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Correlation of RNA Binding Affinity of Avian Oncornavirus p19 Proteins with the Extent of Processing of Virus Genome RNA in Cells

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We purified the p19 proteins from the Prague C strain of Rous sarcoma virus, avian myeloblastosis virus, B77 sarcoma virus, myeloblastosis-associated virus-2(0), and PR-E 95-C virus and measured their binding affinities for 60S viral RNA by the nitrocellulose filter binding technique. The apparent association constants of the p19 proteins from Rous sarcoma virus Prague C, avian myeloblastosis virus, and B77 sarcoma virus for homologous and heterologous 60S RNAs were similar (1.5×10^{11} to 2.6×10^{11} liters/mol), whereas those of myeloblastosis-associated virus-2(0) and PR-E 95-C virus were 10-fold lower. The sizes and relative amounts of the virus-specific polyadenylic acid-containing RNAs in the cytoplasms of cells infected with Rous sarcoma virus Prague C, myeloblastosis-associated virus-2(0), and PR-E 95-C virus were determined by fractionating the RNAs on agarose gels containing methylmercury hydroxide, transferring them to diazobenzyloxymethyl paper and hybridizing them to a 70-nucleotide complementary DNA probe. In cells infected with Rous sarcoma virus Prague C we detected 3.4×10^6-, 1.9×10^6-, and 1.1×10^6-dalton RNAs, in PR-E 95-C virus-infected cells we detected 3.4×10^6-, 1.9×10^6- and 0.7×10^6-dalton RNAs, and in cells infected with myeloblastosis-associated virus-2(0) we detected 3×10^6- and 1.3×10^6-dalton RNAs. Each of these RNA species contained RNA sequences derived from the 5′ terminus of genome-length RNA, as evidenced by hybridization with the 5′ 70-nucleotide complementary DNA. The ratios of subgenomic mRNA's to genome-length RNAs in cells infected with myeloblastosis-associated virus-2(0) and PR-E 95-C virus were three- to five-fold higher than the ratio in cells infected with Rous sarcoma virus Prague C. These results suggest that more processing of viral RNA in infected cells is correlated with lower binding affinities of the p19 protein for viral RNA, and they are consistent with the hypothesis that the p19 protein controls processing of viral RNA in cells.

Several investigators have presented evidence that there are at least three viral mRNA's in the cytoplasm of avian oncornavirus-infected cells, which have sedimentation coefficients of 38S, 28S, and 22S (3, 5, 10, 32). Hybridizations of complementary DNA (cDNA) probes representing specific regions of the viral genome have shown that the 38S RNA represents the entire viral genome, the 28S RNA represents the envelope and sarcoma genes, and the 22S RNA represents the sarcoma gene. The first RNA serves as the mRNA for the group-specific antigens and the β chain of reverse transcriptase (7, 8, 14, 16, 17), the second RNA serves as the mRNA for the envelope glycoprotein (5, 18, 28, 30), and the third RNA may serve as the mRNA for the sarcoma protein (2, 19, 20). The 28S and 22S mRNA's contain at the 5′ end an RNA sequence derived from the 5′ terminus of 38S RNA (9, 15, 21, 32), suggesting that they arise by splicing of the 38S RNA.

The enzymatic mechanism for processing viral RNA in cells is not known. However, we have observed that purified preparations of RNase III cleave the 38S RNA of the Prague C strain of Rous sarcoma virus (RSV Pr-C) into fragments containing polyadenylic acid [poly(A)]; these fragments are similar in size to virus-specific RNAs isolated from infected cells. Moreover, one of the viral group-specific antigens, p19, binds to specific regions of 38S RNA and blocks cleavage of the RNA by purified RNase III (12). These results suggest that p19 may play a role in controlling in vivo processing by preventing cleavage of viral RNA by a RNase III-related splicing enzyme. To provide further support for

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this hypothesis, we examined two RNA tumor viruses which contain increased levels of the major envelope glycoprotein in their virions (29; M. Linial, personal communication). The PR-E 95-C virus has altered molecular weight forms of the p19 protein (24). In this paper we report that the p19 proteins purified from these two viruses have a decreased affinity for 60S viral RNA. Furthermore, the levels of the subgenomic mRNA's found in the cytoplasm of cells infected with these viruses are greater than the levels found in the RSV Pr-C-infected cells.

MATERIALS AND METHODS

Reagents. $^{32}P_i$, (carrier free), [^{3}H]uridine (46.9 Ci/mmol), [^{3}H]dCTP (25.6 Ci/mmol), [α-^{32}P]dCTP (33 Ci/mmol), and [^{3}H]dTTP (18.6 Ci/mmol) were purchased from New England Nuclear Corp. Nitrocellulose filters (type HA; 25 mm; pore size, 0.45 μm) were purchased from Millipore Corp., and glass fiber filters (type E) were purchased from Gelman Instrument Co. Enzo-bond paper was purchased from Enzo Biochemical Co. All other reagents were purchased as previously described (11).

Growth of viruses. Cloned RSV Pr-C and B77 sarcoma virus (subgroup C) were grown in chicken embryo fibroblasts, as described by Smith and Bernstein (25). The RNAs produced by these virions were more than 97% *a* class RNA, as analyzed by gel electrophoresis. Myeloblastosis-associated virus-2 (0) [MAV-2(0)], a nontransforming virus of subgroup B, was plaque purified and grown in chicken embryo fibroblasts as described by Smith et al. (26). An avian sarcoma virus (PR-E 95-C virus) derived by multiple recombination events between RSV Pr-C and Rous-associated virus-0 was a gift from Maxine Linial, Fred Hutchinson Cancer Research Center, Seattle, Wash. PR-E 95-C virus is a transforming virus of subgroup E and was grown in quail embryo fibroblasts. The virus titer in each case was 10^6 infectious units per ml in chronically infected cells.

