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Interaction of mRNA with Proteins in Vesicular Stomatitis Virus-Infected Cells

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The interaction of mRNA with proteins in vesicular stomatitis virus (VSV)-infected cells was studied by photochemical cross-linking in intact cells. The major [^{35}S]methionine-labeled proteins which became cross-linked by UV light to mRNA in uninfected and in VSV-infected HeLa cells were similar and had apparent mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis corresponding to 135, 93, 72, 68, 53, 50, 43, and 36 kilodaltons. The proteins which were cross-linked in vivo specifically to the five mRNAs of VSV were labeled through radioactive nucleotides incorporated only into VSV mRNAs under conditions (5 μg of actinomycin D per ml) in which only VSV mRNAs are labeled. The same major mRNP proteins that became cross-linked to host mRNAs also became cross-linked to VSV mRNAs, although several quantitative differences were detected. Photochemical cross-linking and immunoblotting of cross-linked mRNPs with VSV antiserum demonstrated that in addition to host proteins VSV mRNAs also became cross-linked to the VSV-encoded N protein. The poly(A) segment of both host and VSV mRNAs was associated in vivo selectively with the 72-kilodalton polypeptide. The major proteins of mRNA-ribonucleoprotein complexes are therefore ubiquitous and common to different mRNAs. Furthermore, since the major messenger ribonucleoproteins interact also with VSV mRNAs even though these mRNAs are transcribed in the cytoplasm, it appears that nuclear transcription and nucleocytoplasmic transport are not necessary for mRNA to interact with these proteins.

There is a good deal of evidence that mRNAs in eucaryotic cells are intimately associated with proteins to form ribonucleoprotein complexes (mRNPs) (4, 5, 14, 15, 19, 21, 24, 25, 28, 29, 45, 46, 55). These complexes, rather than the naked mRNAs, are believed to constitute the structural and functional units of mRNAs, and their proteins are likely to play a central role in mRNA formation, transport, translation, stability, and localization. Despite the fact that the potential importance of mRNPs has been recognized for a long time, progress in identifying them has been hampered by the limitations of traditional experimental approaches. The major difficulty in identifying RNPs by conventional isolation techniques is that the criterion of copurification of certain proteins with polynucleotides is, in general, not sufficiently stringent to ascertain that these are genuine RNP components in vivo. This is due to nonspecific interactions between RNA and proteins that are likely to occur after cell fractionation (2). Moreover, genuine RNP proteins may dissociate under the particular fractionation conditions. Photochemical RNA-protein cross-linking in intact cells overcomes these difficulties because it allows the identification of proteins which are in direct contact with heterogeneous nuclear RNA and mRNA in vivo (9, 10, 12, 34, 35, 52–54, 56) and eliminates the nonspecific associations of proteins with RNA since the isolation of the cross-linked RNA-protein complexes can be carried out in the presence of protein denaturants.

The photochemical cross-linking approach relies on the ability of UV light of sufficient intensity to generate photoreactive species of RNA which are extremely reactive with essentially any molecules, including proteins, which are in direct contact with them (47). Cross-linked mRNA-protein complexes can be isolated under protein-denaturing conditions by affinity chromatography on oligo(dT)-cellulose [which selects the mRNA through binding to its poly(A) tail] to ensure that only proteins covalently linked to the mRNA are purified with it (9, 52, 56). The selected cytoplasmic poly(A)$^+$ material can be digested with RNase, and the released proteins can be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9, 52, 56).

In the studies described here we examined the proteins that interact with cytoplasmic poly(A)$^+$ mRNA in normal (uninfected) and in vesicular stomatitis virus (VSV)-infected HeLa cells by photochemical cross-linking. Conditions for UV irradiation of intact cells and for isolation of RNA-protein cross-linked complexes were optimized so as to allow efficient RNA-protein cross-linking and yield maximal recovery of intact RNA. By infection with VSV a new and different set of mRNAs was introduced into the cytoplasm. VSV is a well-characterized, small, single-stranded RNA virus of the rhabdovirus group which encodes five distinct mRNAs (57). Like most host mRNAs, these have a methylated 5′-cap structure (37, 40) and a 3′-poly(A) sequence (13, 48). VSV mRNAs are transcribed in great abundance in the cytoplasm of the infected cell by a virus-encoded polymerase. They can be selectively labeled, and their metabolism can be readily followed. The proteins which are associated specifically with the VSV mRNAs and the effects of VSV infection on mRNP proteins in the infected cell were studied. It is demonstrated that the same proteins which are normally found cross-linked to mRNAs of the host also become cross-linked to the mRNAs of the virus. In addition, a complex of the viral N protein with VSV mRNA previously found in mRNPs from fractionated cells (17, 41) is identified. The poly(A) segment of both host and VSV mRNAs is shown to interact specifically with the 72 kilodalton (kDa) protein (72K protein).

