RNA-Binding Properties of the Matrix Protein (p19^{gag}) of Avian Sarcoma and Leukemia Viruses

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We have reinvestigated the ability of the matrix protein (MA) (p19^{gag}) of avian sarcoma and leukemia viruses to interact with RNA. Previous reports claimed on the one hand that MA can bind tightly and with a high degree of specificity to avian sarcoma and leukemia virus RNA *in vitro* and on the other that it cannot bind to RNA at all. We found that MA purified by any of several methods does bind to RNA, as measured by its ability to cause retention of radioactive RNA on nitrocellulose membranes in a filtration assay. However, this interaction is weak and lacks specificity. The interaction of MA with RNA was barely detectable by classical sedimentation analysis, and from this observation we estimate that the intrinsic MA-RNA association constant is ca. 10^3 M^{-1} , at least 3 orders of magnitude smaller than the constant describing the interaction of the viral nucleocapsid protein (NC) (p12^{gag}) with RNA, ca. 10^6 M^{-1} . Separately purified phosphorylated and nonphosphorylated MA species bound RNA equally. We also found that MA can bind to DNA with an affinity similar to that for RNA. The large quantitative discrepancy between our results and earlier published reports can be traced in part to methods of data analysis.

In all retroviruses, the polypeptide encoded by the *gag* gene gives rise to the internal structural components of the virion. Expression of the *gag* gene itself, in the absence of functional proteins from the other two major genes, *pol* and *env*, can lead to the budding of viruslike particles from the membrane of the infected cell. During the final stages of virion assembly, the *gag* polypeptide is cleaved by the viral protease (PR) (for nomenclature conventions, see reference 12) into the several different structural proteins found in mature infectious particles (retrovirus assembly is reviewed in reference 36). The functions of three of these proteins have been elucidated, at least in part. The nucleocapsid protein (NC) is found tightly associated with the genomic RNA and probably coats the RNA completely. Certain mutations in NC prevent packaging of RNA into virions (18-20). *In vitro*, NC is reported to bind to RNA tightly but nonspecifically (6, 10, 15, 32). In all retroviruses, NC is derived from the ultimate or penultimate domain near the C terminus of the *gag* precursor polypeptide. The capsid protein (CA) is believed to form the regular structure occasionally seen by electron microscopy inside the retroviral membrane. CA is derived from the middle domain of the *gag* precursor. The membrane-associated, or matrix, protein (MA), which in all retroviruses is derived from the N-terminal domain of the *gag* precursor, lies directly underneath the inner face of the lipid envelope (2, 21, 23). In many retroviruses, but not in avian sarcoma and leukemia viruses (ASLV), MA bears a myristic acid moiety at its N terminus (9, 29). In two virus systems, mutations in MA that prevent myristylation have been engineered, and these mutations prevent budding of virus particles (26, 27). In addition to these proteins, PR is derived from the *gag* precursor in some retroviruses, such as ASLV.

In early studies on ASLV, MA (formerly called p19^{gag})

was reported to be associated with the genomic RNA in virus particles (30) and also to bind with high affinity to viral RNA *in vitro* (15, 16). The former conclusion is likely to be in error because of misidentification of the RNA-protein adduct that is formed after UV irradiation. In fact, the major *gag* protein that becomes cross-linked to RNA under UV light has been positively identified as NC by immunological criteria (17) and by peptide mapping (R. B. Pepinsky and V. M. Vogt, unpublished observations). The evidence for MA-RNA interaction *in vitro* was derived from classical nitrocellulose filter binding assays. Purified MA was incubated with radioactive RNA, the mixture was filtered, and the retention of radioactivity on nitrocellulose membranes was used to measure binding. Quantitative analyses of such studies, based on the method of Scatchard, led Leis et al. to claim that MA binds to ASLV genomic RNA with an apparent association constant of ca. 10^{11} to 10^{12} M^{-1} . Binding to rRNA was reported to be orders of magnitude weaker (15), thus leading to the suggestion that MA recognizes specific sequences or structural features of ASLV RNA. Leis et al. speculated that this preference of MA for its cognate RNA plays a role in control of viral RNA splicing (16). In marked contrast to these reports, other workers could not detect any binding of MA to RNA after transfer of the protein from a sodium dodecyl sulfate (SDS)-polyacrylamide gel onto a nitrocellulose membrane (3, 17), under conditions in which the NC-RNA interaction was readily apparent. Indeed, it was claimed that MA is not an RNA-binding protein at all (17).

If confirmed, the selective interaction of any ASLV protein with its cognate viral RNA would provide an important clue to help clarify aspects of the virus replication cycle. To this end and to resolve the discrepancies in the literature, we reinvestigated the ability of MA to bind to RNA. We found that MA purified by any of several methods is indeed capable of causing retention of RNA on nitrocellulose filters. However, the apparent association constant describing this interaction is many orders of magnitude smaller than previously calculated, and the interaction lacks specificity.

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MATERIALS AND METHODS

Purification of viral proteins. MA was purified from avian myeloblastosis virus (AMV), which was obtained as pellets from Life Sciences, Inc. Two basic methods were used. Method A consisted of detergent lysis of the virus followed by two chromatography steps on phosphocellulose (24). Approximately 100 mg (wet weight) of AMV was suspended in 250 μ l of ice-cold 20 mM Tris hydrochloride (pH 7.5)–1% *n*-octylglucoside (OG)–1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was frozen and thawed four times and then incubated on ice for 1 h with occasional mixing. All subsequent manipulations took place at 4°C. The virus solution was diluted 10-fold with 20 mM Tris hydrochloride (pH 7.5) and centrifuged for 10 min at $2,000 \times g$. The supernatant was loaded slowly onto a 4-ml phosphocellulose column (P11; Whatman, Inc.) equilibrated with PC buffer (20 mM Tris hydrochloride [pH 7.5]–5 mM dithiothreitol [DTT]–0.1% OG–0.5 mM PMSF). The flowthrough fraction was reloaded three times to maximize adsorption to the resin. After a 12-ml wash with the same buffer, salt steps of 12 ml each of 100 mM NaCl, 350 mM NaCl, and finally 1 M NaCl in the same buffer were applied. Fractions containing MA (as determined by SDS-polyacrylamide gel electrophoresis (PAGE) were pooled, dialyzed against PC buffer, and then passed through a 200- μ l phosphocellulose column. After a 1-ml wash, 1 ml of each of the same salt steps used previously was used for elution. In both columns, MA appeared in the 350 mM step.

