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Adenovirus Proteins Associated with mRNA and hnRNA in Infected HeLa Cells

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The proteins that interact with cytoplasmic and nuclear polyadenylated RNA in adenovirus type 5 (Ad5) infection of HeLa cells were examined by UV-induced RNA-protein cross-linking in intact cells. The Ad5 100-kilodalton late nonvirion protein (100K protein) was cross-linked to both host and viral polyadenylated cytoplasmic RNA (mRNA). The cross-linking of the 100K protein to mRNA appears to correlate with productive infection, because the protein is not cross-linked to mRNA in abortive infection of wild-type Ad5 in monkey cells (CV-1) even though normal amounts of it are produced. However, when CV-1 cells are infected with Ad5 *hr*404, an Ad5 mutant which overcomes the host restriction to wild-type Ad5 infection in these cells, the 100K protein is cross-linked to mRNA. To identify and obtain antibodies to RNA-contacting proteins, a mouse was immunized with oligo(dT)-selected cross-linked RNA-protein complexes from Ad5-infected cells and the serum was used for immunoblotting experiments. It was found that in addition to the 100K protein, the Ad5 72K DNA-binding protein is also associated with RNA in the infected cells. The 72K DNA-binding protein is cross-linked to polyadenylated nuclear RNA sequences. These findings indicate that adenovirus proteins interact with RNAs in the infected cell and suggest possible mechanisms for the effects of the virus on mRNA metabolism.

mRNA and its nuclear precursor, heterogeneous nuclear RNA (hnRNA), are intimately associated with specific proteins in the cell to form ribonucleoprotein (RNP) complexes. The RNP proteins are likely to be involved in the synthesis, processing, transport, and function of these polynucleotides (for a review, see reference 14a). However, little is known about the role of RNP proteins in these processes and about their function in the regulation of RNA metabolism. Adenovirus-infected cells provide an interesting system in which to examine RNP structure and function; drastic changes take place in the formation of host mRNAs and in mRNA translation (2, 3, 7, 8, 20, 24, 39, 40). At late times in infection, a small number of well-defined viral genes are transcribed in large amounts (59) and viral mRNAs containing 5′-cap- and 3′-poly(A) tail structures indistinguishable from those of host mRNAs are formed (22, 30, 36, 39, 40, 48). Host hnRNAs continue to be transcribed, but newly synthesized spliced host mRNAs do not accumulate in the cytoplasm (3, 7, 8, 19, 24, 41). In the cytoplasm, both host and viral mRNAs are present, but virtually all mRNAs that are translated are viral mRNAs. These changes in host mRNA metabolism are believed to be the result of the expression within the cell of viral gene products during the late phase of infection. Because host transcription products are present but their normal functions or cellular localization is altered, it is likely that these effects are mediated by viral gene products, possibly through interaction with host RNAs or RNP complexes (5, 41, 46, 47).

Several reports have suggested that the adenovirus 100-kilodalton protein (100K protein) is associated with polysomal virus-specific mRNA in infected cells (31, 50, 54). This association resembles that of some of the cellular mRNP proteins in its resistance to dissociation from the RNA by high salt concentrations (51). Van Venrooij and colleagues (56, 57), however, have applied the more stringent criterion of UV cross-linking of intact cells to examine RNPs in adenovirus-infected cells and failed to detect any viral-protein-RNA interactions.

The work presented here demonstrates by photochemical cross-linking in intact cells that adenovirus-coded proteins interact with mRNA and hnRNA in the infected cell. The results indicate that the 100K late nonstructural protein of adenovirus type 5 (Ad5) interacts directly with cytoplasmic polyadenylated RNA (mRNA) and the adenovirus 72K DNA-binding protein (DBP) interacts with nuclear polyadenylated RNA (hnRNA) in the late phase of Ad5 productive infection of human cells.

MATERIALS AND METHODS

Cell culture and virus infection. HeLa cells and African green monkey kidney cells (CV-1) were grown in monolayer culture in Dulbecco modified Eagle medium containing 10% fetal calf serum at 37°C in 5% CO_2 atmosphere. Cultures were supplemented with penicillin-streptomycin and used at subconfluent densities. HeLa cells were infected with wild-type (wt) Ad5 at 50 to 200 PFU per cell by adsorption for 1 h at 37°C in Tris-saline (pH 7.9). Infection of CV-1 cells was done under identical conditions by using wt Ad5 or the host range mutant virus Ad5 *hr*404 (24). Typical infections were for 18 to 22 h at 37°C. Cells were labeled for the last 2 h of infection with [^{35}S]methionine at 20 μCi/ml in methionine-free medium containing 2% dialyzed fetal calf serum. For [^{3}H]nucleoside labeling, cells were incubated in complete medium containing 100 μCi each of [^{3}H]uridine and [^{3}H]adenosine per ml for 3 h. Cell culture materials were from GIBCO Laboratories, and radiochemical reagents were obtained from Amersham Corp.