MATERIALS AND METHODS

Cell culture and labeling. HeLa cells were grown in monolayer culture in Dulbecco modified Eagle medium

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containing 10% fetal calf serum (FCS) at 37°C in 5% CO_2 atmosphere. All cultures were supplemented with penicillin-streptomycin and used at subconfluent densities. Unless indicated otherwise, cells were labeled for 4 h with [^{35}S]methionine at 10 μCi/ml in methionine-free medium containing 2% undialyzed FCS. Where indicated, labeling was in the presence of actinomycin D added 30 minutes before the addition of the [^{35}S]methionine. ^{32}P labeling was carried out similarly in phosphate-free medium with 50 μCi of $^{32}P_i$ per ml. Labeling with ^{3}H-nucleosides was carried out similarly in complete medium with 50 μCi of isotope per ml. Cell culture materials were from GIBCO Laboratories (Grand Island, N.Y.), and radiochemical reagents were obtained from New England Nuclear Corp. (Boston, Mass.).

Virus infection. Cells were infected with the Indiana strain, Glasgow serotype of VSV (from Harvey Lodish, Massachusetts Institute of Technology) at a multiplicity of 10 PFU per cell essentially as previously described (6, 32). Cell monolayers in 100-mm plates were rinsed twice with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (PBS), and the virus was absorbed at room temperature for 1 h in 2 ml of PBS. The virus was removed, and the cells were placed in normal complete medium and transferred to 37°C (time zero of infection). Virus stocks were grown in HeLa cells, and virus titer was determined by plaque assay (59).

UV irradiation. The culture medium was removed, and cell monolayers were washed twice with PBS. Cells were irradiated in PBS at room temperature with a 15-W germicidal lamp (Sylvania G15T8) placed 4.5 cm away from the cell monolayer (9). Irradiation time was 3 min unless indicated otherwise.

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 $MgCl_2$ containing the protease inhibitors 0.5% aprotinin (Sigma Chemical Co., St. Louis, Mo.), 1 μg of pepstatin A per ml, 1 μg of leupeptin per ml, and the RNase inhibitor vanadyl-adenosine (3, 6). 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 a brief low-speed centrifugation, and the cytoplasmic fraction was adjusted to 1 mM EDTA, 1% mercaptoethanol, and 0.5% SDS. After heating at 65°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, Inc., Waltham, Mass.) with constant rocking. The oligo(dT)-cellulose was then packed in a column and washed with >20 column volumes of binding buffer and eluted with 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–0.05% SDS. The eluted fractions were reheated to 65°C for 5 min, and after addition of LiCl to 0.5 M and SDS to 0.5% the oligo(dT)-cellulose chromatography was repeated as described above. The eluted poly(A)$^+$ material was precipitated overnight at −20°C with 3 volumes of ethanol.

RNase digestion of RNPs. The poly(A)$^+$ material was pelleted by centrifugation at 12,500 × *g* and suspended in 75 μl of 10 mM Tris hydrochloride (pH 7.4) containing 1 mM $CaCl_2$, and digestion with RNase was carried out with 25 μg of pancreatic RNase A (Worthington Diagnostics, Freehold, N.J.) per ml and 400 U of micrococcal nuclease (P-L Biochemicals, Inc., Milwaukee, Wis.) 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 the 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 PAGE system containing a final concentration of 12.5% acrylamide as previously described (9). After electrophoresis of ^{35}S-labeled or ^{3}H-labeled proteins, the gels were stained with Coomassie blue and impregnated with 2,5-diphenyloxazole (PPO), and fluorography was performed at −80°C with preflashed X-ray films (26).

RNA was fractionated according to size by electrophoresis through vertical 1.4% agarose gels containing formaldehyde by a modification of previously described procedures (27). The gel buffer contained 1.2 M formaldehyde, 20 mM MOPS (3-[*N*-morpholino]propanesulfonic acid) (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA. Electrophoresis was at 40 mA for 6 to 8 h. The RNA samples were pretreated at 65°C for 5 min in the same buffer containing 50% formamide. The tank buffer, which was the same as the gel buffer, was rapidly circulated between the two electrode compartments.

Antibody production and immunoblotting. VSV was purified from the medium after overnight infection by sedimentation through a 10 to 45% sucrose gradient in 10 mM Tris chloride (pH 7.6)–1 mM EDTA for 60 min at 75,000 × *g*. Purified virions were dissolved in PBS containing 0.5% SDS and boiled. The viral lysate was diluted to 0.1% SDS, and samples were used to immunize a BALB/c mouse. The primary intraperitoneal injection contained equal parts of viral lysate and complete Freund adjuvant. The three subsequent booster injections were with incomplete adjuvant and were done at 2-week intervals. At the end of the immunization protocol, the mouse was sacrificed and the serum was collected. Immunoblotting was carried out as previously described (7).

RESULTS

UV cross-linking of mRNA to proteins in HeLa cells. The proteins which became cross-linked to poly(A)$^+$ mRNA in human HeLa cells after different times of irradiation with UV light are shown in Fig. 1. The predominant [^{35}S]methionine-labeled polypeptides had approximate molecular weights of 135, 93, 72, 68, 53, 50, 43, and 36 kDa. A number of minor polypeptides were also detectable. Under the conditions employed, no proteins were detected if irradiation was omitted, indicating that the signals are specific in that they result from covalent binding of protein to mRNAs in vivo as a consequence of exposure to UV light. Although the amount of cross-linked protein increased with increasing irradiation times (Table 1), few additional protein species became cross-linked to the mRNA beyond 1 min (Fig. 1), suggesting specificity of this group of proteins to become cross-linked to the mRNA. However, after prolonged irradiation, some of the extensively cross-linked poly(A)$^+$ mRNPs could not be recovered by oligo(dT)-cellulose chromatography (Table 1), and polynucleotide breakage increased. A short irradiation time of 3 min with a 15-W 254-nm light source placed 4.5 cm away from the cell monolayer was found to be optimal and was used in all subsequent experiments. Under these conditions, about 87% of the poly(A)$^+$ sequences were recovered compared with unirradiated controls (Table 1), and strand breakage which increases with increasing UV doses was minimal (see Fig. 3). The high recovery of poly(A)$^+$ material indicates that under these conditions of UV irradiation and RNA isolation, an

adequate representation of the entire population of poly(A)$^+$ mRNA, rather than of a selected subset, is obtained. These conditions are therefore suitable to investigate the proteins that interact with a subset of mRNAs.