Method B consisted of chloroform-methanol lysis of the virus (as described elsewhere [16]) followed by chromatography on two sequential cation-exchange columns. The best results were obtained with carboxymethyl-Sephadex (CM-Sephadex; Sigma Chemical Co.) followed by phosphocellulose, but in some preparations, two sequential CM-Sephadex columns were used. Approximately 200 mg of AMV was suspended on ice in 10 ml of 100 mM NaCl–1 mM PMSF. Exactly 21 ml of chloroform-methanol (2:1) was mixed in, and the sample was centrifuged for 1 h at $2,000 \times g$. The chloroform and the aqueous-methanol phases were removed, and the large white precipitate at the interface was dried with nitrogen. This material was extracted with 5 ml of 0.1 M NaPO₄ (pH 7.0)–1% 2-mercaptoethanol–1 mM PMSF for 2 h at 37°C with shaking. The suspension was centrifuged for 10 min at $10,000 \times g$, and the supernatant was removed. Four more cycles of extraction of the pellet and centrifugation were performed, the last two with 2.5 ml of buffer plus 0.2% OG (or in some experiments, Triton X-100). The supernatants were combined and slowly loaded onto a 13-ml CM-Sephadex column, usually in the presence of detergents. After the column was washed, steps of 0.1 and then 1.0 M NaCl in the extraction buffer plus 0.1% OG were applied. MA appeared in the 0.1 M step, while NC appeared in the 1.0 M step. The fractions containing MA were dialyzed against the PC buffer and then loaded onto a 3-ml phosphocellulose column and eluted by the steps described above.

Further chromatographic separation of the phosphorylated and nonphosphorylated forms of MA was accomplished either by DEAE-cellulose chromatography or by chromatography on a Mono-Q column (Pharmacia) with a fast protein liquid chromatography system (Pharmacia). For the DEAE method (24), 35 μ g of MA was dialyzed against 20 mM Tris hydrochloride (pH 7.5)–10 mM DTT–1 mM EDTA–0.05% OG. The dialyzed protein was loaded onto a 150- μ l DE 52 column (Whatman) equilibrated with the same buffer.

The column was washed with 800 μ l of loading buffer and then with 800 μ l of loading buffer plus 100 mM NaCl. Most of the nonphosphorylated species were eluted during the first wash, while the phosphorylated species (contaminated to ca. 25% with nonphosphorylated MA) were eluted during the salt step. For the Mono-Q method, 50 μ g of MA in 3 ml of 20 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–10 mM DTT–0.1% OG was loaded onto a Mono-Q column at room temperature, and elution was carried out with a 20-ml gradient to 250 mM NaCl in the same buffer. The peak of nonphosphorylated MA appeared at 35 mM NaCl. The peak of phosphorylated MA appeared at approximately 70 mM NaCl.

Some experiments also were done with MA purified by high-resolution gel filtration in 6 M guanidine hydrochloride as described elsewhere (34), and MA exhibited similar binding activity in these experiments. Earlier work by Leis and collaborators also used guanidine-purified MA for protein-RNA binding studies (15). NC was obtained either from the 1 M step in procedure A or B described above or else by gel filtration in guanidine. AMV reverse transcriptase (RT) was obtained from Life Sciences or from Boehringer Mannheim Biochemicals. The integration protein (IN) (pp32 or endonuclease) used as a standard for Western blots (immunoblots) was kindly provided by R. Katz.

RNA and DNA binding assays. Nitrocellulose filter binding assays were performed as described elsewhere (15). In brief, standard reaction mixtures (50 μ l) contained 10 mM Tris hydrochloride (pH 7.5), 1 mM DTT, 20 to 50 mM NaCl, several nanograms of radioactive RNA or DNA, and various concentrations of MA. After 20 min of incubation on ice, the reaction mixtures were diluted with 0.5 ml of ice-cold 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl and immediately filtered through 25-mm presoaked nitrocellulose filters (BA 85; Schleicher & Schuell, Inc.) on a large manifold with gentle suction (ca. 0.25 ml/min per filter). Equivalent binding occurred if the dilution step was omitted or if a wash step was added. Gel mobility assays for binding of protein to DNA were done in 10- μ l samples which contained 50 to 200 ng of DNA and from 50 ng to several micrograms of protein, usually at a final NaCl concentration of 140 mM, plus 8 mM Tris hydrochloride (pH 7.5)–0.4 mM EDTA–2 mM DTT–0.04% Triton X-100. After the samples were kept at room temperature for 20 min, 4 μ l of glycerol was added and the samples were electrophoresed on 1% agarose gels in Tris-acetate buffer.