UV irradiation. The culture medium was removed, and cell monolayers were washed twice with phosphate-buffered saline containing Ca^{2+} and Mg^{2+}. Irradiation was done in phosphate-buffered saline at room temperature with a 25-W germicidal lamp (Sylvania model G15T8) placed 4.5 cm from the cell monolayer for 3 min (15).

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Cell fractionation and RNP isolation. After UV irradiation, the phosphate-buffered saline 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 0.5% aprotinin (Sigma Chemical Co.), 1 μg each of pepstatin A and leupeptin per ml, and the ribonuclease inhibitor vanadyl-adenosine at 10 mM. 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% 2-mercaptoethanol, and 0.5% sodium dodecyl sulfate (SDS). After heating at 90°C for 5 min, rapid chilling, and the addition of LiCl to 0.5 M, the cytoplasmic extract was incubated for 15 min with oligo(dT)-cellulose (type 3; Collaborative Research, Inc.) with constant agitation. The oligo(dT)-cellulose was then packed in a column and washed with >20 column volumes of binding buffer (10 mM Tris hydrochloride, pH 7.4; 500 mM LiCl; 1 mM EDTA; 0.5% SDS). The column was eluted in elution buffer (10 mM Tris hydrochloride, pH 7.4, 0.05% SDS) and monitored by liquid scintillation counting or A_{260}. The eluted poly(A)$^+$ material was digested with 50 μg DNase I per ml for 20 min at 37°C, SDS was added to a final concentration of 1%, the sample was reheated to 65°C for 5 min, and the oligo(dT)-cellulose chromatography was repeated. When a poly(A)$^+$ hnRNA fraction from UV-irradiated cells was prepared, procedures were similar except that the nuclear fraction was digested with DNase I (50 μg/ml for 15 min at 37°C) before oligo(dT)-cellulose chromatography. The poly(A)$^+$ material was precipitated with 3 volumes of ethanol at −80°C for 2 h or at −20°C overnight.

RNase digestion. 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 done with 25 μg of pancreatic RNase A (Worthington Diagnostics) and 400 U of micrococcal nuclease (P-L Biochemicals, Inc.) per ml for 60 min at 37°C. To inhibit possible traces of protease, the pancreatic RNase was preboiled, and aprotinin (1%), 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 with 3 volumes of ethanol as described above. Typically, poly(A)$^+$ RNPs from approximately 2 × 10^7 cells were loaded on each lane for [^{35}S]methionine-labeling experiments or immunoblots. This corresponds to 5 to 10 μg of poly(A)$^+$ cytoplasmic RNA as determined by A_{260}. For total cell protein, the equivalent of 2 × 10^5 cells was loaded in each lane.

Gel electrophoresis. Protein samples were electrophoresed on an SDS-containing discontinuous polyacrylamide gel electrophoresis (PAGE) system as previously described (15). The separating gel had a final acrylamide concentration of 12.5%. Samples were prepared by boiling for 3 min in a 0.125 M Tris hydrochloride (pH 6.8) buffer containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and bromophenol blue. After electrophoresis of ^{35}S-labeled material, the gels were stained with Coomassie blue and impregnated with PPO (2,5-diphenyloxazole). Fluorography was done with Eastman Kodak Co. XAR-5 film at −80°C (28).

Preparation of antibodies. Antiserum to total UV cross-linked poly(A)$^+$ RNPs was prepared in a BALB/c mouse by four injections at 2-week intervals of cross-linked RNPs from 10^8 infected HeLa cells in equal volumes of phosphate-buffered saline and Freund adjuvant. Five days after the final injection, the mouse was sacrificed and the serum was collected (1).

Immunoblotting. 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 5 to 10 h. The nitrocellulose blot was treated with nonfat dry milk (13, 16), processed essentially as described by Burnette (9), and exposed to a dilution of polyclonal antiserum. Detection was with ^{125}I-labeled goat anti-mouse immunoglobulin antiserum or with ^{125}I-labeled protein A.