Purification of viral proteins. RSV Pr-C p19 and p12, B77 sarcoma virus p19, avian myeloblastosis virus (AMV) p19, and *Escherichia coli* RNase III were purified as previously described (12). PR-E 95-C virus p19 and p12 and MAV-2(0) p19 and p12 were purified by the method of Green and Bolognesi (4). The PR-E 95-C virus produces two forms of p19, as identified by sodium dodecyl sulfate gel electrophoresis (24). Both forms migrate faster than RSV Pr-C or Rous-associated virus-0 p19 on these gels. The two p19 proteins were separated from one another by chromatography on phosphocellulose p11 (column volume, 2 ml) equilibrated with a solution containing 20 mM morpholineethanesulfonic acid buffer (pH 6.6), 0.2% Triton X-100, and 0.1 mM EDTA (buffer A). The p19 proteins were eluted from the column with a 16-ml linear gradient of 0 to 0.5 M NaCl in buffer A and were detected by retention of ^{32}P-labeled 60S B77 RNA to nitrocellulose filters, as described below. Two peaks of RNA-binding activity were eluted from the column at 0.17 and 0.27 M NaCl. The p19 protein eluting at 0.17 M NaCl contained 70% of the total protein and was used in the experiments described below. These protein fractions were indistinguishable by sodium dodecyl sulfate gel electrophoresis at pH 8.8; they both yielded a single polypeptide band. Also, the binding affinity of each separated protein for 60S PR-E 95-C virus RNA was found to be 1.8×10^{10} liters/mol.

Preparation of labeled RNAs. [^{3}H]uridine-labeled PR-E 95-C virus 60S RNA (140 cpm/pmol of nucleotide) and ^{32}P-labeled MAV-2(0) 60S RNA (454 cpm/pmol of nucleotide) were prepared by the method of Smith et al. (27). ^{32}P-labeled 60S B77 RNA (1,296 cpm/pmol of nucleotide) was a gift from A. Faras, University of Minnesota Medical School.

Preparation of virus-specific cDNA probes. DNA complementary to viral RNA was prepared as described by Leis et al. (13), using 60S RSV Pr-C RNA as the template and oligodeoxythymidylic acid$_{12-14}$ [oligo(dT)$_{12-14}$] as the primer. Incubation was for 16 h at 37°C. The reactions were stopped by adding a twofold excess of EDTA to magnesium ions, and the mixtures were made 0.1% in sodium dodecyl sulfate, extracted with phenol, and dialyzed overnight at 4°C against 500 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The solution was brought to 0.3 M NaOH and incubated for 3 h at 30°C to hydrolyze RNA. The alkali was neutralized with hydrochloric acid, and the DNA was dialyzed against 500 ml of 0.3 M NaCl–20 mM Tris-hydrochloride (pH 7.5) overnight at 4°C. These DNA preparations were more than 95% single stranded, as determined by digestion by nuclease S1. The ^{32}P-labeled 70-nucleotide cDNA probe (cDNA$_{70}$) was prepared as described by Leis et al. (13), using RSV Pr-C 38S RNA and tRNA$_{trp}$ as the template and primer, respectively. After alkaline hydrolysis to remove RNA, the cDNA was fractionated by electrophoresis on 1.6% low-melting-point agarose gels calibrated with *Hae*III-digested simian virus 40 DNA. Bands of DNA were located by autoradiography of the wet gel, excised, and dissolved at 65°C in the buffer used for two-phase hybridization (31).

Preparation of cytoplasmic poly(A)-containing RNA. Poly(A)-containing RNA was isolated from RSV Pr-C- or MAV-2(0)-infected chicken embryo fibroblasts, uninfected chicken embryo fibroblasts, and PR-E 95-C virus-infected quail embryo fibroblasts essentially as described by Krzyzek et al. (9). Virus-infected primary cells were harvested from three roller bottles containing 8×10^8 cells and suspended in a solution containing 0.01 M Tris-hydrochloride (pH 8.0), 0.01 M NaCl, 0.015 M $MgCl_2$, and 0.01 M *N*-ethylmaleimide. Cells were homogenized, and the degree of cell breakage was monitored with a phase-contrast microscope. Nuclei and cell debris were removed by successive centrifugations in a Sorvall SS34 rotor at $700 \times g$ for 4 min and $3,000 \times g$ for 4 min at 4°C. The supernatant fraction was deproteinized four times with an equal volume of phenol at 50°C, and the RNA was precipitated with 2 volumes of ethanol. The RNA was dissolved in 2 to 3 ml of a solution containing 0.4 M NaCl, 2 mM EDTA, 10 mM Tris-hydrochloride (pH 7.5), and 0.5% sodium dodecyl sulfate, heated to 80°C for 2 min, rapidly cooled, and chromatographed on a 10-ml oligo(dT)-cellulose column at room tem-

perature, which was equilibrated with the above-described buffer. The poly(A)-containing RNA which eluted from the column with 0.5% sodium dodecyl sulfate–10 mM Tris-hydrochloride (pH 7.5)–10 mM NaCl was detected by absorbancy at 260 nm. This poly(A)-containing RNA was brought to a final NaCl concentration of 0.4 M, heated to 80°C for 2 min, cooled rapidly, and again fractionated on oligo(dT)-cellulose, as described above. The poly(A)-containing RNA recovered from the second column usually represented between 1 and 2% of the total input absorbance at 260 nm. The RNA was adjusted to 0.1 M NaCl and precipitated with 2 volumes of ethanol. ^{32}P-labeled 38S RSV RNA added to the cells exogenously could be recovered intact after the above-described procedure.