In the binding assay based on sucrose gradient sedimentation, MA and NC were incubated for 20 min at room temperature with or without 40 μ g of the 6-kilobase (kb) tobacco mosaic virus (TMV) RNA in 200 μ l of 20 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–1 mM EDTA–1 mM DTT–20 μ g of bovine serum albumin per ml. The samples were then layered over a 3.8-ml sucrose gradient (5 to 20%) in the same buffer and centrifuged at 4°C for 3 h at 45,000 rpm in an SW60 rotor. Samples of the collected fractions were analyzed for RNA by determining A_{260} and for the presence of viral proteins by immunoblotting after SDS-PAGE with a mixture of antisera to MA and NC. The binding constant for MA and NC was estimated from the following formula (7): $KL = [(P_n/P_0)^{-1/n} - 1]^{-1}$, where K is the intrinsic association constant (expressed as the reciprocal of the molar concentration), L is the concentration of nucleic acid binding sites (expressed as molar concentration), P_0 is the initial concentration of protein in the loaded sample, and P_n is the concentration of protein in the n th fraction of the gradient, where the RNA peak is located. The

FIG. 1. SDS-PAGE of purified MA. Purified MA was electrophoresed on a 17.5% polyacrylamide gel and then stained with Coomassie blue or silver (22) or probed with a monoclonal antibody to MA. (A) Stain. Lane a, 0.5 μ g of MA purified by method A; lane b, total AMV starting material. (B) Stain. Lane c, Marker proteins; lane d, ca. 2 μ g of MA purified by method B; lane e, 1 μ g of nonphosphorylated MA from DEAE column; lane f, 1 μ g of phosphorylated MA from DEAE column; lane g, 100 ng of nonphosphorylated MA from Mono-Q column; lane h, 100 ng of phosphorylated MA from Mono-Q. Lanes a and b are from one gel, and lanes c to h are from a different gel. Lanes a to f were stained with Coomassie blue, and lanes g and h were stained with silver. (C) Immunoblot. Different preparations of MA were subjected to SDS-PAGE, electrophoretically blotted to nitrocellulose, and probed with anti-MA monoclonal antibody 2A7. The blot was developed with horseradish peroxidase-conjugated anti-mouse antibody. Lane i, 500 ng of a sample of MA obtained from J. Leis; lane j, 150 ng of MA from peak fraction of second CM-Sephadex column (variant of method B, with purification in presence of 0.01% Triton X-100); lane k, flowthrough fraction from first CM-Sephadex column (method B, in the presence of 0.01% Triton X-100) containing ca. 200 ng of MA and ca. 75 ng of viral CA; lane l, 1 μ g of MA from peak fraction of second CM-Sephadex column (method B, in the absence of detergent). Silver staining of the same quantity of MA as in lane d showed a pattern of bands nearly identical to that seen in this immunoblot, indicating that most of the stainable bands are MA related.

volume of each fraction is assumed to be 200 μ l, the same as that of the sample loaded onto the gradient. We assume that L is approximately equal to 6,000 times the molar concentration of the 6-kb TMV RNA, i.e., that there are approximately 6,000 overlapping binding sites on the RNA.

Other techniques. The P3-76 transcription plasmid was generated from plasmid p779/-273/#3 (33), which contains a 5' long terminal repeat and Rous sarcoma virus (Schmidt-Ruppin strain) sequences through about nucleotide 850 in the *gag* gene abutted to sequences from the 3' end of the *src* gene and continuing through the 3' long terminal repeat. When expressed in chicken cells, this plasmid generates an RNA that is fully competent to be packaged by a nondefective virus (33). The P3-76 plasmid was cleaved with *Eco*RI (which cuts in the U3 regions of both long terminal repeats), and then the fragment was inserted into the transcription vector pIBI76 (International Biotechnologies, Inc.). Transcription with T7 polymerase generated an RNA that is very similar to the RNA synthesized in vivo. The control plasmid pT7-211, which was provided by E. Moon, contains the rice cytochrome oxidase subunit II gene inserted downstream of the T7 promoter. RNA synthesis in vitro was carried out as specified by the manufacturer. The plasmid pSal 103, obtained from J. T. Parsons, contains a permuted copy of the Rous sarcoma virus (Prague A strain) provirus. End-labeled fragments used in the DNA binding experiments were prepared by standard methods after elution of fragments from agarose gels. TMV RNA was prepared by digestion of TMV with proteinase K in SDS followed by phenol extraction.

Radioactive viral RNAs and rRNAs were prepared from AMV-infected chicken embryo fibroblasts labeled either with 32 P_i or with [3 H]uridine. Ribosomes were prepared by standard methods and phenol extracted, and the resulting

large and small rRNA species were separately purified by sucrose gradient sedimentation. 32 P-labeled AMV was collected at 6- to 8-h intervals for 3 days after an overnight incubation of cells in 100 μ Ci of 32 P_i per 10-cm-diameter plate. The virus was pelleted and then incubated with 500 μ g of pronase per ml plus 1% SDS for 3 min at 37°C before extraction with phenol-chloroform-isoamyl alcohol (24:24:1) by standard procedures. The genomic RNA dimer was purified further by sucrose gradient sedimentation.

Western blotting to detect viral proteins was performed with either (i) polyclonal rabbit antisera to MA or NC (prepared by injecting rabbits with proteins purified by gel filtration in 6 M guanidine) or (ii) the 2A7 monoclonal antibody to MA (25). The rabbit antisera were diluted 1/500, and the monoclonal ascites fluid was diluted 1/2,000. The rabbit anti-IN antibody 4634, which was a gift from R. Katz, was diluted 1/10,000. The blots were developed with horseradish peroxidase conjugates (Bio-Rad Laboratories) or with 125 I-protein A.