Immunoprecipitation of cross-linked 100K protein. UV cross-linked mRNPs from 10^8 adenovirus-infected HeLa cells were prepared by two cycles of oligo(dT)-cellulose chromatography as described above. The mRNPs were precipitated with ethanol, suspended in 10 mM Tris hydrochloride (pH 7.4)–50 mM NaCl–1 mM $CaCl_2$, and digested with 2 U of micrococcal nuclease per ml for 10 min at 30°C. The digestion was stopped by the addition of EGTA to 5 mM, and the detergent Empigen BB (*n*-alkyl-betaine; Albright and Wilson, Ltd.) was added to the sample to a final concentration of 1%. The immunoprecipitations were done with equal volumes of either rabbit anti-100K antiserum (23) or nonimmune rabbit serum for 4 h at 4°C. Immune complexes were removed from the solution by the addition of protein A-agarose beads, and the beads were washed extensively with the same buffer. The agarose beads were then washed twice with T4 polynucleotide kinase reaction buffer (50 mM Tris hydrochloride [pH 7.6], 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA), and the bound RNA fragments were labeled by the addition of 20 U of T4 polynucleotide kinase and 150 μCi [γ-^{32}P]ATP in a final volume of 50 μl for 30 min at 37°C. After extensive washing of the agarose beads in the immunoprecipitation buffer, the bound RNP complexes were removed from the beads in 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA containing 1% SDS and 350 μg of proteinase K per ml. The solution containing the RNA fragments was then passed through oligo(dT)-cellulose to remove any undigested poly(A), and it was phenol extracted and ethanol precipitated. Labeled HeLa mRNA was prepared by end labeling with ^{32}P partially digested poly(A)$^+$ RNA isolated from uninfected HeLa cells by phenol extraction.

Dot blot hybridization. Single-stranded cDNA was prepared from HeLa cell poly(A)$^+$ cytoplasmic RNA with avian myeloblastosis virus (AMV) reverse transcriptase as described previously (33). Ad5 virion DNA was prepared from virus particles isolated by cesium chloride sedimentation (37). Approximately equal amounts of each DNA were denatured by boiling and treatment with 0.5 M NaOH and immobilized on nitrocellulose. Approximately 10^5 cpm of ^{32}P-labeled RNA fragment per ml was hybridized to each filter in 65% formamide–20 mM 1,4-piperazinediethanesulfonic acid (pH 6.4)–0.2% SDS–0.4 M NaCl–100 μg yeast tRNA per ml for 7 h at 50°C. After extensive washing at 65°C in 10 mM Tris hydrochloride (pH 7.6)–0.15 M NaCl–1 mM EDTA–0.5% SDS, the filters were autoradiographed.

RESULTS

The proteins which are associated in vivo with polyadenylated RNA in the cytoplasmic and nuclear fractions of mock-infected and Ad5-infected HeLa cells at 18 h after infection were identified by UV cross-linking in intact cells. The predominant [^{35}S]methionine-labeled polypeptides which are cross-linked to poly(A)$^+$ mRNA (mRNP proteins)

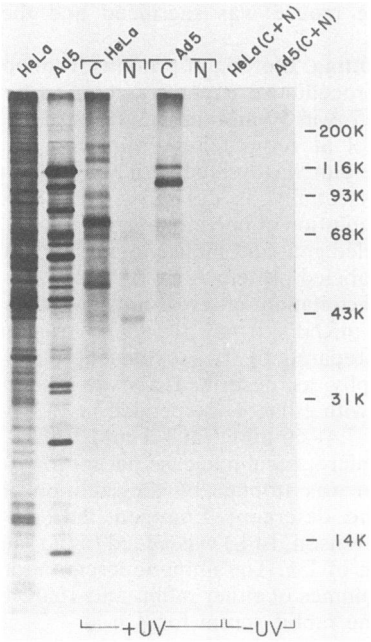

FIG. 1. [^{35}S]methionine labeling of RNPs cross-linked to poly(A)$^+$ RNA late in Ad5 infection. Mock-infected and infected cells were labeled for 4 h with [^{35}S]methionine from 18 to 22 h postinfection in methionine-free media. UV cross-linking, cell fractionation, RNase digestion, and SDS-PAGE are as detailed in Materials and Methods. The lanes designated HeLa and Ad5 contain total cell material from uninfected and infected cells, respectively. The +UV HeLa and Ad5 lanes contain proteins cross-linked to cytoplasmic (C) and nuclear (N) poly(A)$^+$ RNPs. In the absence of UV irradiation before cell fractionation, no labeled polypeptides are isolated with the poly(A)$^+$ RNA (−UV lanes).

in uninfected cells have approximate molecular weights in SDS-PAGE of 135,000, 93,000, 72,000, 68,000, 53,000, 50,000, 43,000, and 36,000 (Fig. 1), (15, 58). The poly(A) segment of the mRNA is selectively cross-linked to the 72K polypeptide (1, 21). These mRNP proteins are nonribosomal (15, 16) and constitute a different set from those which are cross-linked to poly(A)$^+$ hnRNA (hnRNP proteins) (16). When UV cross-linked mRNPs were isolated from late Ad5-infected HeLa cells, a major band at molecular weight 100,000 was seen, which comigrates with the Ad5 nonstructural 100K polypeptide (Fig. 1). Host proteins are associated with viral mRNAs in adenovirus infection (57) but these are only weakly labeled here because labeling of proteins was done at a time when host protein synthesis was greatly repressed. Occasionally, small and variable amounts of a 140K polypeptide were also detected with the cross-linked mRNPs. This band is not immunoreactive with hexon-specific antiserum on immunoblots, and its exact nature is unknown although it is not always observed. No association of adenovirus proteins with nuclear poly(A)$^+$ RNA was detected by [^{35}S]methionine labeling of infected cells late in infection. As a control against nonspecific adsorption of adenovirus proteins to the RNA or to the oligo(dT)-cellulose and to demonstrate that the proteins identified as RNP proteins were indeed covalently bound to the RNA, an identical isolation of RNPs from infected cells was done without prior UV cross-linking. No proteins were detected in material isolated from unirradiated cells under these conditions (Fig. 1).

