not specific to actinomycin D since it also occurred with other inhibitors of transcription (Fig. 1 and 2). These included camptothecin, an inhibitor of rRNA and hnRNA synthesis (22, 56); 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which inhibits hnRNA synthesis (11, 48); and VSV, which at this time postinfection causes shutdown of host cell transcription (36). Cordycepin (3'-deoxyadenosine), which inhibits polyadenylation but apparently not hnRNA synthesis or mRNA emergence to the cytoplasm (57), did not promote the cross-linking of 38K to mRNA. Low levels of actinomycin D (0.04 μg/ml) at which rRNA synthesis is inhibited (40) were not sufficient to bring about this effect on 38K. In fact, labeling of proteins with [<sup>35</sup>S]methionine was carried out in the presence of 0.04 μg of actinomycin D per ml, and since 38K was labeled under such conditions where rRNA synthesis was inhibited, it is not a bona fide ribosomal protein (9, 40, 54), although its association with ribosomes is possible.

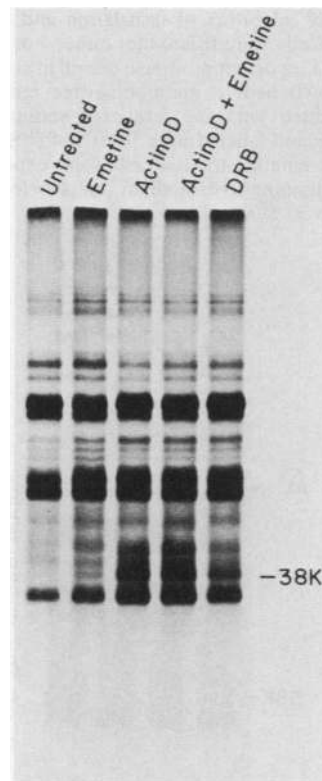


FIG. 2. Proteins cross-linked in vivo to poly(A)<sup>+</sup> mRNA in cells treated with inhibitors of transcription and translation. Cells were labeled with [<sup>35</sup>S]methionine as described in the legend to Fig. 1 and incubated for 2 h in complete medium with 20 μg of emetine per ml, 5 μg of actinomycin D per ml, 5 μg of actinomycin D with 20 μg of emetine per ml, or 75 μM DRB. Cross-linked complexes were isolated, digested, and analyzed by SDS-PAGE as described in the legend to Fig. 1.

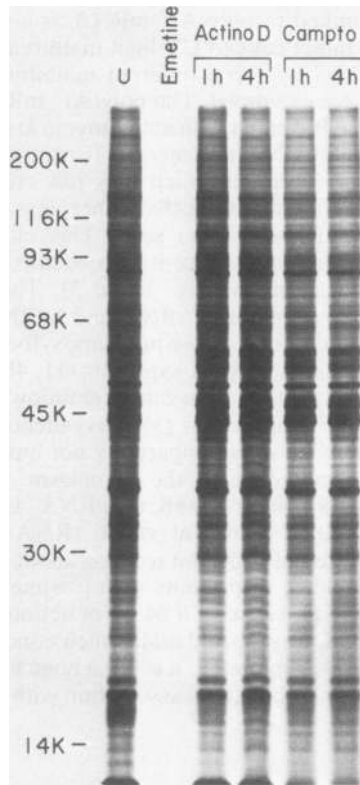


FIG. 3. Effect of inhibitors of translation and transcription on protein synthesis. Cells were treated for either 1 or 4 h with 5  $\mu$ g of actinomycin D or 25  $\mu$ g of camptothecin per ml in complete medium. They were then switched to methionine-free minimum essential medium supplemented with 2% fetal calf serum containing the respective inhibitor and labeled with 10  $\mu$ Ci of [ $^{35}$ S]methionine per ml for 30 min. The emetine-treated cells were exposed to the drug for 15 min in methionine-free medium and labeled similarly with [ $^{35}$ S]methionine for 30 min.

**Association of 38K with mRNA does not require or affect protein synthesis.** The labeling protocols used here were such that the [ $^{35}$ S]methionine was removed several hours before the UV cross-linking. The 38K which became cross-linked to the mRNA was synthesized before the cells were exposed to the inhibitors of transcription; therefore, preformed 38K becomes cross-linked to mRNA. To examine whether continued synthesis of 38K or of another protein(s) is needed for the cross-linkable interaction of 38K with mRNA to take place, we treated cells simultaneously with the protein synthesis inhibitor emetine and with actinomycin D. Inhibition of protein synthesis did not by itself promote the association of 38K with mRNA (Fig. 2). However, actinomycin D promoted the cross-linking of 38K to mRNA even when emetine was present (Fig. 2). Emetine at the concentration used (25  $\mu$ g/ml) completely inhibited protein synthesis in these cells (Fig. 3). The association of 38K with mRNA therefore occurs with preexisting protein and mRNA components, and thus all of the necessary elements for this induction are already present in the cell. In this sense it is entirely a posttranscriptional as well as a posttranslational event.