mRNP proteins in VSV-infected cells. Protein synthesis in the cells at 4.5 h after infection with VSV was markedly different from normal. Massive synthesis of the VSV proteins L, G, N, Ns, and M was observed (Fig. 2, lanes A and B). To examine whether such a change in protein synthesis is accompanied by a change in the mRNP proteins, cells were labeled with [^{35}S]methionine before VSV infection, and labeling was terminated upon infection (at $t = -1$ h). At 4.5 h postinfection the cells were exposed to UV light, and the proteins which became cross-linked to the entire cytoplasmic poly(A)$^+$ fraction in vivo were visualized (Fig. 2, lanes F to H). Since 5 μg of actinomycin D per ml was used in some of the experiments described below it is also included in this experiment. Comparison of the mRNP proteins of mock-infected cells and VSV-infected cells, both treated with actinomycin D (lanes G and H), shows that in VSV-infected cells there is increased cross-linking of several proteins to the mRNA. Most apparent are the increases in the 68K, 60K, and 43K proteins. A slight increase in the cross-linking to mRNA of the 72K protein is also detectable. Some possibly qualitative differences such as the appearance of bands at about 60 and 100 kDa are also seen. Interestingly, actinomycin D treatment of uninfected cells by itself (lane G) brought about a significant increase in the cross-linking of a 38-kDa protein to the mRNA, and VSV infection without actinomycin D also causes the same effect (9). This effect has now been further characterized and shown to be a consequence of a structural change in mRNPs that occurs after treatment of cells with various inhibitors of nuclear transcription (9). The infected cells contain both host and VSV mRNAs (11, 38) so that the total number of mRNA molecules per infected cell is about threefold greater than normal (30). The overall amount of cross-linked protein after infection also increases by about that factor, although it is apparent that this is not the case for some of the individual mRNP proteins such as the 72K protein (Fig. 2, lanes H and G). In contrast, the 68K polypeptide and proteins in the 36 to 40 kDa range become major components of mRNPs after VSV infection. The mRNP proteins which are identified in Fig. 2 (lanes C to H) are not ribosomal proteins, since in these experiments the synthesis of ribosomal proteins was inhibited by actinomycin D (8, 39, 58).

The proteins which were synthesized and became associated with poly(A)$^+$ mRNA during the time of infection are shown in Fig. 2, lanes C to E. No drastic changes were detectable in the mRNP proteins after VSV infection (lanes G and H) in the presence of actinomycin D. However,

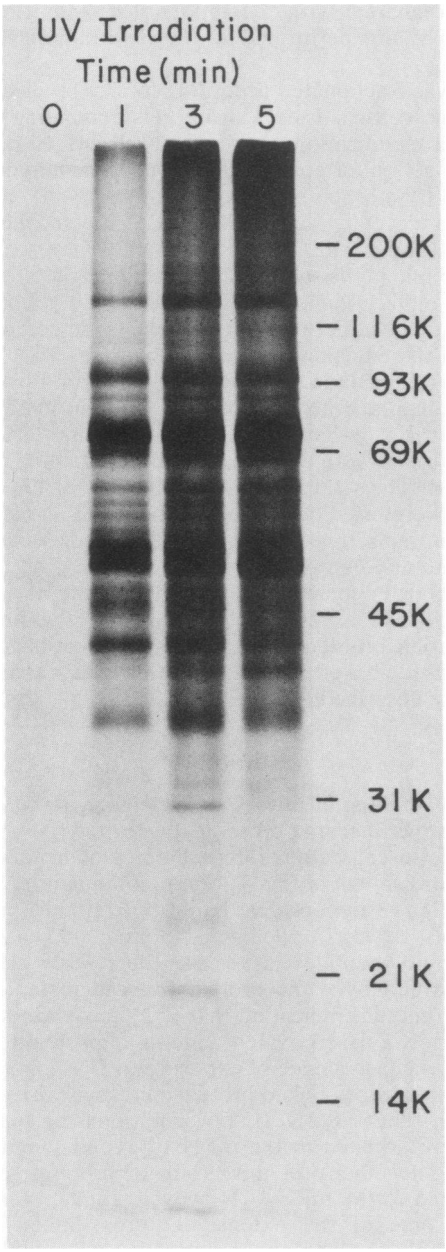

FIG. 1. Electrophoretic pattern of proteins cross-linked in vivo to poly(A)$^+$ mRNA after different UV irradiation times. HeLa cells were labeled for 4 h with [^{35}S]methionine (10 μCi/ml) in methionine-free medium containing 2% FCS in the presence of actinomycin D (0.04 μg/ml) added 30 min before addition of the label. The cell monolayers were washed and irradiated in PBS for the times indicated. Cytoplasmic fractions were prepared, and the poly(A)$^+$ material was selected by chromatography on oligo(dT)-cellulose. After RNase digestion, samples were electrophoresed on a 12.5% polyacrylamide gel and fluorographed. Numbers on right show proteins.