Immunoblotting experiments were done with antiserum specific to the Ad5 100K protein in order to verify the identification of the protein made on the basis of mobility in SDS-PAGE. The polyadenylated RNP fractions prepared for blotting were identical to those described above (Fig. 1), except that the cells were not labeled with [^{35}S]methionine before UV irradiation. The proteins were electrophoretically transferred to nitrocellulose paper after SDS-PAGE and probed with an anti-100K antiserum. An immunoblot of material obtained from infected and mock-infected cells probed with a polyclonal rabbit antiserum to the 100K protein (Fig. 2) demonstrated that the 100K protein which is in contact with mRNA in the infected cells is the nonvirion 100K protein (product of the L4 region of the Ad5 genome). In agreement with the [^{35}S]methionine-labeling data, no signal was detected in the nuclear fraction from infected cells (lane N) or in mock-infected cells.

The proteins cross-linked to RNA can also be detected by incorporating tritium-labeled ribonucleosides into the RNA before UV cross-linking. After ribonuclease digestion and electrophoresis, the RNP proteins are labeled by virtue of the covalent association of [^{3}H]ribonucleotide and protein generated by UV light. This method of labeling is independent of the amino acid content of the RNP proteins and of their individual rates of synthesis (57) and can also be used to distinguish between the proteins cross-linked to different populations of RNA on the basis of the time or conditions of labeling of the RNA (1, 57). At least four major proteins were labeled by [^{3}H]ribonucleosides in uninfected HeLa cells (Fig. 3). These proteins of molecular weights 35,000, 50,000, 68,000, and 135,000 were also the most strongly labeled by [^{35}S]methionine (Fig. 1). In Ad5-infected cells, the 100K protein, in addition to the HeLa cell proteins, was labeled by cross-linking to ^{3}H-labeled RNA in the cell. Furthermore, the 100K protein was labeled whether the RNA was labeled between 0 and 3 h or between 19 and 22 h of infection. In the early labeling (Fig. 3), the cells were labeled for the first 3 h

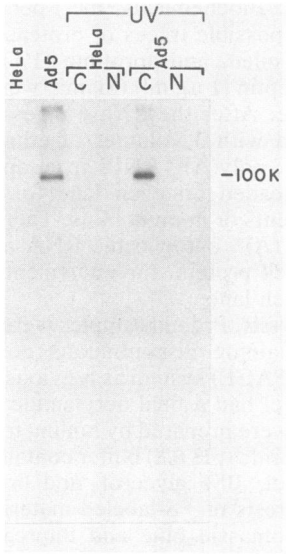

FIG. 2. Immunoblot analysis of UV cross-linked poly(A)$^+$ RNPs probed with polyclonal anti-100K Ad5 serum. The experiment was done as described in the legend to Fig. 1 without labeling, and after SDS-PAGE the proteins were electrophoretically transferred to nitrocellulose paper. The blot was probed with a polyclonal rabbit antiserum for the 100K protein of Ad5. Detection was with an ^{125}I-labeled anti-rabbit immunoglobulin second antibody.

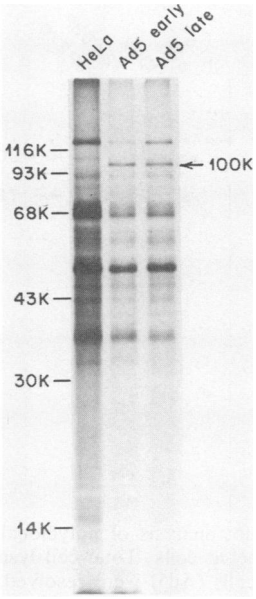

FIG. 3. [^{3}H]ribonucleoside labeling of ribonucleoproteins cross-linked to poly(A)$^+$ cytoplasmic RNA in Ad5 infection. Mock-infected and infected cells were labeled for 3 h with [^{3}H]uridine and [^{3}H]adenosine in complete medium, and cytoplasmic RNPs were isolated as described in Materials and Methods. The lane designated HeLa contains mRNPs isolated from uninfected HeLa cells. The lane designated Ad5 early contains mRNPs isolated from infected cells labeled during the first 3 h of infection, with the label removed for the last 19 h. The lane designated Ad5 late contains mRNPs isolated from infected cells labeled between 19 and 22 h of infection. Twice as much poly(A)$^+$ RNP was loaded on the Ad5 early lane to equalize the counts per minute loaded per lane.