Although the consequences of the altered interaction of 38K with mRNA are not yet known, the transcriptional inhibitors did not drastically affect protein synthesis within the times and doses used in these experiments (Fig. 3). It therefore appears that the altered interaction of 38K with mRNA is not accompanied by a change in protein synthesis as judged by one-dimensional SDS-PAGE.

**Inhibitor-dependent interaction of 38K with mRNA.** Substantial amounts of 38K became cross-linked to mRNA within 20 min of exposure of the cells to actinomycin D (Fig. 4). The amount of 38K cross-linked to mRNA continued to increase and appeared to reach a maximum level within 1 h. The effect was saturable in that longer exposure times did not lead to any further increase. Considering it takes about 15 min for the drug to reach the maximal intracellular level, it

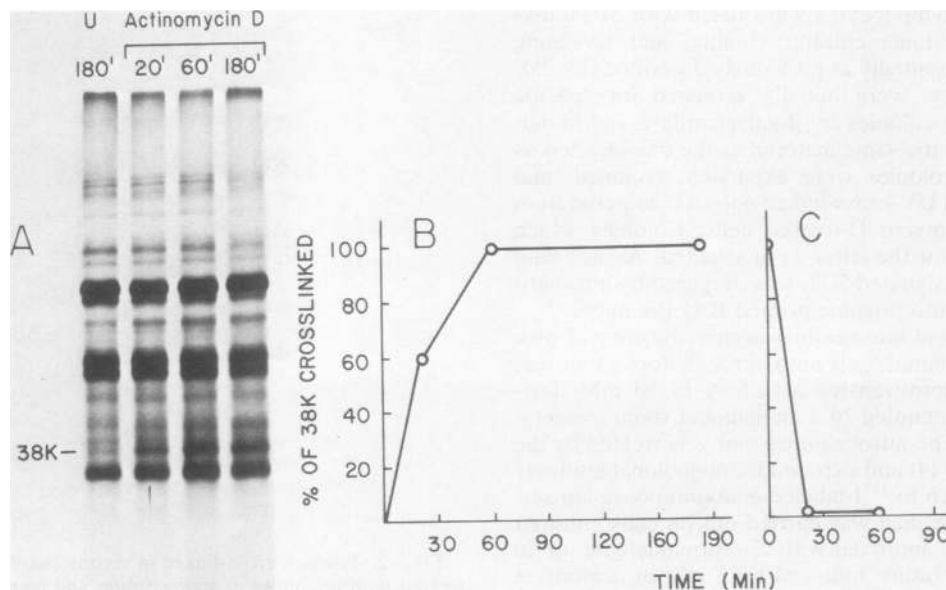


FIG. 4. Time course of the association of 38K with mRNAs after treatment with 5  $\mu$ g of actinomycin D per ml. (A) Cells were pre-labeled with [ $^{35}$ S]methionine for 4 h as described in the legend to Fig. 1 and treated with 5  $\mu$ g of actinomycin D per ml for the indicated times before UV cross-linking and mRNP isolation. (B) Percentage of 38K cross-linked was calculated from densitometric scans (at 600 nm) of the autoradiogram shown in (A). The amount of 38K present at 180 min was taken as 100% of 38K cross-linked. (C) Reversibility of 38K association with mRNPs. Cells labeled as in (A) were treated for 1 h with 25  $\mu$ g of camptothecin instead of actinomycin D, and the drug was removed by replacement with fresh medium. Cross-linking was performed at the indicated times after removal of the drug. Calculations of the percentage of 38K cross-linked were made by densitometry as described for (B).

may be that the real effect is even faster than the data in Fig. 4A reflect. To examine whether the interaction of 38K with mRNA is reversible so that when the inhibitor is removed 38K dissociates from mRNA, camptothecin, rather than actinomycin D, was used. Unlike actinomycin D, the inhibitory effect of camptothecin is instantaneously reversed upon its removal from culture medium (22, 56). The data in Fig. 4C demonstrate that at the earliest point examined, 20 min after removal of camptothecin, there was no detectable 38K cross-linked to mRNA. The onset of interaction of 38K with mRNA is therefore rapid and it is rapidly reversed. The association of 38K with mRNA is thus dependent on the continued presence of the transcriptional inhibitor.

**Amount of 38K cross-linked to mRNA is proportional to the extent of inhibition of mRNA synthesis.** Experiments with different inhibitors suggested that the association of 38K with mRNA may not be an all-or-none effect. Rather, there appeared to be some correlation between the extent to which some treatments caused 38K cross-linking to mRNA and their efficacy as inhibitors of mRNA synthesis. A more quantitative examination of this point was carried out by exposing cells to different concentrations of actinomycin D (Fig. 5). The effect appeared to increase with increasing concentrations of actinomycin D. A close correlation was apparent between the amount of 38K which became cross-linked to mRNA and the extent of inhibition of mRNA