TABLE 1. Effect of time of irradiation on the recovery of poly(A)$^+$ mRNA and on the efficiency of protein-mRNA cross-linking[a]

Time of irradiation (min)	10^6 cpm [^{3}H]uridine (%)	10^5 cpm [^{35}S]methionine	^{35}S cpm/ ^{3}H cpm
0	1.59 (100)	0	0
1	1.47 (92)	4.9	0.03
3	1.39 (87)	22.6	0.16
5	1.07 (68)	32.4	0.30

[a] HeLa cells were labeled simultaneously for 3 h with [^{3}H]uridine (50 μCi/ml) and [^{35}S]methionine (10 μCi/ml) in methionine-free medium supplemented with 2% FCS and containing actinomycin D (0.04 μg/ml). Cells were irradiated for the times indicated, and poly(A)$^+$ mRNPs were isolated by oligo(dT)-cellulose chromatography as described in Materials and Methods, except that only one cycle of heating and chromatography was performed. Samples of the pooled poly(A)$^+$ material were precipitated with 10% trichloroacetic acid onto Gelman Metricel filters, and counts per minute were determined in a Beckman scintillation counter. Channel crosstalk counts per minute of ^{3}H and ^{35}S were subtracted.

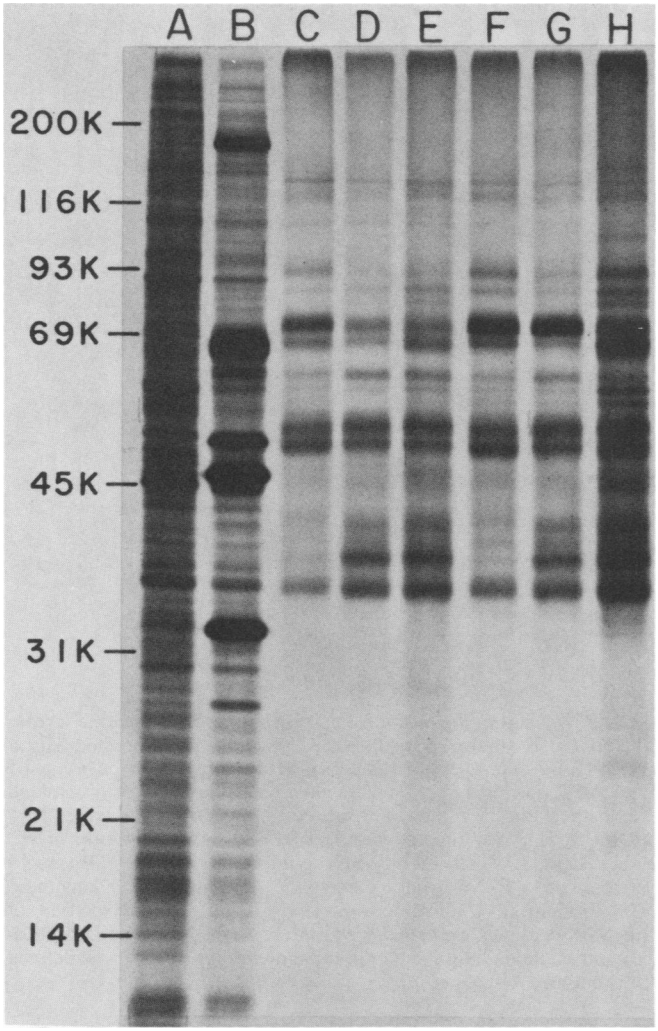

FIG. 2. Electropherogram of the proteins which became cross-linked to poly(A)$^+$ mRNA in VSV-infected and in mock-infected HeLa cells. Cell labeling with [^{35}S]methionine, UV irradiation, mRNP isolation, and SDS-PAGE were as described in Materials and Methods. Lanes A and B, Protein synthesis in control and VSV-infected cells, respectively. Labeling was for 30 min at 4.5 h after infection. Lanes C to E, mRNA-cross-linked proteins which were labeled in the course of infection (between 0.5 and 4.5 h after infection): C, mock infected and labeled with 0.04 μg of actinomycin D per ml; D, mock infected and labeled with 5 μg of actinomycin D per ml; E, VSV infected and labeled with 5 μg of actinomycin D per ml. Lanes F to H, Prelabeled proteins which become cross-linked to poly(A)$^+$ mRNA. Cells were labeled with [^{35}S]methionine for 4 h, infected with VSV, transferred to complete medium without [^{35}S]methionine, and irradiated 4.5 h later; F, mock-infected cells; G, mock-infected cells with 5 μg of actinomycin D per ml; H, VSV-infected cells with 5 μg of actinomycin D per ml. Numbers on left show proteins.

exposure to actinomycin D by itself affected the cross-linking of 38K to mRNA (Fig. 2, lane D) (9), and there was some decrease in the amount of the 72K protein cross-linked to the mRNA. The fact that at least most VSV proteins did not become cross-linked to mRNA although they were the most prominently labeled proteins in the cytoplasm (Fig. 2, lane B; and see below) is further evidence for the specificity of the UV cross-linking.