of infection and the [^{3}H]nucleosides were then removed for the remainder of the infection (19 h). Host cell mRNAs are not preferentially degraded in adenovirus infection and degrade with their normal half-lives (39); therefore, the proteins seen in lane Ad5 early are likely to be associated with HeLa cell mRNA. The same proteins were detected when cells were labeled late in infection, at a time when mRNAs accumulating in the cytoplasm are almost entirely late adenovirus mRNA (3, 24, 41). This suggests that the 100K protein is associated with both host and viral mRNA. It should be noted that the RNA labeled early in the infection, hence host mRNA, is still associated with host cell RNP proteins, suggesting that the virus infection does not cause major disassembly of host cell mRNPs late in infection.

The association of the 100K protein with both host and viral mRNA was shown directly by probing HeLa cDNA

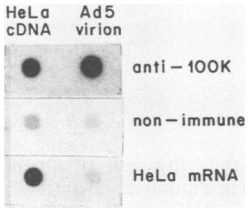

FIG. 4. DNA dot blot analysis of the RNA cross-linked to the 100K protein. RNA fragments isolated by immunoprecipitation were 5′-end labeled with ^{32}P and used to probe DNA immobilized on nitrocellulose. The DNA in each spot is indicated at the top, and the sources of the RNA probes are indicated to the right.

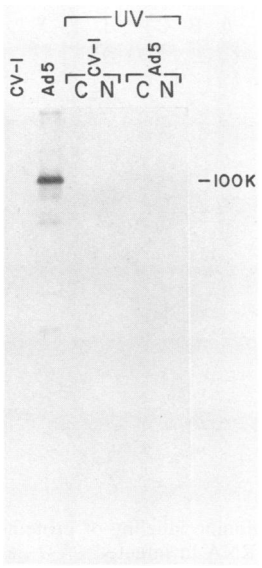

FIG. 5. Immunoblot analysis of proteins cross-linked to poly(A)$^+$ RNA in abortive infection. UV-cross-linked poly(A)$^+$ RNPs were isolated from wt Ad5-infected African green monkey kidney cells (CV-1) and analyzed as described in the legend to Fig. 2. C, Cytoplasmic fractions; N, nuclear fractions.

and adenovirus virion DNA immobilized on nitrocellulose with ^{32}P end-labeled RNA isolated by immunoprecipitation with antiserum to the 100K protein (Fig. 4). UV cross-linked mRNPs isolated from infected cells were partially digested with nuclease, and the fragments covalently associated with the 100K protein were immunoprecipitated and 5′-end labeled with ^{32}P. After removal of the cross-linked protein, the RNA fragments were used to probe DNA dot blots to determine whether HeLa or adenoviral mRNA sequences were precipitated. Figure 4 clearly shows that the 100K protein is associated with both HeLa and adenovirus mRNA.

Infection of monkey cells with C-type human adenoviruses results in abortive infection (18, 25, 26). The 100K protein is produced in CV-1 cells in amounts similar to those produced in human cells, and hexon trimer complexes form as efficiently in these cells as in HeLa cells (11). However, whereas the 100K protein is found predominantly in the cytoplasm of infected HeLa cells late in infection, at comparable times in monkey CV-1 cells it is detected essentially only in the nucleus (11, 20, 38). Immunoblotting with anti-100K serum shows (Fig. 5) that although the 100K protein was made in CV-1 cells, no 100K protein was cross-linked to mRNA or to hnRNA by UV irradiation. The restriction of monkey cells for the productive infection by human adenoviruses can be overcome by mutations in the 72K DBP (27). To determine whether the lack of direct cross-linkable interaction between the 100K protein and mRNA is a consequence of an intrinsic property of CV-1 cells and to examine the possible correlation between 100K-RNA cross-linking and productive lytic infection, CV-1 cells were infected with *hr*404 (an Ad5 mutant), which overcomes the host range restriction and which productively infects CV-1 cells (27). The results presented in Fig. 6 show that although the 100K protein is produced in the same quantity in wt Ad5 and *hr*404-infected cells, the 100K protein can be cross-linked to RNA only in the host range mutant infection. The amount of 100K protein cross-linked to RNA in the *hr*404