Fractionation of poly(A)-containing RNA by electrophoresis. The poly(A)-containing RNA was fractionated by electrophoresis through 1% agarose horizontal slab gels (30 by 16.5 by 0.3 cm) containing 5 mM methylmercury hydroxide (1). Electrophoresis was at 150 V for 12 to 14 h at 4°C. The electrophoresis buffer was circulated during the run. Unlabeled HeLa cell rRNA's were included as molecular weight markers and were located by excising the marker tracks and staining with ethidium bromide (1 μg/ml) in 0.5 M ammonium acetate.

Transfer of RNA to DBM paper. Poly(A)-containing RNA was transferred from the agarose gels to diazobenzyloxymethyl (DBM) paper (Enzo-bond), as described by Wahl et al. (31). RNA was fragmented as described by Wahl et al. (31) to facilitate transfer to the DBM paper.

Two-phase hybridization. The [^{32}P]DNA probes were hybridized to RNA covalently bound to DBM paper as described by Wahl et al. (31). The $cDNA_{70}$ probe, which was isolated by agarose gel electrophoresis, was used without further purification by melting the agarose gel fraction into the hybridization buffer at 65°C. The $cDNA_{70}$ probe was 80-fold in excess of the virus RNA sequences on the DBM paper. For sequential hybridization, the first DNA probe was removed from the paper by washing with 99% formamide–10 mM Tris-hydrochloride (pH 7.5) for 20 min at 65°C. Single-phase hybridizations were carried out as previously described (13).

Nitrocellulose filter assay for measuring binding of RNA to proteins. The nitrocellulose filter binding assay was as previously described (12). The apparent association constants were calculated by plotting the ratio of the amount of bound RNA to the amount of free RNA against the amount of bound RNA, using the method of Scatchard (22), as modified by Hizi et al. (6). The association constants were calculated from the negative slopes of the lines in these plots and are expressed in liters per mole.

RESULTS

Two of the group-specific antigens of RSV p12 and p19, bind viral RNA (23). However, they differ in their binding specificity; p12 binds nonselectively to all polyanions, whereas p19 binds preferentially to regions of RNAs that contain RNase III-sensitive sites (12). Furthermore, RSV p19 is able to protect 38S RSV RNA from cleavage by RNase III in vitro, whereas p12 cannot. The specific RNA-binding properties of p19 have led us to postulate that this protein may affect the amount of processing of genome-length (38S) RNA into the smaller mRNA's which code for the envelope glycoprotein and the sarcoma protein in infected cells. To provide further support for this hypothesis, we purified p19 proteins from three avian oncornaviruses, RSV Pr-C, B77 sarcoma virus, and AMV, which have similar levels of envelope glycoprotein in their virions, as well as two avian oncornaviruses, MAV-2(0) and PR-E 95-C virus, which have higher levels of envelope glycoprotein in their virions relative to levels of the group-specific antigens. We measured the binding affinity of these p19 proteins for 60S viral RNA by using the nitrocellulose filter binding assay (6). The retention of ^{32}P-labeled 60S B77 sarcoma virus RNA to nitrocellulose filters by MAV-2(0) and RSV Pr-C p19's is shown in Fig. 1. These data were replotted by the method of Scatchard (22) (Fig. 2). The apparent association constant of RSV Pr-C p19 for 60S B77 RNA was 2.6×10^{11} liters/mol, whereas the apparent association constant of MAV-2(0) p19 was 10-fold lower (Table 1). By using the same technique, the apparent association constants for the p19's of B77 sarcoma virus, AMV, and PR-E 95-C virus were calculated (Table 1). The binding affinities of B77, AMV, and RSV Pr-C p19 proteins for 60S B77 RNA were very similar. Thus, there does not appear to be any detectable difference in the binding affinities of the p19 proteins of different viruses for 60S B77 RNA, as measured by this technique. These results are in agree-

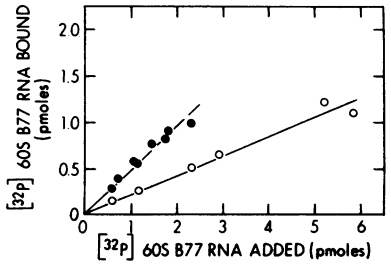

FIG. 1. *Binding of ^{32}P-labeled B77 RNA to RSV Pr-C and MAV-2(0) p19 proteins. Increasing amounts of ^{32}P-labeled 60S RNA (1,272 cpm/pmol of nucleotide) were incubated with either 2 μg of RSV Pr-C p19 or 0.8 μg of MAV-2(0) p19, as described in the text. After 15 min at 0°C, the reaction mixture was diluted with 1 ml of 10 mM Tris-hydrochloride (pH 7.5)–10 mM NaCl and passed through a nitrocellulose filter. Symbols: ●, binding of 60S B77 RNA to RSV Pr-C p19; ○, binding of 60S B77 RNA to MAV-2(0) p19.*