The intensity of the bands of the major mRNP proteins did not markedly decrease when the cells were labeled during infection (0.5 to 4.5 h). Since the inhibition of host protein synthesis by VSV was marginal during the 1.5 h after infection, significant labeling of these proteins could occur (Fig. 2, lane E). The continued synthesis and association with mRNA of all of these proteins after host mRNA production was blocked by actinomycin D (Fig. 2, lanes D and E) suggest that these newly synthesized proteins must become associated with older, preformed mRNAs or with newly synthesized VSV mRNAs.

Proteins in contact with the mRNAs of VSV. The experimental strategy used so far examined the entire population of poly(A)$^+$ mRNPs at the same time. It did not, therefore, distinguish between proteins that are cross-linked to host mRNAs and those that are cross-linked to VSV mRNAs. To address this issue, the proteins which are associated in vivo specifically with the mRNAs of VSV were examined. In addition to labeling of the proteins by [^{35}S]methionine, the proteins which become cross-linked to mRNA can also be identified by labels incorporated through the RNA because the cross-linked nucleotide(s) remains linked to the protein after RNase digestion (36). To identify the proteins which are cross-linked to VSV mRNAs, VSV-infected cells were labeled in the presence of 5 μg of actinomycin D per ml (18). Under these conditions, host poly(A)$^+$ mRNA synthesis was completely inhibited, whereas substantial amounts of label accumulated in VSV poly(A)$^+$ mRNAs (Table 2) (18). Thus, when VSV-infected cells are treated with such levels of actinomycin D, the only poly(A)$^+$ RNA into which radioactive nucleotides are incorporated are VSV mRNAs.

When the infected cells were exposed to UV light, VSV poly(A)$^+$ mRNAs, like host mRNAs in uninfected cells, became cross-linked to protein. This could be seen by electrophoresis of the poly(A)$^+$ material on a denaturing formaldehyde-agarose gel (Fig. 3). Most of the UV-cross-linked VSV mRNAs did not penetrate the agarose gel (Fig. 3, lane A). However, after proteinase K digestion of the cross-linked proteins before electrophoresis (lane B), the VSV mRNAs were recovered and appeared to have the same mobility as that of deproteinized, phenol-extracted VSV mRNAs from unirradiated cells (lane C). Some of the VSV mRNAs from UV irradiated cells which migrated normally (lane A) appeared not to be cross-linked to protein under the irradiation conditions used. The data in Fig. 3 also indicate that UV irradiation at the doses used in this work does not cause significant breakage of the mRNA.

The labeled nucleotides in VSV mRNAs make it possible to specifically tag the proteins that become cross-linked to

TABLE 2. Effect of actinomycin D on incorporation of $^{32}P_i$ into poly(A)$^+$ mRNA in uninfected and VSV-infected cells[a]

Cells	Actinomycin D (μg/ml)	^{32}P in poly(A)$^+$ fraction (cpm/10^6 cells) (%)
Uninfected	0.04	17,200 (100)
Uninfected	5	390 (2)
VSV infected	5	25,400 (148)

[a] VSV-infected and uninfected HeLa cells were labeled with $^{32}P_i$ in phosphate-free medium containing 2% FCS and the indicated amount of actinomycin D between 0.5 and 4.5 h postinfection. Poly(A)$^+$ mRNA was extracted from the cytoplasmic fraction with phenol in the presence of 10 mM vanadyl-adenosine (3, 6) after digestion with proteinase K. Samples of the poly(A)$^+$ fraction were precipitated with trichloroacetic acid, and radioactivity was determined by liquid scintillation counting.

them. Because some of the mRNP proteins may be phosphorylated, tagging of the proteins that interact with VSV mRNAs was carried out with ^{3}H-nucleosides instead of $^{32}P_i$ (Fig. 4).

During the course of VSV infection, preexisting host mRNAs remain stable in the cytoplasm but translate poorly (11, 30, 31, 38). By labeling the cells with the tritiated nucleosides before infection, the proteins that cross-link to host mRNAs during the infection can be identified. HeLa cells were labeled for 4 h with all four tritiated nucleosides before infection, and the radioactive nucleosides were removed at $t = -1$ h of infection and exposed to UV light 4.5 h later. The mRNP proteins labeled in this experiment (Fig. 4, lane A) were cross-linked mostly to HeLa mRNAs at 4.5 h of infection. This suggests that the untranslated host mRNAs are still complexed with RNP proteins and that these resemble what is seen in uninfected cells (lane B). Lane C shows the proteins that were cross-linked to VSV mRNA by labeling infected cells in the presence of actinomycin D as described above. The overall protein pattern was quite similar in uninfected, prelabeled infected, and infected cells with some quantitative differences. The most obvious qualitative difference was in the labeling of a protein of approximately 50 kDa which was cross-linked to VSV mRNAs (lane C, indicated by arrow). No corresponding band was seen in uninfected cells, and some signal which may correspond to the same protein was present in the prelabeled infected cells (lane A). The increased cross-linking of another protein of 38 kDa to both host and VSV mRNAs has been previously described (9). In the absence of UV irradiation, no proteins were cross-linked to the RNA, although residual RNA which was resistant to RNase was consistently seen (lane D).