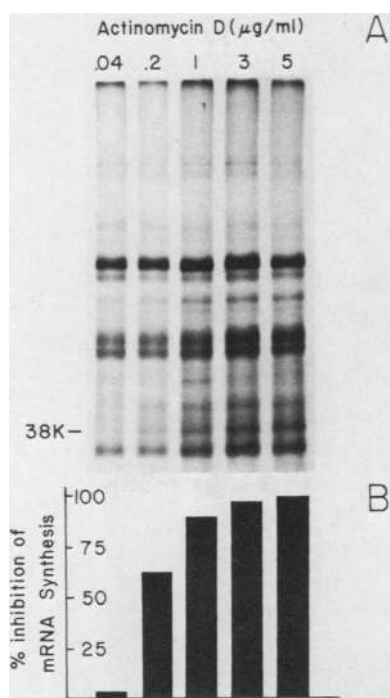


FIG. 5. Dose response of the association of 38K with mRNA. (A) Cells were prelabeled for 4 h as described in the legend to Fig. 1 and treated with various concentrations of actinomycin D for 2 h. UV cross-linked mRNPs were then isolated, and the proteins were analyzed as described in the legend to Fig. 1. (B) Inhibition of cytoplasmic poly(A)<sup>+</sup> mRNA synthesis by different concentrations of actinomycin D. Cells were treated with 1.5 µg of ethidium bromide per ml and the concentrations of actinomycin D indicated in the lanes above the bars for 30 min in complete medium. [<sup>3</sup>H]uridine was added to a concentration of 50 µCi/ml, and incubation was continued at 37°C for 90 min. Poly(A)<sup>+</sup> mRNA was isolated from the cytoplasmic fraction by extraction with phenol, and radioactivity in trichloroacetic acid precipitates was determined by liquid scintillation counting.

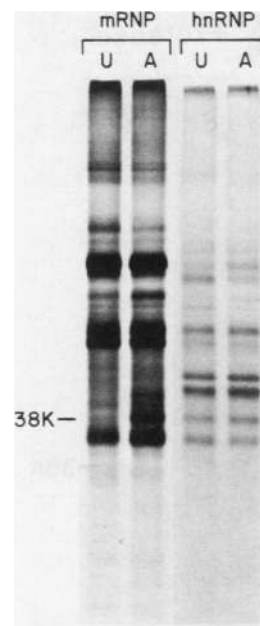


FIG. 6. Comparison of proteins cross-linked in vivo to poly(A)<sup>+</sup> mRNA and poly(A)<sup>+</sup> hnRNA after treatment with 5 µg of actinomycin D per ml. Cells were labeled with [<sup>35</sup>S]methionine as before and treated with 5 µg of actinomycin D per ml for 2 h. Cross-linking and isolation of RNPs was performed as described in the text. Abbreviations: U, untreated; A, after treatment with actinomycin D.

synthesis. This effect was saturable and reached the same maximal level not only for each inhibitor used, but once attained by one it did not increase any further by addition of another inhibitor (data not shown).

The proteins associated with hnRNA, like those associated with mRNA, can also be cross-linked to it by UV irradiation of intact cells. Unlike poly(A)<sup>+</sup> mRNPs, no major changes were seen in proteins cross-linked to poly(A)<sup>+</sup> hnRNA. One of the hnRNPs comigrated with 38K of mRNPs from actinomycin D-treated cells (Fig. 6). A one-dimensional V8 protease partial peptide map of this polypeptide from mRNPs of actinomycin D-treated cells and from control hnRNPs indicated extensive similarity between the major peptide fragments of the two proteins (Fig. 7). The 38K protein which becomes cross-linked to poly(A)<sup>+</sup> mRNAs in actinomycin D-treated cells may therefore be related to a minor but consistent component of poly(A)<sup>+</sup> hnRNPs in untreated cells.

**Monoclonal antibodies to 38K and its cellular localization.** To characterize 38K, it was desirable to raise antibodies to it. Attempts were made to find a cellular fraction in which it was enriched. In the course of these studies, poly(A)<sup>+</sup> mRNPs were prepared from polyribosomes by oligo(dT)-cellulose chromatography, and the major proteins in the 38,000-molecular-weight region were compared by peptide mapping to the UV-cross-linked 38K protein from actinomycin D-treated cells. It was found that when polyribosomal mRNPs were prepared at salt concentrations lower than 500 mM and the oligo(dT)-cellulose columns were eluted first with 25% formamide followed by 50% formamide, the latter fraction contained a major protein of molecular weight 38,000 which was identical to 38K by peptide mapping. This protein was present in similar amounts in the poly(A)<sup>+</sup> mRNA fraction from control and from actinomycin D-treated cells, and no other significant effects of actinomycin D on the composition of un-cross-linked mRNPs were detectable (Fig. 8). The 38K protein from poly(A)<sup>+</sup> polyribosomal