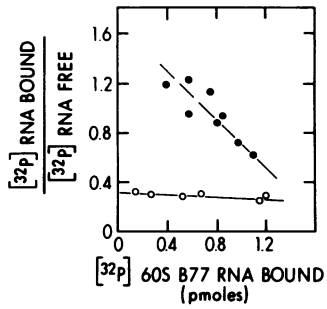

FIG. 2. *Scatchard plot of the data presented in Fig. 1. The amount of free 60S RNA was calculated by subtracting the amount of bound 60S RNA from the total 60S RNA. Symbols: ●, binding of 60S B77 RNA to RSV Pr-C p19; ○, binding of 60S B77 RNA to MAV-2(0) p19.*

TABLE 1. *Association constants of viral proteins for 60S B77 RNA*

Protein	Association constant (liters/mol)[a]
RSV Pr-C p19	2.6×10^{11}
B77 sarcoma virus p19	2.0×10^{11}
AMV p19	1.5×10^{11}
PR-E 95-C virus p19	1.9×10^{10}
MAV-2(0) p19	2.5×10^{10}
RSV Pr-C p12	1.2×10^{11}
PR-E 95-C virus p12	2.1×10^{11}
E. coli RNase III	4.5×10^{10}

[a] These values were calculated from Scatchard plots and represent the amount of binding of 60S B77 RNA (1,272 cpm/pmol of nucleotide) to RSV Pr-C p19 (1.9 μg), B77 sarcoma virus p19 (1.1 μg), AMV p19 (1.3 μg), PR-E 95-C virus p19 (0.1 μg), MAV-2(0) p19 (0.18 μg), RSV Pr-C p12 (0.8 μg), PR-E 95-C virus p12 (0.1 μg), and *E. coli* RNase III (0.85 U) as described in the text.

ment with results found previously with 60S RSV Pr-C RNA (12). In contrast, the apparent association constants of PR-E 95-C virus and MAV-2(0) p19 proteins for 60S B77 RNA were similar to each other, but 10-fold lower than those of the above-described proteins (Table 1). The PR-E 95-C virus protein used was the 0.17 M eluant protein from the phosphocellulose column.

Binding of PR-E 95-C virus p19 to PR-E 95-C virus 60S RNA. To rule out the possibility that the lower binding affinities of MAV-2(0) and PR-E 95-C virus p19 proteins for 60S B77 RNA were related to virus-specific nucleotide sequences, we repeated the binding experiments with 60S PR-E 95-C virus RNA and 60S MAV-2(0) RNA. Figure 3 shows the retention of ^{3}H-labeled 60S PR-E 95-C virus RNA to nitrocellulose filters in the presence of PR-E 95-C virus p19 and RSV Pr-C p19. Since the specific activity of the ^{3}H-labeled PR-E 95-C virus RNA was 1/10 that of the ^{32}P-labeled B77 RNA used in the experiment described above (Fig. 1), the amount of RNA bound with increasing amounts of input RNA reached a plateau with both proteins. The Scatchard plots of the data in Fig. 3 are shown in Fig. 4, and the calculated apparent association constants are summarized in Table 2. The binding affinity of PR-E 95-C virus p19 for homologous RNA was again 10-fold lower than the binding affinity of the heterologous RSV protein for PR-E 95-C virus 60S RNA. A similar relationship was found for the binding of MAV-2(0) p19 and RSV Pr-C p19 for 60S MAV-2(0) RNA (Table 2). These results indicate that the lower binding affinities of PR-E 95-C virus p19 and MAV-2(0) p19 for RNA are properties of these proteins and not of the RNA used.

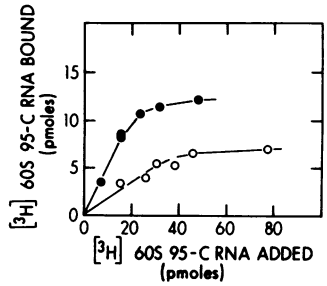

FIG. 3. *Binding of ^{3}H-labeled PR-E 95-C virus 60S RNA to PR-E 95-C virus and RSV Pr-C p19 proteins. Increasing amounts of ^{3}H-labeled PR-E 95-C virus 60S RNA (140 cpm/pmol of nucleotide) were incubated with either 2 μg of RSV Pr-C p19 or 0.1 μg of PR-E 95-C virus p19, as described in the legend to Fig. 1. Symbols: ●, binding of 60S RNA to RSV Pr-C p19; ○, binding of 60S RNA to PR-E 95-C virus p19.*

Binding of other proteins to 60S RNA. The p12 protein is the other group-specific antigen which binds to RNA. Therefore, we measured the binding affinities of the PR-E 95-C virus and MAV-2(0) p12 proteins for viral RNA to determine whether their association constants were also lower. We found that the apparent association constant of PR-E 95-C virus p12 for 60S B77 RNA was 2×10^{11} liters/mol, the same as the binding affinity of RSV Pr-C p12 for B77 RNA (Table 1). The binding affinity of MAV-2(0) p12 for 60S MAV-2(0) RNA was similar (1.1×10^{11} liters/mol) (Table 2). The binding affinity of feline leukemia virus p10 for 60S RSV RNA was 1.3×10^{11} liters/mol (12). Thus, we did not detect differences in the binding affinities of the p12 proteins from these viruses. In a separate set of experiments, the apparent association constant of *E. coli* RNase III for 60S B77 RNA was determined to be 4.5×10^{10} liters/mol.