RESULTS

We purified MA from AMV by one of several methods, as described in Materials and Methods. The purity of the resulting protein was assessed by Coomassie blue and silver staining of SDS-polyacrylamide gels and by immunoblotting with polyclonal and monoclonal antibodies. An example of the results of a typical preparation is shown in Fig. 1A, lane a. MA is known to migrate as a doublet; the upper band is phosphorylated, while the lower band is not (8). Although only one major doublet band is apparent in the Coomassie-stained profile shown, numerous minor bands in the molecular weight range of 12,000 to 35,000 appear after silver

FIG. 2. Nitrocellulose filter binding of MA and NC to [3 H]RNA. Different concentrations of purified viral proteins were added to labeled RNAs, and then the mixtures were filtered through nitrocellulose membranes and counted. (A) MA plus 70S viral RNA or 28S rRNA. MA was purified by method A. Each sample contained 1.5 ng of 70S or 1.4 ng of 28S RNA in 50 μ l of mixture. The final NaCl concentration was 20 mM. (B) MA or NC plus 18S rRNA. MA was purified by method B, while NC was prepared by gel filtration in 6 M guanidine. Samples contained 0.9 ng (MA reactions) or 1.8 ng (NC reactions) of RNA. The final NaCl concentration was 10 mM. Each point represents the average of duplicate determinations.

staining (for example, Fig. 1B, lane h). Most or all of these minor bands also are recognized by a monoclonal antibody to MA, as can be seen for different MA fractions (Fig. 1C). This is consistent with the findings of several laboratories that MA is proteolytically processed in various ways in vivo and perhaps in vitro during isolation. Both longer (Pr32 and p23 [24, 35]) and shorter (p19f [24, 31]) forms of MA have been described elsewhere. All of these polypeptide fragments appear to be nested fragments containing the same N terminus. Thus, the preparation of purified MA used for most of our experiments at a high level of resolution is in fact a collection of related polypeptides, of which the major species composes about 90%.

To assess the ability of MA to interact specifically with ASLV RNA, we initially used the same nitrocellulose filter binding assay described by Leis and collaborators (15). This technique, which was originally used to characterize the interaction of *Escherichia coli* tRNA synthetases (37) and *E. coli* lac repressor (28) with DNA, is based on the finding that proteins, but not RNA or double-stranded DNA, bind to nitrocellulose at low to moderate ionic strengths. It is common practice to assume that a single protein molecule bound to a radioactive RNA or DNA molecule is sufficient to cause retention of the nucleic acid on the filter. We incubated 3 H-labeled 70S viral RNA (dimeric; ca. 14 kb) or chicken 28S rRNA (ca. 4.6 kb) with different concentrations of MA and then filtered the mixtures through membranes. We verified by immunoblotting that under the conditions of these assays, at least 99% of the free MA was retained on the nitrocellulose filter. An example of the results from such an experiment is shown in Fig. 2. All of the RNA of each species remained on the filter at high concentrations of MA. The viral RNA was retained on the filter at a threefold-lower protein concentration than the rRNA was, implying a threefold-higher apparent association constant. However, since the viral RNA is also larger by the same factor, this is the expected result if the binding reaction lacks specificity; the larger RNA has more sites available for binding. In several experiments, the protein concentration at which half-max-

imal binding of 70S viral RNA occurred ranged from about 50 to 150 ng/50 μ l of reaction mixture (ca. 60 to 180 nM MA). As discussed below, this implies an apparent association constant for 70S RNA of ca. 5×10^6 to 15×10^6 M $^{-1}$ (which is equivalent to an intrinsic binding constant of ca. 10^3 M $^{-1}$, assuming a total lack of binding specificity), or 4 to 5 orders of magnitude lower than the value given by Leis et al. (15).

We have repeated these experiments with different preparations of p19, as outlined in Materials and Methods, and with several additional types of RNAs. These include 18S rRNA and RNAs synthesized in vitro from ASLV templates as well as from nonviral templates. Transcription of one of the viral templates used (P3) yields a small RNA that contains all the known sequences involved in packaging of RNA into virions (33). In all of these cases, the protein concentration at half-maximal binding was proportional to the size of the RNA and independent of whether its origin was viral or nonviral. Thus, we infer, in contrast to a previously published report (15), that MA-RNA interaction is devoid of specificity.

Using protocols similar to that described in the legend to Fig. 2, we investigated several parameters that might affect the ability of MA to retain RNA on membranes. Binding varied by a factor of less than 2 between pH 6.3 and 8.3, was not significantly affected by the presence of Mg $^{2+}$ or EDTA, and was relatively independent of ionic strength. In a typical experiment, the half-maximal binding of 70S viral RNA ranged from 180 nM in 20 mM NaCl to 380 nM in 200 mM NaCl (data not shown). The lack of selectivity for viral RNA compared with that for rRNA did not change at the elevated ionic strength.

The conclusion that MA binds to RNA in vitro is critically dependent on the purity of the protein preparation. It might be argued that instead of MA, a minor contaminating protein with a high affinity for RNA is the actual binding species. Several control experiments were performed to address this issue. In the first experiment, MA was purified by independent methods; the final specific binding activity was similar in each case. In the second experiment, we considered the

FIG. 3. Filter binding at low MA/RNA ratios. 32 P-labeled transcripts were generated with T7 RNA polymerase in vitro from a viral template (*Pvu*I-cut P3-76) or a nonviral control template (*Sal*I-cut pT7-211). The sizes of these RNAs are 1.36 and 0.6 kb, respectively. Each reaction contained 400 ng of MA purified by method B and various amounts of RNA. The final NaCl concentration was 56 mM. At the highest RNA concentration, the 400 ng (23 pmol) of MA retained 600 ng (3 pmol) of the nonviral RNA on the filter.

possibility that the known RNA-binding protein NC (formerly called p12^{gag}), which is present in virions in an amount equimolar with that of MA, could be responsible for the activity observed in MA preparations. We assayed the level of NC contamination in the preparations of MA used for these experiments by immunoblotting SDS-polyacrylamide gels in which 1 μ g of MA per lane had been electrophoresed. In different preparations which exhibited similar binding activity, NC was found to be present at levels ranging from less than 0.1 to 0.5%. This amount of NC cannot explain the retention of RNA on membranes, since the activity of purified NC itself is similar to that of MA in this assay (Fig. 2B). Leis and collaborators also concluded that these two *gag* proteins are similarly effective in retaining RNA on membranes (15). Using immunoblot analyses again, we attempted to quantify the levels of the other known virus-encoded nucleic acid-binding proteins, RT and IN, in preparations of MA. Both RT and IN are derived from the *pol* gene and are present in whole virions at about 20- to 50-fold-smaller amounts than the *gag* proteins. Neither IN (less than 0.1% of MA) nor RT (less than 0.5% of MA) could be detected in the MA purified by method B, although both *pol* proteins could be detected in cruder fractions.