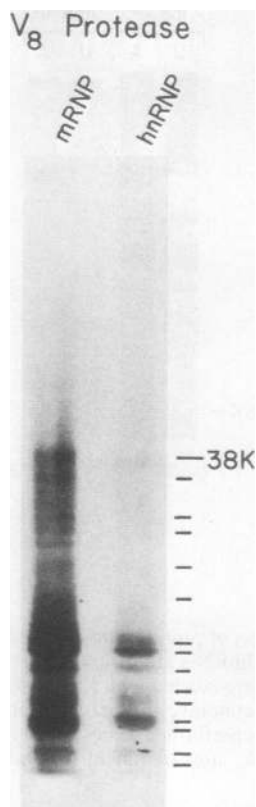


FIG. 7. One-dimensional partial peptide map of [ $^{35}\text{S}$ ]methionine-labeled 38K cross-linked by UV light to mRNA and to hnRNA. Sample preparation was as described in the legend to Fig. 6, and digestion was carried out with V8 protease (0.1  $\mu\text{g}$  per sample) by the method of Cleveland et al. (8).

material eluted with 50% formamide was excised from SDS-polyacrylamide gels and injected into mice for preparation of monoclonal antibodies. Hybridoma colonies were screened by  $\beta$ -galactosidase-linked assays and by immunoblotting with UV-cross-linked poly(A) $^+$  mRNPs from control and actinomycin D-treated cells as antigens. One of the colonies, designated 5C2, which was strongly positive only for material from actinomycin D-treated cells, was recloned. An immunoblot of ascites fluid of 5C2 is shown in Fig. 9. The antibody recognized a 38K protein in polyribosomal material and a 38K protein as well as a smaller protein of about 36K in total cell material. The amount of the band at 36K was variable, and it is likely to be a fragment of 38K. A diffuse stain was seen in the 38K region in UV cross-linked poly(A) $^+$  mRNPs from actinomycin D-treated cells but not from control cells. The increase in molecular weight of the UV cross-linked form is expected because the protein contains one or more RNase-resistant covalently bound nucleotides. Immunoblotting experiments with 38K turned out to be quite difficult because of some unusual properties of the protein. Only a small percentage of the protein could be electrophoresed out of the polyacrylamide gel and onto nitrocellulose paper even after very long transfer times and at several different pHs in the absence of SDS because the protein precipitates in situ once SDS is removed. If, however, SDS was included in the electrotransfer buffer, 38K was completely electroeluted from the gel but did not bind to the nitrocellulose paper. The binding of 38K to nitrocellulose was very poor, and the small amount which could be

transferred from the gel often tended to smear rather than form sharp bands. This was particularly so for the UV-cross-linked 38K. Attempts to use 5C2 to immunoprecipitate 38K extracted from cells with ionic detergents have so far been unsuccessful.

Immunofluorescence staining data with 5C2 are shown in Fig. 10. The monoclonal antibody stained both the cytoplasm and the nucleus. The cytoplasmic pattern was of fine dots essentially all over the cytoplasm (Fig. 10A). Some nuclear signal was also seen but it was weaker than the cytoplasmic signal and was non-nucleolar. Most of the 38K protein was retained with a Triton X-100 insoluble matrix and displayed a complex and heterogeneous pattern (Fig. 10B). In cells exposed briefly to hypertonic medium so as to disaggregate polyribosomes (43), most of the 38K protein appeared in large clusters in the cortex of the cytoplasm (Fig. 10C). The immunofluorescence patterns did not coincide with one particular recognizable cellular structure.

## DISCUSSION

The findings presented here demonstrate that treatment of cells with either of several different inhibitors of transcription causes a change in the physical state of cytoplasmic poly(A) $^+$  mRNPs. This is reflected by a change in the interaction of a 38K protein, and possibly also of other proteins, with the mRNA in vivo. The observations rely on

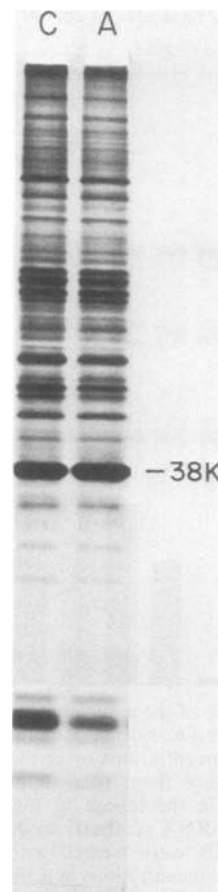


FIG. 8. [ $^{35}\text{S}$ ]methionine-labeled polyribosomal proteins from a 50% formamide eluate of an oligo(dT)-cellulose column prepared as described in the text from control HeLa cells (C) and from cells pretreated for 2 h with 5  $\mu\text{g}$  of actinomycin D per ml (A).

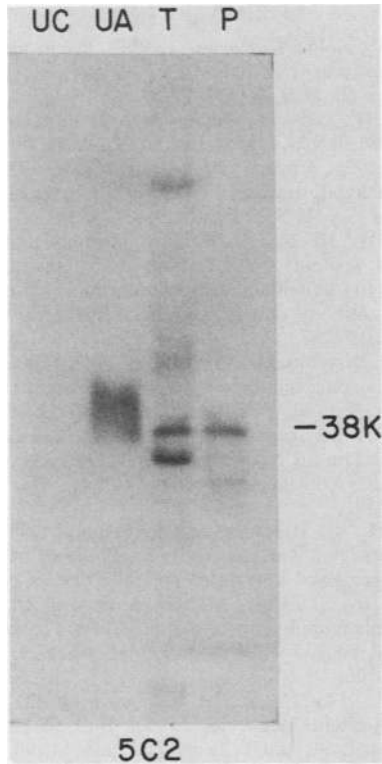


FIG. 9. Immunoblot with monoclonal 5C2 hybridoma ascites fluid. Abbreviations: UC, UV cross-linked poly(A)<sup>+</sup> mRNPs from control cells; UA, UV cross-linked poly(A)<sup>+</sup> mRNPs from actinomycin D-treated cells (5  $\mu$ g/ml for 2 h); T, total cell material; P, polyribosomal fraction after selection on an oligo(dT)-cellulose column as detailed in the legend to Fig. 8.