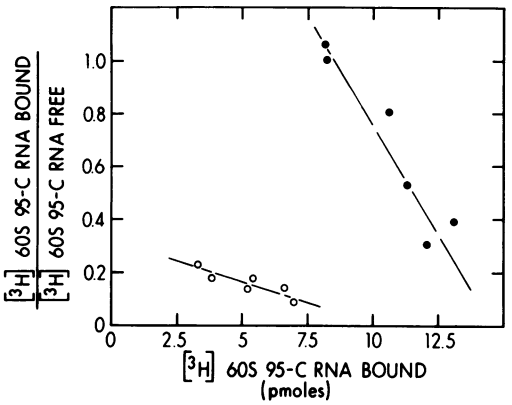

FIG. 4. *Scatchard plot of the data presented in Fig. 3. Symbols: ●, binding of 60S PR-E 95-C virus RNA to RSV Pr-C p19; ○, binding of 60S PR-E 95-C virus RNA to PR-E 95-C virus p19.*

TABLE 2. *Association constants of viral proteins for PR-E 95-C virus and MAV-2(0) 60S RNAs*

Protein	60S RNA	Association constant (liters/mol)[a]
PR-E 95-C virus p19	PR-E 95-C virus	1.8×10^{10}
RSV Pr-C p19	PR-E 95-C virus	1.1×10^{11}
MAV-2(0) p19	MAV-2(0)	4.1×10^{10}
RSV Pr-C p19	MAV-2(0)	2.4×10^{11}
MAV-2(0) p12	MAV-2(0)	1.1×10^{11}

[a] The values were calculated from Scatchard plots and represent the amount of binding of ^{3}H-labeled PR-E 95-C virus RNA (140 cpm/pmol of nucleotide) and ^{3}H-labeled MAV-2(0) 60S RNA (454 cpm/pmol of nucleotide) to PR-E 95-C virus p19 (0.1 μg), RSV Pr-C p19 (1.9 μg), MAV-2(0) p19 (2.1 μg), and MAV-2(0) p12 (0.3 μg), as described in Table 1, footnote *a*.

Size of virus-specific RNAs isolated from the cytoplasm of infected cells. The data presented above show that the p19 proteins purified from MAV-2(0) and PR-E 95-C virus have lower binding affinities for viral RNA than B77 sarcoma virus, RSV, and AMV p19 proteins. If p19 suppresses the cleavage of the 38S RNA into subgenomic mRNA species in cells, the ratio of smaller mRNA species to genome-length RNA should be greater in MAV-2(0)- and PR-E 95-C virus-infected cells than in RSV Pr-C-infected cells. We identified the viral RNA species in RSV Pr-C-, MAV-2(0)-, and PR-E 95-C virus-infected cells by fractionating the cytoplasmic poly(A)-containing RNA on agarose gels containing methylmercury hydroxide. After transfer onto DBM paper, the virus-specific RNAs were detected by hybridization to oligo(dT)-primed RSV Pr-C cDNA probes, as described above. The sizes of the viral RNAs were estimated by plotting relative migration distances against logs of molecular weights, using 28S and 18S HeLa cell rRNA's as markers. As Lee et al. (10) found, there were three RNA species in RSV Pr-C-infected cells; the molecular weights of these RNAs were of 3.4×10^6, 1.9×10^6, and 1.1×10^6 (data not shown). When preparations of RSV Pr-C contained transformation-defective virus, two additional RNA species (molecular weights, 3.0×10^6 and 1.3×10^6) were found. The RNAs isolated from MAV-2(0)- and PR-E 95-C virus-infected cells are shown in Fig. 5. MAV-2(0)-infected cells contained two RNA species, which

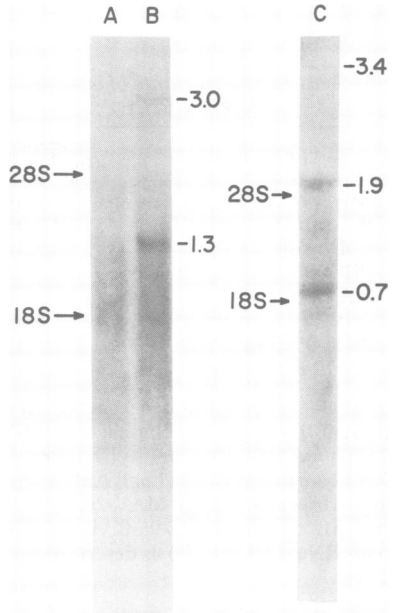

FIG. 5. *Sizes of virus-specific poly(A)-containing RNAs from the cytoplasm of infected cells. RNAs were isolated from the cytoplasm of uninfected and MAV-2(0)-infected chicken embryo fibroblasts and PR-E 95-C virus-infected quail embryo fibroblasts, fractionated on 1% agarose gels containing 5 mM methylmercury hydroxide, and transferred to DBM paper as described in the text; 10 nmol of each RNA was applied to the agarose gels. Virus-specific RNA was detected by incubating the paper with 2×10^6 cpm of oligo(dT)-primed RSV Pr-C cDNA for 48 h at 65°C, washed as described by Wahl et al. (31), dried, and subjected to autoradiography with Kodak X-OMat X-ray film, using Lightning-Plus screens at −70°C for 7 days. The PR-E 95-C virus RNA was fractionated on a separate agarose gel. The arrows indicated the positions of the 28S and 18S HeLa cell rRNA's. Lane A, Cytoplasmic RNA from uninfected chicken embryo fibroblasts; lane B, cytoplasmic RNA from MAV-2(0)-infected chicken embryo fibroblasts; lane C, cytoplasmic RNA from PR-E 95-C virus-infected quail embryo fibroblasts.*

had molecular weights of 3.0×10^6 and 1.3×10^6 (Fig. 5, lane B). MAV-2(0) is a nontransforming virus with a *b* type RNA genome (26). The larger RNA species represents genome-length RNA, whereas the smaller species represents envelope mRNA (see below). None of these RNA species was detected in uninfected cells (Fig. 5, lane A). In PR-E 95-C virus-infected cells, we detected three RNA species, which had molecular weights of 3.4×10^6, 1.9×10^6, and 0.7×10^6 (Fig. 5, lane C). Since PR-E 95-C virus was derived from RSV Pr-C, we assumed that the three RNA species corresponded to the RSV PR-C RNAs of comparable sizes. For clarity, Table 3 shows the different virus RNAs detected and their probable coding capabilities.