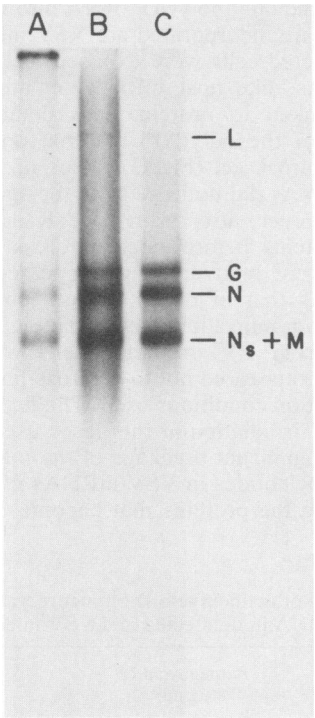

FIG. 3. Autoradiogram of formaldehyde-agarose gel electrophoresis of poly(A)$^+$ material from VSV-infected cells. Cells were labeled with $^{32}P_i$ in phosphate-free medium containing 5 μg of actinomycin D per ml between 0.5 and 4.5 h after infection and irradiated, and the cytoplasmic poly(A)$^+$ fraction was prepared as described in Materials and Methods. Lane A, Sample from UV-irradiated cells. Lane B, Same as in lane A except that the sample was treated with proteinase K (350 μg/ml, 30 min at 37°C) before electrophoresis. Lane C, Sample from unirradiated cells prepared by extraction with phenol (6). L, G, N, Ns, and M are VSV proteins.

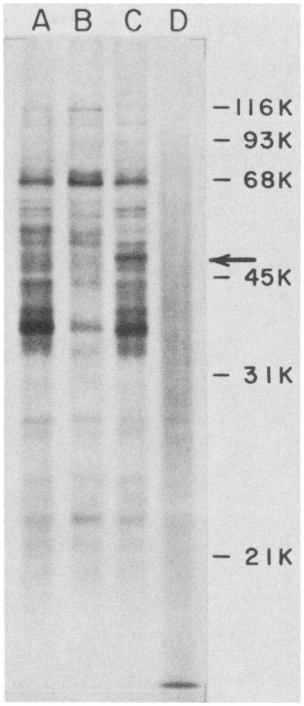

FIG. 4. Autoradiogram of the proteins which become cross-linked to ^{3}H-labeled poly(A)$^+$ mRNA from VSV-infected HeLa cells. HeLa cells were labeled for 4 h before infection or between 0.5 and 4.5 h during infection with 50 μCi each of all four tritiated ribonucleoside precursors per ml. Actinomycin D was added to 5 μg/ml to all VSV-infected samples 30 min before addition of the label. Lanes: A, cells labeled before infection with VSV; B, mock-infected cells; C, VSV-infected cells; D, VSV-infected cells with no UV irradiation. UV irradiation (lanes A to C) and isolation of mRNPs were performed 4.5 h postinfection as described in Materials and Methods. Numbers on right show proteins. Arrow is defined in the text.

VSV N protein is cross-linked to mRNA. To examine the possibility that some of the proteins that were cross-linked to mRNA after infection such as the 35K and 50K polypeptides are VSV proteins, polyclonal antiserum was prepared against purified denatured VSV virions and used to probe an immunoblot of cross-linked RNPs. The antiserum is specific for VSV proteins and detected well the G, Ns, N, and M proteins in total lysates from infected HeLa cells (Fig. 5, lane B) but not mock-infected cells (lane A). On longer exposure of immunoblots with this antiserum, a band probably corresponding to the L protein of VSV was also present. The identification of the N and Ns proteins was confirmed by in vitro translation of VSV mRNAs separated on a methyl mercury hydroxide-agarose gel (data not shown). Immunoblot analysis of UV-cross-linked poly(A)$^+$ RNPs from mock-infected (Fig. 5, lane C) and VSV-infected cells (lane D) demonstrated that a small amount of the VSV N protein was cross-linked to RNA. In the absence of UV light, no N protein was isolated with the RNA (lane E). The possibility that some L protein also cross-linked to mRNA

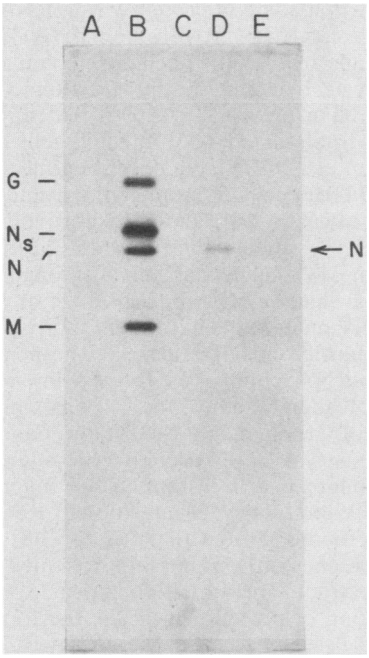

FIG. 5. Immunoblot of mRNPs isolated from mock-infected and VSV-infected cells (4.5 h postinfection) probed with anti-VSV serum. UV-cross-linked mRNPs were isolated from mock-infected or VSV-infected HeLa cells as described in the text. Lanes: A, total cell material from mock-infected cells; B, total cell material from VSV-infected cells; C, poly(A)$^+$ mRNPs from mock-infected cells; D, poly(A)$^+$ mRNPs from VSV-infected cells; E, poly(A)$^+$ mRNPs from VSV-infected cells without UV irradiation. G, Ns, N, and M are VSV proteins.

cannot be ruled out because the weak response of the serum for this protein may preclude its detection. mRNPs previously isolated from VSV-infected cells have been shown to contain the viral N protein (17, 22, 41). The 50K protein cross-linked to VSV mRNA as detected by ^{3}H-nucleoside labeling (Fig. 4) comigrated with the VSV N protein.