the ability of UV light to generate photoreactive species of RNA and thus bring about cross-linking of mRNA to proteins which are in direct contact with it in the intact cell (10, 34, 35, 50, 51, 53). The observed cross-linking of 38K to mRNA after treatment of cells with inhibitors of transcription may be a consequence of a complete "on-and-off" phenomenon or of an altered mode of interaction of 38K with mRNA so that it becomes more favorable to cross-linking by UV light. Although we consider the former to be more likely, the latter possibility must also be considered since the 38K protein can be isolated *in vitro* with polyribosomal poly(A)<sup>+</sup> mRNA from untreated cells even without prior UV cross-linking. This may reflect a genuine association of 38K with mRNA *in vivo*, but it may be a nonspecific one which occurs *in vitro* in the course of cell fractionation. In either case, the UV cross-linking data indicate that a physical change has taken place in mRNPs in the treated cells. The effect seems to be universal in vertebrates since the same response is seen in different cell types, including those of monkeys, mice, and hamsters (data not shown).

What is the mechanism by which inhibitors of mRNA formation effect a change in cytoplasmic mRNPs? Perhaps the most straightforward one is that all of the examined effectors also interact directly with mRNAs (or mRNPs) in the cytoplasm to cause a change in the mRNPs which promotes the cross-linking of the mRNA to 38K. An entirely different mechanism would be that the inhibitors exert their effect through their known action on nuclear transcription, processing, or transport of RNA polymerase II products and

that this nuclear effect is transduced to the mRNPs in the cytoplasm. That is, a feedback-type response in which the state of mRNA formation is communicated to the cytoplasm. Initially, a direct effect of the inhibitors on mRNA may be easier to envision. However, several observations which may be of relevance are not entirely consistent with it. The different inhibitors are structurally different and, to the