In the third control experiment, we sought to establish a maximum binding activity in the MA preparation. When increasing amounts of a small, in vitro-transcribed RNA were added to a given amount of protein, about 20% of the input RNA (600 ng) was retained on the filter at the highest level of RNA added (3 μ g) (Fig. 3). The levels of retention were the same for viral and nonviral species. For the smaller, nonviral RNA, 600 ng corresponds to 3 pmol. Thus, in the experiment whose results are shown in Fig. 3, there must have been at least 3 pmol of an RNA-binding protein in the reaction. Since the binding curves were still horizontal at 3 μ g of RNA input, higher RNA concentrations would have yielded even more picomoles of RNA retained. The amount of input protein was about 400 ng, or 23 pmol, of MA. Hence, an RNA-binding species must have been at least 13% as abundant as MA. Since there is no evidence of a contam-

FIG. 4. Filter binding of phosphorylated or nonphosphorylated MA to $[^{32}$ P]rRNA. Phosphorylated (MA + P) and nonphosphorylated (MA - P) MA species were purified on a Mono-Q column. The 28S and 18S rRNAs were purified separately and then pooled for this assay. Each reaction mixture contained 57 ng of radioactive RNA. The final NaCl concentration was 40 mM.

inating protein species at this level, we conclude that MA itself must be the binding species.

About half of the MA molecules in a virion are phosphorylated (8), and these modified molecules can be distinguished by their slower mobility in SDS-PAGE. We used two methods to separate the major phosphorylated and nonphosphorylated species in native form, both based on anion-exchange chromatography. Figure 1B shows stained profiles of the resulting MA fractions from columns of DEAE-cellulose (lanes e and f) and Mono-Q on a fast protein liquid chromatography system (lanes g and h). The first MA fraction to elute from each column was the nonphosphorylated species, which appeared as a single band. The second fraction to elute contained the major phosphorylated species and an assortment of minor MA-related species, which we assume are also phosphorylated. Although in this experiment insufficient protein was available to generate complete binding curves, it is apparent from Fig. 4 that the affinities of MA and phosphorylated MA for RNA are similar to each other and to the affinity estimated for the MA mixture used as starting material. The retention of full binding activity after this additional purification step further argues against the possibility that a contaminant is responsible for the activity.

We sought an independent method to measure the binding of MA to RNA, i.e., a method that would be insensitive to contaminants and also could lead to a quantitative estimate of an intrinsic binding constant. The method chosen was that described by Draper and von Hippel (7), which is based on sedimentation. A large excess of RNA is mixed with a small amount of protein, the mixture is layered onto a sucrose gradient, and centrifugation is performed until the RNA has moved approximately halfway down the gradient. The amount of protein is assayed in each fraction of the gradient. The percentage of total protein that cosediments with RNA is then used to estimate the binding constant. The results of an experiment of this type are shown in Fig. 5. MA (300 ng) and NC (200 ng; used as an internal marker) were mixed together with or without TMV RNA (ca. 6 kb, 40 μ g) and

FIG. 5. Sedimentation analysis of MA and NC in the presence or absence of RNA. (A) TMV RNA added; (B) no RNA. The peak of RNA in panel A is indicated by the arrows. The numbers are the fraction numbers from the bottom of the gradient (total of 19 fractions). Lane L contained a fraction of the mixture loaded onto the sucrose gradient.

then subjected to centrifugation as described in the legend to Fig. 5. Samples of the collected fractions were analyzed for RNA by determining the A_{260} and for the two ASLV *gag* proteins by immunoblotting with a mixture of monospecific sera against MA and NC. The control profile for sedimentation in the absence of RNA is shown in Fig. 5B; both proteins remained near the top of the gradient. In the presence of RNA (Fig. 5A), at least 95% of the NC cosedimented with RNA, since the protein profile matched the A_{260} profile (not shown; arrows indicate RNA peak). From this result, it is not possible to obtain an accurate association constant for the NC-RNA interaction, since this method is reliable quantitatively only when about 10 to 90% of the protein is dragged into the gradient by the RNA. Lower RNA concentrations would have to be used to obtain an accurate estimate. However, we estimate from this result that the intrinsic binding constant of NC for RNA must be in the range of 10^6 M^{-1} (expressed as moles of nucleotide, which approximately equals moles of binding sites). In contrast to NC, only a very small fraction (perhaps 0.1% or less) of the MA cosedimented with RNA in this and other experiments. These numbers translate roughly into an intrinsic binding constant of 10^3 M^{-1} (between 0.8×10^3 and $3 \times 10^3 \text{ M}^{-1}$ if the amount of MA cosedimenting with RNA is in the range of 0.01 to 1%; see formula in Materials and Methods and reference 7). Thus, as demonstrated by a direct binding assay that relies on no assumptions about purity and minimal assumptions about the nature of RNA-protein association, MA interacts with RNA at least 3 orders of magnitude more weakly than NC does.