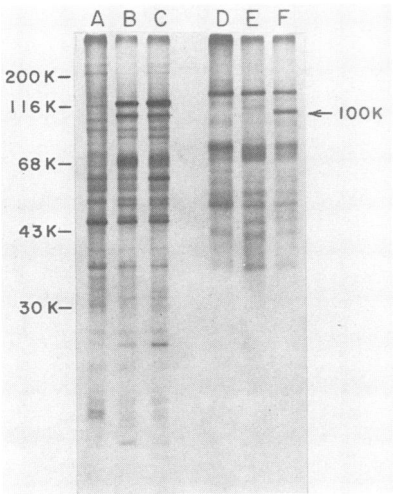

FIG. 6. [^{35}S]methionine labeling of proteins cross-linked to cytoplasmic poly(A)$^+$ RNA in infected CV-1 cells. Labeling of cells and preparation of UV-cross-linked RNPs was as described in the legend to Fig. 1 except that CV-1 cells were used instead of HeLa cells. Protein synthesis (lanes A through C) and the proteins cross-linked to cytoplasmic poly(A)$^+$ RNA (lanes D through F) is shown in mock-infected CV-1 cells (lanes B and E), and *hr*404-infected CV-1 cells (lanes C and F).

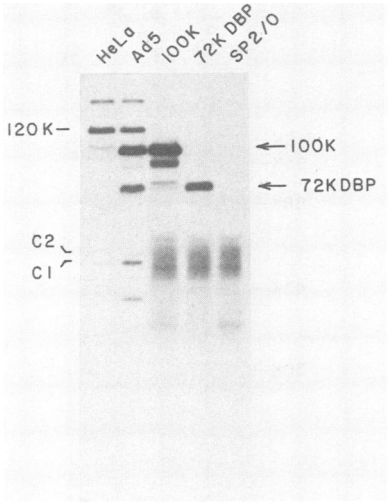

FIG. 7. Immunoblot analysis of polyclonal serum to poly(A)$^+$ RNPs from Ad5-infected cells. Total cell lysates from uninfected (HeLa) or infected cells (Ad5) were resolved on SDS-PAGE and transferred to nitrocellulose. Immunoprecipitations from total cell lysate of infected cells with anti-100K or anti-72K sera are in adjacent lanes. Lane SP2/0 is an immunoprecipitation with ascites fluid prepared by intraperitoneal injection of a mouse with the mouse myeloma line SP2/0. The blot was probed with a dilution of the mouse antiserum to total UV-cross-linked poly(A)$^+$ RNPs from Ad5-infected HeLa cells.

infection is roughly equivalent to the amount cross-linked to HeLa mRNA in a wt Ad5 infection. The cross-linking of 100K to mRNA therefore correlates with productive infection. The shutoff of host cell protein synthesis in these infections is not very dramatic (approximately 60%); consequently, most of the CV-1 RNP proteins are also labeled. Since the specific mutation which overcomes the host range restriction in *hr*404 is in the 72K DBP, we investigated whether the 100K protein and the 72K protein might interact in the cell. Immunoprecipitations under a variety of conditions with several antisera to both the 100K and 72K proteins failed to show any direct association between the two proteins.

The initial detection of the 100K protein-mRNA cross-link was possible because substantial amounts of the protein become cross-linked and because it labels extremely well with [^{35}S]methionine. Since this may not be the case for other virus RNP proteins, another general approach has been devised to identify proteins which may be cross-linked to poly(A)$^+$ RNPs in infected cells (1). UV cross-linked RNP complexes were prepared from Ad5-infected HeLa cells and used to immunize mice. The serum from an immunized mouse was tested by immunoblotting on total proteins from Ad5-infected and mock-infected cells. The results of an immunoblotting experiment (Fig. 7) indicate that antibodies in the mouse serum clearly recognize the 100K protein and also a 72K protein in infected but not in uninfected cells. The identification of the adenovirus 100K and 72K proteins was confirmed by immunoprecipitation with specific antisera for each protein from total infected cell lysate followed by immunoblotting the precipitate with the mouse serum. The specificity of this procedure is demonstrated by the fact that no response is detected to other abundant and immunogenic Ad5 proteins such as hexon and penton base. Substantial immunological response is also seen to several host RNP proteins including the 120K hnRNP protein (16) and the hnRNP C proteins 41K and 43K (13, 16). Although similar cell equivalents of protein were loaded on the gel, a stronger signal for the C proteins was detected in the Ad5-infected sample. This suggests that the C proteins accumulate in the cell during infection or that some posttranslational modification increases the affinity of the antibody for them.