Amounts of virus-specific RNAs isolated from the cytoplasm of infected cells. The amount of each RNA species detected on DBM paper was measured by hybridization to the $cDNA_{70}$ probe, which represented the 70-nucleotide sequence from the $tRNA_{trp}$ DNA initiation site to within 30 nucleotides of the 5′ terminus of RSV Pr-C RNA. This cDNA is complementary to part of the spliced 5′-terminal sequence and is present only once in each mRNA species (9). Therefore, the amount of radioactivity hybridized to each RNA species should reflect the amount of the RNA present. The RSV Pr-C $cDNA_{70}$ probe used in these experiments was 3.4 and 94% resistant to nuclease S1 after hybridization in the absence and presence of excess 60S RSV Pr-C RNA, respectively. In a separate experiment, 40% of the RSV Pr-C $cDNA_{70}$ probe was found to be resistant to nuclease S1 after hybridizing to 60S MAV-2(0) RNA. When this experiment was repeated with $cDNA_{100}$, which contained the $cDNA_{70}$ sequence plus the 30 nucleotides at the 5′ terminus of 38S RSV Pr-C RNA, 51% of the DNA probe was resistant to nuclease S1. These results indicate that MAV-2(0) and RSV Pr-C share in part the same 5′ terminal sequence; 65% of the $cDNA_{70}$ probe was resistant to nuclease S1 after hybridization to 60S PR-E 95-C virus RNA.

The $cDNA_{70}$ probe (in 80-fold excess) was hybridized to poly(A)-containing RNA coupled to DBM paper, as described above. The paper was washed thoroughly and cut into strips, and the radioactivity was measured with a liquid scintillation spectrometer. We detected the same

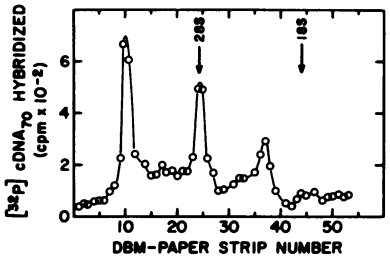

FIG. 6. *Hybridization of $cDNA_{70}$ to RSV Pr-C RNA from infected cells. Chicken embryo fibroblasts were infected with freshly cloned stock of RSV Pr-C, and the cytoplasmic poly(A)-containing RNA was isolated and fractionated as described in the legend to Fig. 5; 10 nmol of RNA was fractionated on the agarose gel. The amount of virus-specific RNA present on the DBM paper was determined by hybridizing to 1.2×10^6 cpm of the $cDNA_{70}$ probe (2,500 cpm/pmol) for 72 h at 65°C, washing, cutting into 2-mm strips, and counting in a liquid scintillation spectrometer.*

TABLE 3. *Amount of virus-specific RNA isolated from infected cells*

Mol wt of RNA ($\times 10^6$)	Virus	Coding capacity[a]	Relative abundance[b]			
			RSV Pr-C	RSV Pr-C and td RSV Pr-C[c]	PR-E 95-C virus	MAV-2(0)
3.4	RSV Pr-C PR-E 95-C	gag,pol,env,src	1	1	1	
3.0	td RSV Pr-C MAV-2(0)	gag,pol,env		1.1		1
1.9	RSV Pr-C PR-E 95-C	env,src	0.7	0.3	2.3	
1.3	td RSV Pr-C MAV-2(0)	env		0.3		2.1
1.1	RSV Pr-C	src	0.3	0.6		
0.7	PR-E 95-C	src			2.1	

[a] Abbreviations: gag, group-specific antigens; pol, RNA-dependent DNA polymerase; env, envelope glycoprotein; src, sarcoma gene product.

[b] The relative abundances of RNAs were calculated from the hybridization data presented in Fig. 6 through 8. The RSV Pr-C and PRE 95-C virus 3.4×10^6-dalton RNAs and the MAV-2(0) 3.0×10^6-dalton RNA were set equal to 1.

[c] td, Transformation defective.