We found that MA from ASLV is able to bind not only to RNA but also to double-stranded DNA. This result is presented in Fig. 6A, which shows a gel mobility assay in which radioactive DNA from a cloned Rous sarcoma virus provirus was mixed with different concentrations of MA and then electrophoresed in an agarose gel. As with RNA, we

FIG. 6. Agarose gel band shift assay showing interaction of MA or RT with DNA. End-labeled DNA was mixed with MA purified by method B or with RT and incubated for 20 min, and the reaction mixture was layered into a well in a 1% agarose gel. After electrophoresis, the dried gel was autoradiographed. The numbers at the sides of the gels are the sizes of the fragments in kilobases. (A) pSal103 (a permuted Rous sarcoma virus provirus cloned in pBR322) was cut with *EcoRI*, yielding the bands indicated. Lanes 0, No protein added; lane a, 50 ng of MA; lane b, 100 ng of MA; lane c, 200 ng of MA; lane d, 400 ng of MA; lane e, 800 ng of MA. The NaCl concentration in the samples was 140 mM. (B) P3-76 (a plasmid clone of a deleted provirus) was cut with *EcoRI*, yielding the bands indicated. Lanes 0, No protein added; lane a, 200 ng of MA; lane b, 400 ng of MA; lane c, 800 ng of MA; lane d, 40 ng of RT; lane e, 80 ng of RT; lane f, 160 ng of RT; lane g, 330 ng of RT; lane h, 660 ng of RT; lane i, 1.3 μg of RT. The NaCl concentration in the samples was 140 mM.

could detect no specificity for this interaction. In particular, similarly sized DNA species that contained parts of the proviral DNA or no viral sequences were retarded equally by the protein. Preparations of MA derived from different purification methods showed similar specific activities, as did phosphorylated and nonphosphorylated species of MA (not shown). MA-DNA interaction was also detectable by filter binding assays similar to those performed for RNA. In these experiments, half-maximal binding for DNA fragments of sizes comparable to those of rRNAs was obtained in the range of 75 ng of MA per reaction, and thus we conclude that the affinity of the protein for double-stranded DNA is similar to that for RNA. Experiments such as the one described in the legend to Fig. 3 showed that, as in the case of RNA, a

minor contaminant in the MA preparation could not account for the DNA-binding activity. For example, in one experiment, at high DNA concentrations, 23 pmol of MA retained 2.5 pmol of DNA on the filter.

NC also was able to cause mobility shifts of DNA fragments. Per microgram, NC and MA exhibited a similar potency in this assay. By contrast, CA (formerly called p27^{gag}) did not interact with DNA. When a crude extract of disrupted purified virus was subjected to chromatography on a Mono-S fast protein liquid chromatography column, the major DNA-binding peaks, as determined by the gel mobility shift assay, coincided with the peaks for MA and NC (not shown), suggesting to us that most of the DNA-binding activity in ASLV can be attributed to these two *gag* proteins.

Since RT is a known DNA-binding protein and since it was a contaminant in the less pure of our MA preparations, we compared the abilities of purified RT and MA to cause mobility shifts of radioactive DNA fragments. Increasing quantities of RT yielded a qualitatively different shift of the labeled DNA from that induced by MA (Fig. 6B). While MA caused a gradual smearing upwards, RT binding to DNA led to retention of the DNA in the well. The amount of RT needed for this mobility shift (ca. 160 to 330 ng in this experiment; Fig. 6B, lanes f and g) was roughly comparable to the amount of MA needed for the test DNA fragment to shift (ca. 400 ng; lane b). Such an amount of RT vastly exceeds the amount of any contaminating RT that might have been present in a typical assay with MA.

DISCUSSION

We have reexamined the ability of ASLV MA (p19^{gag}) to interact with nucleic acids *in vitro*. In our experiments, MA bound to both RNA and DNA with similar affinities and without sequence specificity. The binding was weak, being barely detectable in a sedimentation assay with large quantities of a defined RNA. We performed several control experiments to rule out the possibility that the observed filter binding could be attributed to contaminating viral proteins such as NC or the RT and IN products of the *pol* gene. The case for MA as the binding species rests on two additional observations. First, different purification procedures for MA yielded preparations with similar specific binding activities. These procedures included gentle methods that rely only on lysis with nonionic detergents and on ion-exchange chromatography as well as procedures that may cause protein denaturation. Second, at high RNA concentrations, the molar ratio of RNA retained to input MA protein was substantial (13%), being much higher than the level of potential viral or nonviral contaminants.

The filter binding analyses can be used to obtain an approximate association constant (K_A) for the simple reaction $MA_{free} + RNA_{free} \rightleftharpoons MA-RNA$. The constant is given by the equation $apparent\ K_A = [MA-RNA]/[MA_{free}] \cdot [RNA_{free}]$. Under conditions in which $[MA_{total}] \gg [RNA_{total}]$, $[MA_{free}]$ is approximately equal to the total $[MA]$ added to the reaction. Thus the apparent K_A is simply the reciprocal of the concentration of total protein at which one-half of the labeled RNA is bound to the filter, i.e., where $[RNA_{free}] = [MA-RNA]$. The one critical assumption in this analysis is that a single protein molecule is sufficient to retain an RNA molecule of any size on the membrane. The specific activity of the RNA plays no role in the calculation as long as the molar RNA concentration is kept much lower than the molar protein concentration. In most of our filter binding experi-

ments, the ratio of moles of protein to moles of RNA ranged from 10^2 to 10^4 . This simple treatment is widely used to calculate the association constants of proteins that bind in a sequence-specific manner to nucleic acids. Accurately estimating a K_A for a protein without sequence specificity, such as MA, requires minor modifications in the calculations (1), since some of the retained RNA molecules may harbor more than one protein. In this case, the initial slope of the binding curve can be used to calculate the K_A . Using these modifications, we calculate from the average of numerous experiments that the apparent K_A for the binding of MA to 70S ASLV RNA is 5×10^6 to $15 \times 10^6\ M^{-1}$. Assuming that there are approximately 1.4×10^4 overlapping binding sites on the 14-kb 70S RNA, i.e., that binding is completely random and can occur at any place on the polynucleotide, we can thus obtain an estimate for the intrinsic K_A (the constant that describes the interaction of protein with a single site), which is about 3.5×10^2 to $11 \times 10^2\ M^{-1}$. This value is in agreement with the affinity roughly estimated from the sedimentation experiment, in which barely detectable binding of MA to TMV RNA was observed (ca. $10^3\ M^{-1}$).