Immunoblotting experiments with a monoclonal antibody to the Ad2 72K DBP (42) (Fig. 8) confirmed that the adenovirus 72K protein is cross-linked to poly(A)$^+$ RNA. Subsequent immunoblotting experiments demonstrated that

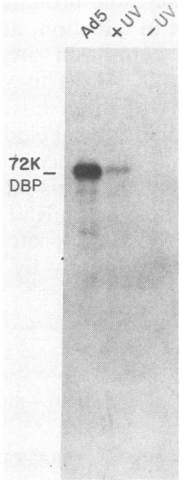

FIG. 8. Immunoblot analysis of proteins cross-linked to poly(A)$^+$ RNA by using a monoclonal antibody to the 72K DBP. UV cross-linked poly(A)$^+$ RNPs were prepared as described in the legend to Fig. 2, and immunoblotting was done with a monoclonal culture supernatant from a hybridoma-producing antibody to the 72K DBP of Ad2. Small amounts of the 72K protein can be cross-linked to RNA in the presence (+UV) but not the absence (−UV) of UV irradiation.

the observed cross-linking was to nuclear poly(A)$^+$ RNA (data not shown). The amount of the DBP which was cross-linked is rather small, and under our labeling and cross-linking conditions it could not be detected by labeling with [^{35}S]methionine. The observed cross-linking is not a consequence of cross-linking to DNA, because the nuclear material was extensively digested with RNase-free DNase I before oligo(dT)-cellulose chromatography. Also, when RNase T2 was substituted for micrococcal nuclease in the RNase digestion step, the same 72K signal was observed. Preliminary experiments suggest that, unlike the 100K protein, the 72K DBP is cross-linked to RNA in wt Ad5-infected monkey CV-1 cells.

DISCUSSION

The major findings of this work are that the 100K nonvirion protein (product of L4) and the 72K DBP (product of E2A) of Ad5 are associated with mRNA and with hnRNA, respectively, in the infected cell in vivo. These two proteins along with several host proteins make up the RNP particles within the cell late in infection.

Previous studies have examined the RNP complexes in adenovirus-infected cells. Lindberg and Sundquist (31, 50) and Tasseron-de Jong et al. (51) have shown that the adenovirus 100K polypeptide is associated with polysomal virus-specific RNPs from infected cells late in infection. This association resembles that of the major host cell mRNA-associated polypeptides (74K and 48K) in its resistance to dissociation by high salt (31, 50, 51, 54). A major shortcoming of the methods used in these studies is that the possibility of nonspecific association of 100K with RNPs during cell fractionation could not be ruled out. Furthermore, the 100K protein in RNPs isolated in this manner may be associated not directly with the RNA but through protein-protein interactions. The direct association of proteins with RNA in intact cells can be identified by photochemical RNA-protein cross-linking (13, 15–17, 21, 34, 35, 56–58).

UV cross-linking of protein to RNA in intact cells has recently been used by van Venrooij and colleagues (56, 57) to study RNPs in Ad2-infected cells. However, in contrast to the earlier studies cited above (31, 50, 51, 54), no adenovirus proteins, including the 100K, were found to be associated either with mRNPs or hnRNPs. To identify the RNA-contacting proteins, van Venrooij and colleagues have labeled the RNA with tritiated nucleosides before the UV cross-linking and relied on the label in the RNase-resistant cross-linked nucleotide to tag the proteins (57). Exclusively host proteins were seen cross-linked to both the total mRNA population and to hybridization-selected adenoviral RNA sequences (57). None of the previous studies has examined the fate of host cell mRNPs during adenovirus infection.

In the work described here, we have used UV cross-linking with [^{35}S]methionine labeling of proteins, [^{3}H]ribonucleoside labeling of the RNA, and immunoblotting and have demonstrated that the Ad5 100K protein and the 72K DBP are associated with polyadenylated cytoplasmic and nuclear RNA, respectively. ^{3}H-labeling of the RNA before cross-linking suggests that the 100K protein is associated with both host mRNA and viral mRNA in the infected cell. The late viral mRNAs are also associated with host cell RNP proteins, and preexisting host mRNAs remain associated with host cell RNP proteins even late in the infection, suggesting that the infection does not cause a general dissociation of host mRNPs. These results are in conflict with a previous report which suggested that only host cell proteins were in contact with viral mRNA (57). In our study, with complete infection, the 100K protein labeling with the ^{3}H-ribonucleosides was consistently observed. The efficiency of infection is a possible explanation for the discrepancy between our results and those of van Venrooij and colleagues (57). The amount of 100K protein which is cross-linked to mRNA is similar to if not greater than the amount of the major host mRNA-associated proteins such as the 72K poly(A)-binding protein. However, UV cross-linking and selective digestion of the mRNA with pancreatic RNase and RNase T_1 suggest that the 100K protein is not associated with the polyadenylate portion of the mRNA (S. Adam and G. Dreyfuss, unpublished results). The protein can be easily detected in our RNP preparations by Coomassie blue staining of the gels. We estimate that this is approximately 1% of the total 100K proteins present in the cell. The specificity of the cross-linking of the 100K to RNA is supported by the observation that other abundant Ad5 proteins do not become cross-linked to the RNA in these cells. Furthermore, in abortive infection of monkey CV-1 cells, the 100K protein is not cross-linked to RNA, even though normal amounts of the protein are present. When the same cells are infected with a mutant virus, *hr*404, which overcomes the host range restriction to productive infection, the 100K protein is cross-linked to mRNA in similar amounts to those seen in wt Ad5 infection of HeLa cells. Since the 100K protein is aberrantly localized to the nucleus in wt Ad5-infected monkey cells and the protein can therefore no longer be cross-linked to mRNA, the protein-mRNA association may be important for productive infection. In addition, the fact that it does not cross-link to nuclear RNA although it is a very abundant nuclear protein during late infection of CV-1 with wt Ad5 underscores the specificity of the interaction of the 100K protein with mRNA.