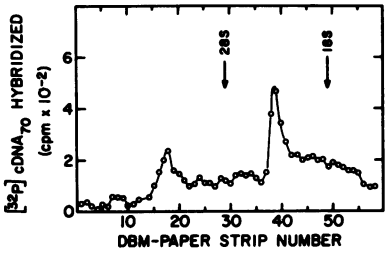

FIG. 7. *Hybridization of $cDNA_{70}$ to MAV-2(0) RNA from infected cells. The ^{32}P-labeled $cDNA_{70}$ probe (8×10^4 cpm; 1,000 cpm/pmol) was hybridized to the DBM paper shown in Fig. 5, lane B, for 72 h at 65°C, and the amount of radioactivity hybridized to viral RNA was determined as described in the legend to Fig. 6.*

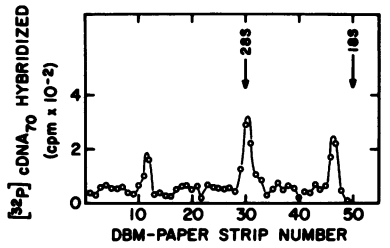

FIG. 8. *Hybridization of $cDNA_{70}$ to PR-E 95-C virus RNA from infected cells. The ^{32}P-labeled $cDNA_{70}$ (8×10^4 cpm; 1,000 cpm/pmol) was hybridized to the DBM paper shown in Fig. 5, lane C, for 72 h at 65°C, and the amount of radioactivity hybridized to viral RNA was determined as described in the legend to Fig. 6.*

size classes of viral RNAs with $cDNA_{70}$ and with oligo(dT)-primed cDNA (Fig. 6 through 8). Cells infected with a freshly cloned stock of RSV Pr-C which was free of transformation-defective virus contained three RNA species (Fig. 6). The 3.4×10^6-, 1.9×10^6-, and 1.1×10^6-dalton species were present in a ratio of 1:0.7:0.4 (Table 3). A similar analysis of the RNAs isolated from cells infected with RSV Pr-C containing transformation-defective virus yielded a ratio of 1:1.1:0.3:0.3:0.6 for the 3.4×10^6-, 3.0×10^6-, 1.9×10^6-, 1.3×10^6-, and 1.1×10^6-dalton RNA species (data not shown). In each case, the most abundant RNA species detected was genome-length RNA (Table 3).

In contrast, in cells infected with either PR-E 95-C virus or MAV-2(0), the genome-length RNA was the least abundant species. The ratio of the MAV-2(0)-specific RNAs was 1:2.1 for the 3.0×10^6- and 1.3×10^6-dalton species (Fig. 7). The ratio of the PR-E 95-C virus-specific RNAs was 1:2.3:2.1 for the 3.4×10^6-, 1.9×10^6-, and 0.7×10^6-dalton species (Fig. 8). These results are also summarized in Table 3. By comparing the amounts of envelope mRNA's and the amounts of sarcoma mRNA's to genome-length RNAs (Table 3), we found that the subgenomic mRNA's of MAV-2(0) and PR-E 95-C virus are three- to fivefold more abundant relative to genomic RNA than the subgenomic mRNA's from RSV Pr-C-infected cells. If we use the ratio of the distribution of RSV Pr-C RNAs containing the transformation-defective virus RNAs (see above), the MAV-2(0) and PR-E 95-C virus subgenomic mRNA's are four to eightfold more abundant than genome-length RNA, respectively.

DISCUSSION

There are three known pathways for the utilization of 38S RSV Pr-C RNA after transcription in cells: (i) it is packaged in virions as a 60S RNA complex; (ii) it is translated directly to express the group-specific antigens and the RNA-dependent DNA polymerase; and (iii) it is processed into 28S and 22S RNAs for translation of the envelope and the sarcoma gene products, respectively. The 38S, 28S, and 22S RNAs have molecular weights of 3.4×10^6, 1.9×10^6, and 1.1×10^6, respectively, and have been isolated from polysomes from infected cells (10). Hybridization studies with gene-specific probes have indicated that the 28S RNA represents the 3′ one-half of 38S RNA, whereas the 22S RNA represents the 3′ one-third of 38S RNA (5, 32). A unique feature of the processing of the 38S RNA into the 28S and 22S RNAs has been the finding that at least 100 nucleotides from the 5′ terminus of 38S RNA are spliced onto the smaller RNAs (8, 15, 21, 32).

The recognition that there are two pools of viral mRNA's in cells (unprocessed and processed RNAs) has raised a question as to whether there is a mechanism that controls the flow of RNA from the former to the latter. This type of control may be important for viruses, since genome-length 38S RNA is used both for progeny and for translation of some of the viral proteins.

We have proposed previously that the viral protein p19 controls the level of processing of 38S RNA into 28S and 22S RNAs in cells by binding to processing sites on 38S RNA and preventing their cleavage by the processing enzyme (12). The binding of p19 to the virus RNA may represent one of the first steps in virion assembly. The evidence which supports this hypothesis is as follows.

(i) RNase III cleaves 38S RNA at sites similar to the processing sites. We have found that *E. coli* RNase III cleaves 38S RSV Pr-C RNA in vitro into poly(A)-containing RNA species of 1.8×10^6 and 1.0×10^6 daltons, as analyzed on 1%

agarose gels containing methylmercury hydroxide (S. Johnson, unpublished data). In addition, RNase III cleaves 35S MAV-2(0) RNA (3.0×10^6 daltons) into poly(A)-containing RNA species of 1.05×10^6 and 0.86×10^6 daltons. The in vitro-produced RNA species are approximately 100,000 daltons (350 nucleotides) smaller than the virus-specific mRNA's detected in infected cells (Fig. 5 through 7), with the exception of the smallest MAV-2(0) RNA. Based on this size difference, we have speculated that RNase III recognizes splicing cleavage sites on viral RNA but probably does not catalyze the ligation reaction. RNase III has also been shown to cleave other viral RNAs. For instance, vesicular stomatitis virus 40S RNA is cleaved by RNase III into five RNA species which are similar in size to the known vesicular stomatitis virus mRNA's found in cells (33). RNase III also cleaves poliovirus RNA. The cleavage sites appear to be intercistronic, as determined by oligonucleotide mapping. An RNase III fragment that encodes the poliovirus polymerase has been isolated and translated in vitro to yield a polypeptide the size of the virus polymerase (M. Stewart, R. Crouch, and J. Maizel, Virology, in press). Apparently, *E. coli* RNase III recognizes some feature of viral RNAs (probably secondary structure) that is present in processing sites.