In contrast to those for MA-RNA interaction, the calculated association constants for NC-RNA interaction are not self-consistent when filter binding and sedimentation analysis are compared. By sedimentation analysis, NC associates with RNA with a 1,000-fold-higher affinity than MA does, while by filter binding, the association shows similar affinity. The intrinsic K_A estimated by sedimentation, ca. $10^6\ M^{-1}$, is the correct value; it is based on fewer assumptions, and it is consistent with careful independent measurements by other groups. Smith and Bailey (32) and Karpel et al. (10) used fluorescence quenching to calculate that murine and avian retrovirus NCs have an intrinsic K_A for RNA binding of about 1×10^6 to $5 \times 10^6\ M^{-1}$. Filter binding experiments with NC lead to grossly incorrect estimates of binding affinity, probably because the assumption that one bound protein molecule is sufficient to retain one RNA molecule on the filter is faulty. In preliminary experiments (M. Sakalian and V. Vogt, unpublished results), we found that even when dozens of NC molecules are bound to one RNA molecule, under some conditions full retention of the complex on membranes is not achieved.

We originally undertook this study because of the reports from Leis and collaborators that MA could bind specifically and with high affinity to viral RNA (15, 16). These workers claimed that the apparent K_A for MA binding to 70S viral RNA was 10^{11} to $10^{12}\ M^{-1}$, i.e., 10^4 to 10^5 times higher than we calculate, while the K_A for binding to rRNA was less than $10^6\ M^{-1}$. A possible explanation for the lack of binding of rRNA is that this RNA was degraded to small fragments, since for nonspecific binding the apparent K_A is strictly size dependent. The very large discrepancy between our calculated apparent K_A for viral RNA and that calculated by Leis et al. is due in part to errors in data handling. When they are presented or can be extrapolated from the information given, the raw binding data in the earlier papers actually are similar to ours; that is, the concentration of MA that yielded 50% retention of radioactive RNA was in the range of 100 nM. However, the authors then plotted their data by the method of Scatchard to derive an apparent K_A . Scatchard analysis is a way of measuring the approach to saturation (titration) of a protein species by the ligand that binds to that protein. However, in the experiments of Leis et al., as in our own experiments, the binding species (MA) was in vast excess (ca. 100- to 10,000-fold) over the ligand (RNA), not vice versa as would be required to obtain saturation of the protein

by the ligand. Thus, the physical meaning of the derived curves shown in the papers of Leis et al. (15, 16), directly implying titration of the protein by the RNA, is obscure. It is unclear to us how such curves could be generated. The values for apparent association constants for NC binding to various RNAs (11, 13–15), also calculated by the same workers by the Scatchard method, appear to be similarly flawed. In the latter case, however, the calculated apparent K_A appears to be fortuitously correct, perhaps because of a compensating error in the assumption that a single NC molecule is able to retain a high-molecular-weight RNA on a membrane.

It could well be that the weak in vitro binding of MA to RNA and to DNA lacks biological significance. However, although its affinity for RNA is low, at the high concentration of RNA found in a mature or nascent virus particle (over 1 mg of RNA per ml), MA might indeed interact with the viral RNA. Virus particles are assembled from the *gag* precursor molecules, which are cleaved only late in the budding process. One could speculate that the MA and NC domains in the *gag* precursor cooperate in the RNA-binding reaction that leads to specific incorporation of genomic RNA into the virion. It is also possible to envision a role for the interaction of MA and DNA during or after reverse transcription in the newly infected cell. The affinity of MA for DNA is not high, but it is in the same range as that of some other proteins known or widely speculated to interact with DNA, for example, RT (see above) and the protein product of the *v-myc* oncogene (5). Little is now known about the structure of retroviral cores that are active in reverse transcription in vivo, except that they contain CA (4). Genetic or more careful biochemical approaches are needed to investigate these possible functions.