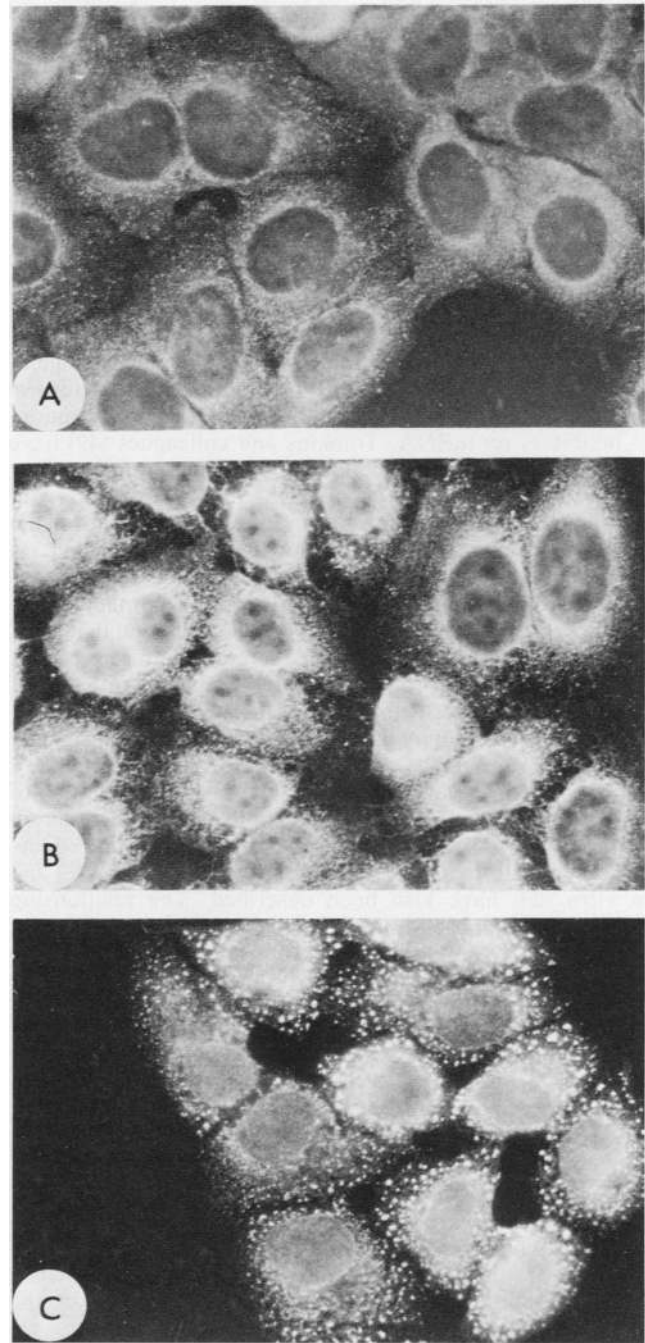


FIG. 10. Immunofluorescence of HeLa cells stained with 5C2. (A) Untreated cells. (B) Cells extracted with 0.5% Triton X-100 in 10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] buffer, pH 6.8, containing 100 mM KCl, 2.5 mM MgCl<sub>2</sub>, and 300 mM sucrose for 5 min on ice (7) in the presence of aprotinin, leupeptin, and pepstatin A. (C) Cells treated with 175 mM NaCl for 15 min before fixation.

extent that it is known, are believed to have different mechanisms and sites of action. Actinomycin D is known to intercalate into DNA but not into RNA *in vitro* (15, 19, 41, 47, 55). Camptothecin is not as well studied, but it also appears to intercalate into DNA (15). DRB, unlike actinomycin D and camptothecin, does not appear to prevent hnRNA transcription by intercalation into DNA but causes either proximally induced premature termination of nascent hnRNA or inhibition of initiation of hnRNA synthesis (11, 12, 37, 48). The inhibition of host transcription observed within 3 to 4 h after VSV infection is probably the result of the action of the 48-nucleotide leader sequence (36). Therefore, the observed effect of all these inhibitors which have such diverse characteristics may not simply be the result of a direct interaction with RNA *in vivo*. The close dose-response relationship of the effect of actinomycin D on mRNA synthesis and on the association of 38K with mRNA and the saturability of the effect also support this view. The possibility of a change in the physical state of mRNPs as a consequence of inhibition of transcription or of other nuclear events in mRNA formation or transport merits consideration.

Although the function of 38K or the physiological significance of its interaction with mRNA is not yet known, several previous reports suggest unexpected effects of transcriptional inhibitors on mRNA. Tomkins and colleagues (49) have described the "superinduction" of tyrosine aminotransferase in which the normal decay of the translatable mRNA of the induced protein which occurs after the removal of the inducer was prevented by actinomycin D. Cereghini et al. (6) observed that addition of actinomycin D to starved ascites cells prevented the loss of actin mRNA that normally takes place. Seghal et al. (45) and Gupta et al. (18) found that DRB treatment of cells induced to secrete interferon causes superinduction or prolonged and sustained synthesis of interferon even after withdrawal of the stimulus. An enhancing effect of actinomycin D on the levels of translatable mRNA in rat myoblast cultures has also been reported (23). All of these observations are consistent with stabilization of translatable mRNA and prevention of its normal degradation upon exposure to inhibitors of transcription. Possible effects of actinomycin D on mRNA translation *in vivo* (39, 42) and *in vitro* (29) have also been described. The relationship between these observations and the altered interaction of 38K with mRNA is not known, but it may be that 38K is involved in mRNA stabilization. If this is the case, 38K may play a role in mRNA stabilization also under normal conditions. One typical example of a natural system where transcription is arrested and mRNA is stabilized for long periods of time is the unfertilized egg. It is now possible to imagine that one effector (e.g., inhibitor of transcription) can affect both transcription and the state of formed mRNPs independently or else the two events are somehow coupled. Regardless of their mechanism and physiological significance, the observations described here indicate that experiments in which actinomycin D or other inhibitors of transcription are used should be interpreted with caution when questions about the behavior of formed mRNA in the cytoplasm are addressed.

#### ACKNOWLEDGMENTS

We thank Ermone J. Hussissian and Terry Nakagawa for excellent technical assistance.

This work was supported by grants from the National Science Foundation, the Leukemia Foundation, Inc., and the Searle Leadership Fund.