Several adenovirus mutants which fail to correctly form hexon trimers have been mapped to the gene for the 100K protein (23, 29, 45, 49, 53). Cepko and Sharp (10, 11) have shown that in abortively infected monkey CV-1 cells, hexon trimer formation is normal. Together with the finding that the 100K protein is not cross-linked to mRNA in abortively infected monkey CV-1 cells, this suggests that the cross-linkable interaction of the 100K protein with mRNA is a manifestation of a function of this protein in a process other than hexon morphogenesis (10, 12). At least one mutant in the 100K was found, H5*ts*18 (45), which displays a different phenotype from other 100K mutants in that it has no effect on hexon transport, although virus particles do not form. This mutant, unlike all the other 100K mutants, is defective in the induction of interferon at restrictive temperature (53).

The late phase of adenovirus infection of human cells is characterized by a dramatic switch in the pattern of protein synthesis in the cell that begins at about the same time host cell DNA replication is inhibited (3, 7, 8, 24, 39). This rapid switch in protein synthesis cannot be explained by simple competition between the accumulating viral transcripts and the host cell mRNA for the translational apparatus (39), for although viral mRNA levels increase rapidly during late infection until nearly all of the mRNA entering polysomes is of viral origin (30), host mRNA is not preferentially degraded in adenovirus-infected cells and persists throughout the infection (39). Control of translation late in infection appears to involve at least two distinct mechanisms. Adenovirus VAI RNA is necessary for translation in general in late infection as it prevents inactivation of the function of eucaryotic initiation factor 2 (46, 47) but cannot itself be responsible for the shutoff of host protein synthesis (52). The mechanism by

which preferential inhibition of host cell translation is achieved is not known. Late-region-specific mRNAs containing the adenovirus tripartite leader sequence initiate translation severalfold more efficiently than mRNAs lacking this sequence late after infection (32), but this alone does not explain the observed rapid shutoff of host translation. We demonstrate here that the adenovirus 100K protein is associated with both host and adenovirus mRNA late in infection. Therefore, the 100K protein is a likely candidate for a viral gene product which might be involved in the inhibition of host translation in adenovirus-infected cells. This could be accomplished if, in binding to all the mRNA present in the cell, the 100K protein decreases the translational efficiency of all mRNA. The increased translational efficiency of the tripartite leader sequence containing late adenovirus mRNAs however, would overcome this decrease with the net effect that only viral mRNAs would be translated. It is interesting that the 100K protein is the first viral late protein to be translated at the onset of the late phase of infection (6). We have previously identified a similar viral mRNA-protein complex from vesicular stomatitis virus-infected cells by UV cross-linking, consisting of the viral N protein and viral mRNAs (1). Rosen et al. (43, 44) have demonstrated that this viral mRNP inhibits translation in vitro and may be involved in the inhibition of host protein synthesis in the infected cell. Similarly, the 100K-mRNA complex may be involved in the inhibition of protein synthesis during adenovirus infection. Viral protein-mRNA complexes should provide interesting insights into the mechanisms by which viruses interact with the host cell gene expression apparatus and which may modify cellular function.

We also demonstrate here that the E2a gene-encoded 72K DBP is associated with nuclear poly(A)$^+$ RNA late in infection. The amount of DBP which is cross-linked to RNA is much less than that of the 100K protein, and it can be detected only by immunoblotting. The adenovirus DBP is a multifunctional protein which is involved in diverse processes including DNA replication and mRNA metabolism (4, 27, 55). The observations described here demonstrate that in addition to its interactions with DNA, this protein also interacts with RNA in the infected cell. Although the functional significance of this interaction is not presently known, it is interesting that mutants in the 72K DBP allow expression of the adenovirus late genes in monkey cells (27). Preliminary experiments suggest that the 72K DBP is cross-linked to RNA in wt Ad5-infected monkey cells. We have not been able to detect any physical associations between the 72K DBP and the 100K proteins to explain the apparent relationship between them suggested by the experiments in abortively infected cells. While this manuscript was in preparation, a report by Cleghorn and Klessig (14) was published which also presents evidence that the Ad5 72K DBP is an RNA-binding protein.

Finally, the immunological approach which we have described here for the identification of the proteins which are cross-linked to polyadenylated RNA in Ad5-infected cells should be generally useful for studying nucleic acid-binding proteins.

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