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LITERATURE CITED

- Bailey, J. M. 1979. Interpretation of nitrocellulose filter assays of protein-nucleic acid binding. *Anal. Biochem.* **93**:204–206.
- Barbacid, M., and S. A. Aaronson. 1978. Membrane properties of *gag* gene-encoded p15 protein of mouse C-type RNA tumor viruses. *J. Biol. Chem.* **253**:1408–1414.
- Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub. 1980. The detection of DNA-binding proteins by protein blotting. *Nucleic Acids Res.* **8**:1–20.
- Bowerman, B., P. O. Brown, J. M. Bishop, and H. E. Varmus. 1989. A nucleoprotein complex mediates the integration of retroviral DNA. *Genes Dev.* **3**:469–478.
- Dang, C. V., H. van Dam, M. Buckmire, and W. M. F. Lee. 1989. DNA-binding domain of human c-Myc produced in *Escherichia coli*. *Mol. Cell. Biol.* **9**:2477–2486.
- Davis, J., M. Scherer, W. P. Tsai, and C. Long. 1976. Low-molecular-weight Rauscher leukemia virus protein with preferential binding for single-stranded RNA and DNA. *J. Virol.* **18**:709–718.
- Draper, D. E., and P. H. von Hippel. 1979. Measurement of macromolecular equilibrium binding constants by a sucrose gradient band sedimentation method. Application to protein-nucleic acid interactions. *Biochemistry* **18**:753–760.
- Erikson, E., J. S. Brugge, and R. L. Erikson. 1977. Phosphorylated and non-phosphorylated forms of avian sarcoma virus polypeptide p19. *Virology* **80**:177–185.
- Henderson, L. E., H. C. Krutzsch, and S. Oroszlan. 1983. Myristyl amino terminal acylation of murine retrovirus proteins: an unusual post-translational protein modification. *Proc. Natl. Acad. Sci. USA* **80**:339–343.
- Karpel, R. L., L. E. Henderson, and S. Oroszlan. 1987. Interaction of retroviral structural proteins with single stranded nucleic acids. *J. Biol. Chem.* **262**:4961–4967.
- Katz, R. A., X.-D. Fu, A. M. Skalka, and J. Leis. 1986. Avian retrovirus nucleocapsid protein pp12, produced in *E. coli*, has biochemical properties identical to unphosphorylated viral protein. *Gene* **50**:361–369.
- Leis, J., D. Baltimore, J. M. Bishop, J. Coffin, E. Fleissner, S. P. Goff, S. Oroszlan, H. Robinson, A. M. Skalka, H. M. Temin, and V. Vogt. 1988. Standardized and simplified nomenclature for proteins common to all retroviruses. *J. Virol.* **62**:1808–1809.
- Leis, J., and J. Jentoft. 1983. Characteristics and regulation of interaction of avian retrovirus pp12 protein with viral RNA. *J. Virol.* **48**:361–369.
- Leis, J., S. Johnson, L. S. Collins, and J. A. Traugh. 1984. Effects of phosphorylation of avian retrovirus nucleocapsid protein pp12 on binding of viral RNA. *J. Biol. Chem.* **259**:7726–7732.
- Leis, J. P., J. McGinnis, and R. W. Green. 1978. Rous sarcoma virus p19 binds to specific double-stranded regions of viral RNA: effect of p19 on cleavage of viral RNA by RNase III. *Virology* **84**:87–98.
- Leis, J. P., P. Scheible, and R. E. Smith. 1980. Correlation of RNA binding affinity of avian oncornavirus p19 proteins with the extent of processing of virus genome RNA in cells. *J. Virol.* **35**:722–731.
- Méric, C., J.-L. Darlix, and P.-F. Spahr. 1984. It is Rous sarcoma virus protein p12 and not p19 that binds tightly to Rous sarcoma virus RNA. *J. Mol. Biol.* **173**:531–538.
- Méric, C., and S. P. Goff. 1989. Characterization of Moloney murine leukemia virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein. *J. Virol.* **63**:1558–1568.
- Méric, C., E. Gouilloud, and P.-F. Spahr. 1988. Mutations in Rous sarcoma virus nucleocapsid protein p12 (NC): deletions of Cys-His boxes. *J. Virol.* **62**:3328–3333.
- Méric, C., and P.-F. Spahr. 1986. Rous sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S RNA dimer formation and packaging. *J. Virol.* **60**:450–459.
- Montelaro, R. C., S. J. Sullivan, and D. P. Bolognesi. 1978. An analysis of type-C retrovirus polypeptides and their associations in the virion. *Virology* **84**:19–31.
- Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105**:361–363.
- Pepinsky, R. B., and V. M. Vogt. 1979. Identification of retrovirus matrix proteins by lipid-protein cross-linking. *J. Mol. Biol.* **131**:819–837.
- Pepinsky, R. B., and V. M. Vogt. 1984. Fine-structure analyses of lipid-protein and protein-protein interactions of *gag* protein p19 of the avian sarcoma and leukemia viruses by cyanogen bromide mapping. *J. Virol.* **52**:145–153.
- Potts, W., and V. M. Vogt. 1987. Epitope mapping of monoclonal antibodies to *gag* protein p19 of avian sarcoma and leukemia viruses. *J. Gen. Virol.* **68**:3177–3182.
- Rein, A., M. R. McClure, N. R. Rice, R. B. Luftig, and A. M. Schultz. 1986. The myristylation site in Pr65^{gag} is essential for virus particle formation in Moloney murine leukemia virus. *Proc. Natl. Acad. Sci. USA* **83**:7246–7250.
- Rhee, S. S., and E. Hunter. 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. *J. Virol.* **61**:1045–1053.
- Riggs, A. D., H. Suzuki, and S. Bourgeois. 1970. Lac repressor-operator interactions. I. Equilibrium studies. *J. Mol. Biol.* **48**:67–83.

29. **Schultz, A. M., and S. Oroszlan.** 1983. In vivo modification of retroviral *gag* gene-encoded polypeptides by myristic acid. *J. Virol.* **46**:355–361.
30. **Sen, A., and G. J. Todaro.** 1977. The genome-associated, specific RNA binding proteins of avian and mammalian type-C viruses. *Cell* **10**:91–99.
31. **Shealy, D. J., A. G. Mosser, and R. R. Rueckert.** 1980. Novel p19-related protein in Rous-associated virus type 61: implications for avian *gag* gene order. *J. Virol.* **34**:431–437.
32. **Smith, B. J., and J. M. Bailey.** 1979. The binding of an avian myeloblastosis virus basic 12,000 dalton protein to nucleic acids. *Nucleic Acids Res.* **7**:2055–2072.
33. **Sorge, J., W. Ricci, and S. H. Hughes.** 1983. *cis*-Acting RNA packaging locus in the 115-nucleotide direct repeat of Rous sarcoma virus. *J. Virol.* **48**:667–675.
34. **Vogt, V. M., R. Eisenman, and H. Diggelmann.** 1974. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. *J. Mol. Biol.* **96**:471–493.
35. **Vogt, V. M., R. B. Pepinsky, and L. E. Southard.** 1985. Primary structure of p19 species of avian sarcoma and leukemia viruses. *J. Virol.* **56**:31–39.
36. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1982. RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Yarus, M., and P. Berg.** 1967. Recognition of tRNA by aminoacyl tRNA synthetases. *J. Mol. Biol.* **28**:479–490.