RNP complex of the poly(A) segment. To identify which, if any, of the cross-linked proteins are associated specifically with the poly(A) tract, poly(A) segments were prepared from UV-cross-linked poly(A)$^+$ mRNPs by digestion with pancreatic and T_1 RNases [which degrade the mRNA except for the poly(A) segment (23)] and isolated by chromatography on oligo(dT)-cellulose. Size analysis of the remaining poly(A)$^+$ preparation by electrophoresis on polyacrylamide gels showed that the poly(A) obtained from irradiated cells was similar to the poly(A) obtained by a similar procedure from unirradiated cells, indicating that the UV cross-linking did not cause any polynucleotide breakage or hinder the RNase digestion (data not shown). The repurified poly(A) segment from UV-irradiated cells was treated with micrococcal nuclease, and the released proteins were analyzed by SDS-PAGE. Similar experiments were carried out to determine if the poly(A) regions of VSV mRNAs are associated with proteins similar to host poly(A).

The RNP proteins that are cross-linked to the total and to the poly(A) tail of mRNA were labeled with tritiated nucleosides (Fig. 6). By labeling the cells with [^{3}H]uridine or [^{3}H]adenosine separately, nucleotide cross-linking preference can be determined for the individual proteins. Lanes A and B show the proteins that cross-linked to mRNA in mock-infected cells labeled with [^{3}H]uridine or [^{3}H]adenosine. There was a slight preference for proteins to cross-link to uridine, although the major proteins were cross-linked to both uridines and adenosines. Isolation of the poly(A) portion of RNA from cells labeled with [^{3}H]adenosine demonstrated, in agreement with the results of the [^{35}S]methionine labeling (S. A. Adam, Y. D. Choi, and G. Dreyfuss, unpublished data), that a single protein of 72,000 molecular weight is labeled (lane C). The other mRNP proteins are, therefore, associated with other portions of the mRNA. Analysis of uridine- or adenosine-labeled VSV mRNA was similar to that of the host, with most proteins cross-linked to both uridine and adenosine (lanes D and E). One notable exception was the protein at 72 kDa, which in VSV-infected cells did not label by cross-linking to uridine. This protein, however, was labeled by cross-linking to adenosine and was the major protein on the poly(A) tail of VSV mRNAs (lane F). This suggests that the 72K protein is associated with other regions of the mRNA in addition to the poly(A) regions in HeLa mRNAs but interacts only with the poly(A) portion of VSV mRNAs. Alternatively, the 72K protein could interact with other regions of the VSV message which do not contain much uridine.

DISCUSSION

We examined the interaction of proteins with mRNA using UV cross-linking in living cells under normal culture conditions and after infection with VSV. Irradiation and RNA

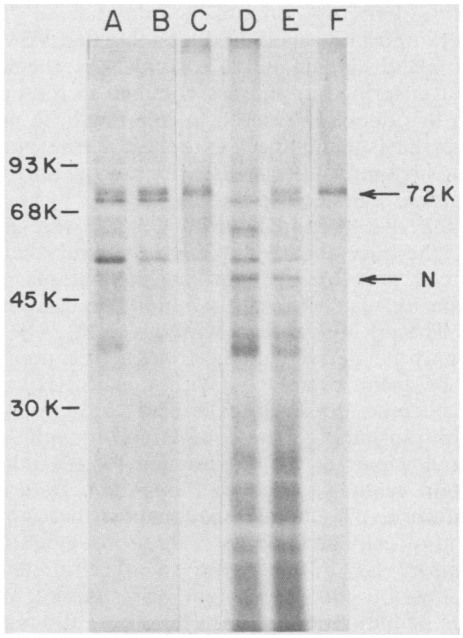

FIG. 6. Proteins cross-linked to the poly(A) portion of HeLa and VSV mRNAs. Mock-infected cells were labeled with 50 μCi of [^{3}H]adenosine or [^{3}H]uridine per ml in the presence of 0.05 μg of actinomycin D per ml for 4 h. VSV-infected cells were labeled similarly in the presence of 5 μg of actinomycin D per ml between 0.5 and 4.5 h postinfection. Labeled mRNPs were isolated as described in the text. Lanes: A, [^{3}H]uridine-labeled mock-infected poly(A)$^+$ mRNPs; B, [^{3}H]adenosine-labeled mock-infected poly(A)$^+$ mRNPs; C, [^{3}H]adenosine-labeled mock-infected poly(A) segment; D, [^{3}H]uridine-labeled VSV poly(A)$^+$ mRNPs; E, [^{3}H]adenosine-labeled VSV poly(A)$^+$ mRNPs; F, [^{3}H]adenosine-labeled VSV mRNAs poly(A) segment. N, VSV protein. Other numbers show proteins.