#### LITERATURE CITED

- Baer, B. W., and R. D. Kornberg. 1983. The protein responsible for the repeating structure of cytoplasmic poly(A)-ribonucleoprotein. *J. Cell. Biol.* **96**:717-721.
- Baltimore, D., and A. S. Huang. 1970. Interaction of HeLa cell proteins with RNA. *J. Mol. Biol.* **47**:263-272.
- Blobel, G. 1973. A protein of molecular weight 78,000 bound to the polyadenylate region of eukaryotic messenger RNA. *Proc. Natl. Acad. Sci. U.S.A.* **70**:924-928.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**:195-203.
- Butcher, P. D., and H. R. V. Arnstein. 1983. Efficient translation and polyribosome binding of  $^{125}\text{I}$ -labeled rabbit globin messenger ribonucleoprotein. *FEBS Lett.* **153**:119-124.
- Cereghini, S., T. Geoghegan, I. Bergmann, and G. Brawerman. 1979. Studies on the efficiency of translation and on the stability of actin mRNA in mouse sarcoma ascites cells. *Biochemistry* **18**:3153-3159.
- Cervera, M., G. Dreyfuss, and S. Penman. 1981. Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV-infected HeLa cells. *Cell* **23**:113-120.
- Cleveland, D., S. Fisher, M. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**:1102-1106.
- Craig, N. C. 1971. On the regulation of the synthesis of ribosomal proteins in L-cells. *J. Mol. Biol.* **55**:129-134.
- Economides, I. V., and T. Pederson. 1983. Structure of nuclear ribonucleoprotein: heterogeneous nuclear RNA is complexed with a major sextet of proteins *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **80**:1599-1602.
- Egyhazi, E., A. Pigon, and L. Rydlander. 1982. 5,6-Dichlororibofuranosylbenzimidazole inhibits the rate of transcription on initiation in intact *Chironomus* cells. *Eur. J. Biochem.* **122**:445-451.
- Fraser, N. W., P. B. Seghal, and J. E. Darnell. 1978. DRB-induced premature termination of late adenovirus transcription. *Nature (London)* **272**:590-592.
- Galfre, G., and C. Milstein. 1982. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* **73**:1-46.
- Girard, M., and D. Baltimore. 1966. The effect of HeLa cell cytoplasm on the rate of sedimentation of RNA. *Proc. Natl. Acad. Sci. U.S.A.* **56**:999-1003.
- Goldberg, I. H., and P. A. Friedman. 1971. Antibiotics and nucleic acids. *Annu. Rev. Biochem.* **40**:775-805.
- Greenberg, J. R. 1980. Proteins crosslinked to messenger RNA by irradiating polyribosomes with UV light. *Nucleic Acids Res.* **8**:5685-5701.
- Greenberg, J. R. 1981. The polyribosomal mRNA-protein complex is a dynamic structure. *Proc. Natl. Acad. Sci. U.S.A.* **78**:2923-2926.
- Gupta, S. L., B. Y. Rubin, and S. L. Holmes. 1981. Regulation of interferon action in human fibroblasts: transient induction of specific proteins and amplification of antiviral response by actinomycin D. *Virology* **111**:331-340.
- Hurwitz, J., J. J. Furth, M. Malamy, and M. Alexander. 1962. The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of the enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by actinomycin D and proflavin. *Proc. Natl. Acad. Sci. U.S.A.* **48**:1222-1230.
- Jain, S. K., M. G. Pluskal, and S. Sarkar. 1979. Thermal chromatography of eukaryotic messenger ribonucleoprotein particles on oligo(dT)-cellulose. *FEBS Lett.* **97**:84-90.
- Jeffrey, W. R. 1978. Composition and properties of messenger ribonucleoprotein fragments containing and lacking polyadenylate. *Biochim. Biophys. Acta* **521**:217-228.
- Kessel, D. 1971. Effect of camptothecin on RNA synthesis in leukemia L1210 cells. *Biochim. Biophys. Acta* **246**:225-232.
- Kessler-Ickson, G., R. H. Singer, and D. Yaffe. 1978. The