(ii) p19 blocks the cleavage of 38S RNA by RNase III (12). In agreement with the results described above, AMV p19 has been shown by direct observation in an electron microscope to ind to poliovirus RNA at sites to which RNase III binds (R. Lenk et al., personal communication).

(iii) RSV Pr-C p19 binds to a variety of unprocessed precursor RNAs with apparent association constants ranging from 10^9 to 10^{10} liters/mol, whereas it does not bind or binds weakly (apparent association constants, 10^7 liters/mol) to mature or processed RNAs (12).

(iv) p19 is the first protein to be translated in response to 38S RNA since it is at the amino terminus of the group-specific antigen precursor polypeptide.

To provide further support for a role for p19 in regulating processing of 38S viral RNA, we studied the RNA-binding properties of RSV Pr-C, MAV-2(0), and PR-E 95-C virus p19 proteins and analyzed the sizes and amounts of virus-specific poly(A)-containing mRNA's from the cytoplasm of cells infected with these viruses. The other viruses have higher levels of envelope glycoprotein in their virions than RSV Pr-C (24, 29). If p19 inhibits the processing of genome-length RNAs to smaller RNAs, we predict that (i) the p19 proteins from MAV-2(0) and PR-E 95-C virus have lower binding affinities for viral RNA than RSV Pr-C p19, (ii) there is no detectable difference in the binding affinities for 60S RNA by the p12 proteins from all three viruses, and (iii) the levels of all of the processed mRNA's are increased relative to genome-length RNA in MAV-2(0)- and PR-E 95-C virus-infected cells compared with RSV Pr-C-infected cells.

These predictions have been verified. We found that (i) the MAV-2(0) and the PR-E 95-C virus p19 proteins have 10-fold lower binding affinities for viral RNA than p19 purified from RSV Pr-C, (ii) there is no difference in the binding affinities for RNA of the p12 proteins from the different viruses, and (iii) in MAV-2(0)- and PR-E 95-C virus-infected cells, the level of processed mRNA's is increased relative to genome-length RNA compared with RSV Pr-C-infected cells.

We detected three viral RNAs in RSV Pr-C-infected cells; these RNAs had molecular weights of 3.4×10^6, 1.9×10^6, and 1.1×10^6, which was in agreement with the results of Lee et al. (10). In MAV-2(0)-infected cells we detected 3.0×10^6- and 1.3×10^6-dalton RNA species. The former is genome-length RNA, whereas the latter RNA is the envelope mRNA, based on the following evidence. Translation of the two RNAs in a low-background reticulocyte cell-free protein-synthesizing system followed by immunoprecipitation and sodium dodecyl sulfate gel electrophoresis indicated that the 3.0×10^6-dalton RNA gave rise to a 76,000-dalton polypeptide which was recognized by antiserum to the avian sarcoma virus group-specific antigens. The 1.3×10^6-dalton RNA gave rise to a 60,000-dalton polypeptide that was recognized by antisera raised against AMV envelope glycoprotein (Scheible, unpublished data). We detected 3.4×10^6-, 1.9×10^6-, and 0.7×10^6-dalton species in PR-E 95-C virus-infected cells. It is likely that these RNAs correspond to the RNAs found in RSV Pr-C-infected cells since (i) PR-E 95-C virus is derived from RSV Pr-C, (ii) it is a transforming virus, and (iii) the number and sizes of the RNA species found in PR-E 95-C virus-infected cells are similar to the number and sizes of the RSV Pr-C RNAs. Each of the subgenomic RNA species described above contains RNA sequences derived from the 5′ and 3′ termini of genome-length RNA.

We measured the concentration of mRNA species isolated from chronically infected cells by hybridization to a $cDNA_{70}$ probe, which was complementary to part of the spliced 5′ RNA sequence and was present at a level of only one copy per RNA. Thus, the amount of radioactive

probe hybridized should have been proportional to the number of poly(A)-containing RNA molecules present regardless of the size of the RNA. We have expressed these data as a ratio of the amounts of the different size classes of RNA to avoid problems in determining absolute amounts of mRNA's in cells. To determine the absolute amounts of mRNA's in infected cells, we would require information about the number of viable cells in culture, the number of particles produced per cell, the ratio of infectious to noninfectious virus particles, the recovery of RNA from infected cells, the number of virus-specific RNA molecules containing poly(A), and the number of proviral equivalents per cell. In the present work we examined the steady-state level of genomic and subgenomic virus mRNA's in chronically infected cells. This approach provided an estimate of the relative abundance of virus-specific mRNA's in cells chronically infected with three different viruses. We found three- to five-fold more subgenomic mRNA's than genome-length RNAs in MAV-2(0)- and PR-E 95-C virus-infected cells than in RSV Pr-C-infected cells. Therefore, it appears that there is enhanced processing of genome-length to subgenome-length mRNA's in MAV-2(0)- and PR-E 95-C virus-infected cells. This correlates with the existence of p19 proteins with lower binding affinities for viral RNA in these two strains. These results are consistent with the hypothesis that the p19 protein controls processing of viral RNA in cells. Further work along these lines will involve a correlation of the appearance of p19 with the appearance of subgenomic mRNA molecules early after infection of cells with different viruses.

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