selection conditions are described which optimize cross-linking efficiency and yield of RNA-protein complexes while minimizing RNA strand breakage. The major finding is that the same mRNP proteins that become cross-linked to host mRNAs also become cross-linked to VSV mRNAs. This suggests that these proteins are common to all mRNAs. This conclusion is supported by the findings of vanVenrooij et al. (54) who have recently shown that adenovirus mRNAs are also associated with at least two host proteins in the infected cell. Each individual copy of a particular mRNA, however, may not necessarily be associated with all of the mRNP proteins at the same time, particularly since individual mRNAs can be found in the cell in a number of different compartments (e.g., in polyribosomal or postpolyribosomal fractions and in the cytoskeletal or soluble fractions [6]). In each of these compartments, an mRNA may be associated with a different subset of the mRNP proteins (43).

The poly(A) tail of both host and VSV mRNAs interacts in the cell selectively with the 72K protein. Of the cross-linked proteins the 72K protein is thus the only one for which a selective binding sequence on the mRNA has been identified. A similar observation was recently reported by Greenberg and Carroll (16), while this manuscript was in preparation. The relationship of this protein to protein(s) of similar molecular weight described by others as interacting with poly(A) in vitro (1, 4, 24, 45, 46) is presently uncertain, but they are quite likely to be the same. Proteins which comigrate in SDS-PAGE with the poly(A)-binding 72K protein of HeLa as well as with the other major mRNPs are also found in other vertebrate species (19; S. A. Adams, Y. D. Choi, and G. Dreyfuss, unpublished data).

Another important aspect of the finding that VSV mRNAs bind host mRNPs is that unlike host mRNAs, the mRNAs of VSV are transcribed in and are confined to the cytoplasm. Although this does not rule out a role for these proteins in mRNA synthesis and nuclear-cytoplasmic transport, it does argue that such nuclear processes are not the only routes by which mRNAs can acquire these proteins.

Assuming that the mRNPs have some role in mRNA function, the fact that VSV mRNAs bind them in the infected cell may have important implications as to the mechanism by which the virus inhibits translation of host mRNAs. The mRNP proteins with which the VSV mRNAs interact may be derived either from a free pool of these proteins or from complexes with host mRNAs. If host mRNAs become deprived of mRNP proteins they may become untranslatable. The competition for mRNP protein could provide part of the explanation for the inhibition of host protein synthesis after VSV infection. Data from several laboratories (11, 30, 31, 50) suggest that inhibition of translation of cellular mRNAs is due to competition at the initiation step of protein synthesis of viral and cellular mRNAs possibly for a constant and limiting number of ribosomes or initiation factors. The observations presented here are consistent with such a mechanism since VSV mRNAs clearly compete for the same proteins and may displace them from some host mRNAs. However, there may also be qualitative changes in host mRNPs (Fig. 2, lane H) which contribute to inhibition of host protein synthesis by other, more selective mechanisms (20, 33, 44, 50, 51).

The identification in the infected cell of cross-linked complexes of the viral N protein with VSV mRNAs (and possibly also with host mRNAs) raises another possible mechanism for the shutoff of protein synthesis. The N protein is known to be associated with the virion RNA to form the nucleocapsid, and we cannot completely rule out the possibility that some N protein-virion RNA cross-linked complexes are carried over with the poly(A)$^+$ RNA-protein fraction through the oligo(dT)-cellulose column even though no 42S RNA is detected in this fraction. Grubman and Shafritz (17) have previously shown that mRNP particles isolated from fractionated VSV-infected cells contain the N protein. Rosen et al. (41) have described the isolation from VSV-infected CHO cells of an mRNP particle that contains all five VSV mRNAs and, almost exclusively, the viral N protein. In these studies the VSV mRNPs were clearly resolved from VSV nucleocapsids. Although the work by Grubman and Shafritz (17) and Rosen et al. (41) virtually eliminated VSV nucleocapsid from the sucrose gradients and used highly purified mRNP fractions, the possibility exists that the N-mRNA complexes formed in vitro after cell fractionation. Taken together, the UV cross-linking findings and the data of Grubman and Shafritz (17) and Rosen et al. (41) strongly suggest that N protein-VSV mRNA complexes exist in the infected cell. Although the function of the N protein-mRNA interaction is not known, Rosen et al. (42) have recently demonstrated that the N-VSV mRNA particles inhibit protein synthesis in rabbit reticulocyte lysate and wheat germ extract. The inhibition appears to be at the first step of initiation of protein synthesis, the formation of the ternary complex eIF-2 · GTP · Met-tRNA (42). The N protein-mRNA complex may therefore be involved in the shutoff of total protein synthesis in VSV-infected cells.

Viral protein-mRNA interactions in general are potentially of great importance because they give rise to new RNP forms which may modify elements of host pathways and facilitate viral functions. In addition to VSV mRNA-N protein complexes the only other viral protein-mRNA complexes so far identified in vivo are those of the adenovirus 5 proteins 100K and 72K which interact with mRNA and heterogeneous nuclear RNA, respectively (S. A. Adam and G. Dreyfuss, unpublished results). A mRNP complex containing the NS protein of reovirus with reovirus mRNA has been recently identified in vitro (49).

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