- capacity of polyadenylated RNA from myogenic cells treated with actinomycin D to direct protein synthesis in a cell-free system. *Eur. J. Biochem.* **88**:403-410.
24. Kish, V. M., and T. Pederson. 1976. Poly(A)-rich ribonucleoprotein complexes of HeLa cell messenger RNA. *J. Biol. Chem.* **251**:5888-5894.
  25. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (London)* **256**:495-497.
  26. Kumar, A., and T. Pederson. 1975. Comparison of proteins bound to heterogeneous nuclear RNA and messenger RNA in HeLa cells. *J. Mol. Biol.* **96**:353-365.
  27. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of <sup>3</sup>H and <sup>14</sup>C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**:335-341.
  28. Leibowitz, R. 1971. The effect of ethidium bromide on mitochondrial DNA synthesis and mitochondrial DNA structure in HeLa cells. *J. Cell Biol.* **51**:116-122.
  29. Leinwand, L., and F. H. Ruddle. 1977. Stimulation of in vitro translation of messenger RNA by actinomycin D and cordycepin. *Science* **197**:381-383.
  30. Liautard, J. P., B. Setyono, E. Spindler, and K. Kohler. 1976. Comparison of proteins bound to the different functional classes of messenger RNA. *Biochim. Biophys. Acta* **425**:373-383.
  31. Lindberg, U., and B. Sundquist. 1974. Isolation of messenger ribonucleoproteins from mammalian cells. *J. Mol. Biol.* **86**:451-468.
  32. Lodish, H. F., and R. A. Weiss. 1979. Selective isolation of mutants of vesicular stomatitis virus defective in production of the viral glycoprotein. *J. Virol.* **30**:177-189.
  33. Martin, T. E., J. M. Pullman, and M. E. McMullen. 1980. Structure and function of nuclear and cytoplasmic ribonucleoprotein complexes. In D. M. Prescott and L. Goldstein (ed.), *Cell biology: a comprehensive treatise*. Academic Press, Inc., New York.
  34. Mayrand, S., and T. Pederson. 1981. Nuclear ribonucleoprotein particles probed in living cells. *Proc. Natl. Acad. Sci. U.S.A.* **78**:2208-2212.
  35. Mayrand, S., B. Setyono, J. R. Greenberg, and T. Pederson. 1981. Structure of nuclear ribonucleoprotein: identification of proteins in contact with poly(A)<sup>+</sup> heterogeneous nuclear RNA in living HeLa cells. *J. Cell. Biol.* **90**:380-384.
  36. McGowan, J. J., S. V. Emerson, and R. R. Wagner. 1982. The plus-strand leader RNA of VSV inhibits DNA-dependent transcription of adenovirus and SV40 genes in a soluble whole-cell extract. *Cell* **28**:325-333.
  37. Mittleman, B., R. Zandomeni, and R. Weinmann. 1983. Mechanism of action 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. *J. Mol. Biol.* **16**:461-473.
  38. Ovchinnikov, L. P., A. S. Voronina, A. S. Stepanov, N. V. Velistina, and A. S. Spirin. 1968. Informosome-like complexes formed on addition of RNA to animal cell homogenates. *Mol. Biol. (Moscow)* **2**:601-609.
  39. Palmiter, R. D., and R. T. Schimcke. 1973. Regulation of protein synthesis in chick oviduct. *J. Biol. Chem.* **248**:1502-1512.
  40. Perry, R. P., and D. E. Kelley. 1970. Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. *J. Cell Physiol.* **76**:127-140.
  41. Reich, E., and I. H. Goldberg. 1964. Actinomycin and nucleic acid function. *Prog. Nucleic Acid Res. Mol. Biol.* **3**:183-234.
  42. Revel, M., and H. Hiatt. 1964. The stability of liver messenger RNA. *Proc. Natl. Acad. Sci. U.S.A.* **51**:810-818.
  43. Saborio, J. L., S. S. Pong, and G. Koch. 1974. Selective and reversible inhibition of initiation of protein synthesis in mammalian cells. *J. Mol. Biol.* **85**:195-211.
  44. Schwartz, H., and J. E. Darnell. 1976. The association of protein with the polyadenylic acid of HeLa cell messenger RNA: evidence for a "transport" role of 75,000 molecular weight peptide. *J. Mol. Biol.* **104**:833-851.
  45. Seghal, P. B., I. Tamm, and J. Vilcek. 1976. Regulation of human interferon production. I. Superinduction by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. *Virology* **70**:532-541.
  46. Setyono, B., and J. R. Greenberg. 1981. Proteins associated with poly(A) and other regions of mRNA and hnRNA molecules as investigated by crosslinking. *Cell* **24**:775-783.
  47. Sobell, H. M., S. C. Jain, T. D. Sakore, and C. E. Nordman. 1971. Stereochemistry of actinomycin-DNA binding. *Nature (London) New Biol.* **231**:200-205.
  48. Tamm, I., and P. B. Seghal. 1978. Halobenzimidazole ribosides and RNA synthesis of cells and viruses. *Adv. Virus Res.* **22**:187-258.
  49. Tomkins, G. M., B. B. Levenson, J. D. Baxter, and L. Dethlefsen. 1972. Further evidence for post-transcriptional control of inducible tyrosine aminotransferase synthesis in cultured hepatoma cells. *Nature (London) New Biol.* **239**:9-14.
  50. Van Eekelen, C. A. G., E. C. M. Mariman, R. J. Reinders, and W. J. Van Venrooij. 1981. Adenoviral hnRNA is associated with host cell proteins. *Eur. J. Biochem.* **119**:461-467.
  51. Van Venrooij, W. J., T. Riemen, and C. A. G. Van Eekelen. 1982. Host proteins are associated with adenovirus specific mRNA in the cytoplasm. *FEBS Lett.* **145**:62-65.
  52. Vincent, A., S. Goldenberg, and K. Scherrer. 1981. Comparisons of the proteins associated with duck-globin mRNA and its polyadenylated segment in polyribosomal and repressed free messenger ribonucleoprotein complexes. *Eur. J. Biochem.* **114**:179-193.
  53. Wagenmakers, A. J. M., R. J. Reinders, and W. J. Van Venrooij. 1980. Crosslinking of mRNA to proteins by irradiation of intact cells with ultraviolet light. *Eur. J. Biochem.* **112**:323-330.
  54. Warner, J. R. 1966. The assembly of ribosomes in HeLa cells. *J. Mol. Biol.* **19**:383-398.
  55. Wells, R. D., and J. E. Larson. 1970. Studies on the binding of actinomycin D to DNA and DNA model polymers. *J. Mol. Biol.* **49**:319-342.
  56. Wu, R. S., A. Kumar, and J. R. Warner. 1971. Ribosome formation is blocked by camptothecin, a reversible inhibitor of RNA synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **68**:3009-3014.
  57. Zeevi, M., J. R. Nevins, and J. E. Darnell. 1982. Newly formed mRNA lacking polyadenylic acid enters the cytoplasm and the polyribosomes but has a shorter half-life in the absence of polyadenylic acid. *Mol. Cell. Biol.* **2**:517-525.
  58. Zyber, E., C. Vesco, and S. Penman. 1969. Selective inhibition of the synthesis of mitochondria-associated RNA by ethidium bromide. *J. Mol. Biol.* **44**:195-204.