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Characteristics and Regulation of Interaction of Avian Retrovirus pp12 Protein with Viral RNA

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We investigated the interaction of the avian retrovirus pp12 protein with viral RNA to assess its possible role in virion assembly. Using chemical modification techniques, we found that reagents specific for lysine or arginine residues inactivated the RNA-binding capacity of the protein. The binding of pp12 to 60S viral RNA was also strongly affected by pH (pK_{app} of 5.5); the affinity for viral RNA decreased by as much as 40-fold after protonation of one or more titratable groups on the protein. When the protein was cleaved by cyanogen bromide, each of the two polypeptide products bound to RNA (with low affinity), but pH dependence was lost. Thus, an intact protein was required for this effect. Since histidine and phosphoserine residues have pK_a values close to the pK_{app} of the pp12-RNA interaction, they were studied to determine whether they were involved in this process. Each of the two histidyl residues in pp12 had pK_a values of 6.2, as determined by proton nuclear magnetic resonance titrations, values too high to account for the pK_{app} of binding. The involvement of phosphoserine residues, which have pK_a values similar to the pK_{app}, was investigated by removal of phosphate from pp12. When phosphate groups were chemically or enzymatically removed from the avian myeloblastosis virus, Rous sarcoma virus (Pr-C), and PR-E 95C virus pp12 proteins, the K_{app} for binding 60S viral RNA was reduced 100-fold at pH 7.5. Thus, it seems possible that phosphorylation of the pp12 protein could favor viral nucleocapsid formation by increasing its affinity for the viral RNA genome. Dephosphorylation could provide for its release from the viral RNA during reverse transcription after viral infection of cells.

Avian retroviruses contain a small basic phosphoprotein, pp12, associated with the viral RNA in the virion core structure. Even though this is an abundant protein in the virion, little is known about its biological function in virus replication. The protein is translated as part of a 76,000-dalton *gag* precursor polypeptide that contains all five of the structural proteins of the virus. Shortly after synthesis, this *gag* precursor polypeptide becomes phosphorylated at serine residues in regions corresponding to both pp12 and pp19 proteins (7, 13, 16). The precursor is cleaved proteolytically by the viral p15 protein to yield the five structural proteins of the sizes found in mature virions (5, 8, 24).

The complete amino acid sequence of the pp12 protein purified from Rous sarcoma virus (RSV) (Pr-C) was determined previously (K. Misono, S. Farida, J. Leis, and T.Vanaman, Fed. Proc. **39**:1611, 1980) and closely agrees with the predicted sequence determined from nucleotide sequencing (20). It contains 89 amino acid residues with a molecular mass of 9,369 daltons. The molecule contains clusters of basic residues which are evenly distributed throughout the polypeptide chain. Some of these residues are presumed to be involved in the nonselective binding of this protein to single-stranded regions of the virus and other RNAs (14). The equivalent protein (p10) purified from Rauscher leukemia virus has similar polynucleotide-binding properties (19). Cyanogen bromide cleavage of the avian protein yields two polypeptide fragments, each of which binds to viral RNA (Misono et al., Fed. Proc., 1980).

In this report, we further analyze the polynucleotide-binding properties of the pp12 protein from three separate avian retroviruses and present evidence that the protein preferentially binds to single-stranded polynucleotides, that the intact pp12 protein exists in two RNA-binding states such that conversion from one state to the other is regulated by the degree of phosphorylation of the protein, and that both lysine and arginine residues are involved in binding polynucleotides.

MATERIALS AND METHODS

Reagents. $^{32}P_i$ (carrier free), [^{3}H]uridine (46.9 Ci/mmol), and [^{3}H]thymidine (26 Ci/mmol) were pur-

chased 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 Sciences, Inc. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) dihydrate was obtained from Pierce Chemical Co. Phenylglyoxal dihydrate was obtained from Aldrich Chemical Co. *N*-Ethylmaleimide was obtained from Sigma Chemical Co. Bacterial alkaline phosphatase (BAP) (50 U/mg) was purchased from Worthington Diagnostics and heated to 70°C for 10 min before use. *Bam*HI (30,769 U/ml) was purchased from New England Biolabs. Avian myeloblastosis virus (AMV), RSV (Pr-C), and PR-E 95C virus were grown as previously described (3, 12, 15).

Purification of pp12 protein. The pp12 protein was purified from RSV (Pr-C), AMV, or PR-E 95-C virus as previously described (12). The viral structural proteins were solubilized from chloroform-methanol-extracted virions with 0.1 M sodium phosphate (pH 7) in the presence of 0.1% Triton X-100–0.1% 2-mercaptoethanol. The pp12 protein was purified from this mixture by chromatography on carboxymethyl cellulose in the presence of 0.1 M sodium phosphate (pH 7)–1 mM 2-mercaptoethanol. The bound pp12 protein was eluted with 1 M NaCl in the same phosphate buffer, dialyzed, lyophilized, and used in the experiments described below. The pp12 protein obtained from this purification procedure was used to determine the amino acid sequence of the protein (Misono et al., Fed. Proc., 1980).

^{32}P-radiolabeled RSV (Pr-C) pp12 protein was prepared from 0.8 g of virus collected from cells grown in the presence of $^{32}P_i$ (carrier free) as previously described (22). The ^{32}P-labeled pp12 protein eluted from the carboxymethyl-Sephadex column (optical density at 280 nm, 2.2; 6.76×10^6 cpm) was further purified by adsorption to a 60S AMV RNA-cellulose column equilibrated with 10 mM sodium phosphate (pH 7)–1 mM 2-mercaptoethanol (12). After washing the column with equilibration buffer containing 0.1 M NaCl, we eluted the bound pp12 protein with equilibration buffer containing 0.5 M NaCl. The pp12 protein obtained from the column had a specific activity of 10^6 cpm per optical density unit at 280 nm. This protein was dialyzed against 0.5 mM sodium phosphate (pH 7.0)–1 mM 2-mercaptoethanol, lyophilized, and used for the studies described below.

Preparation of labeled RNA and DNA. ^{32}P-labeled 60S RSV (Pr-C) RNA (50 to 409 cpm/pmol), [^{3}H]uridine-labeled double-stranded reovirus RNA (16.6 cpm/pmol) and [^{3}H]uridine-labeled reovirus mRNA (2.7 cpm/pmol) were prepared as previously described (14). [^{3}H]thymidine-labeled replicative form (RF) I pBR322 DNA (2 cpm/pmol) was prepared by the method of Clewell and Helinski (2). RF III pBR322 DNA was prepared by incubating 16.8 μg of RF I DNA with 307 U of *Bam*HI for 60 min at 38°C. The DNA was denatured by heating to 100°C for 5 min.

Cyanogen bromide cleavage of RSV (Pr-C) pp12 protein. Cyanogen bromide cleavage of the RSV (Pr-C) pp12 protein (3.3 μg) was done by the procedure previously described for the pp19 protein (12). The two cyanogen bromide fragments of the pp12 protein were separated from one another by gel filtration on a 52-ml Sephadex G-50 (superfine) column equilibrated with 1 mM HCl. The purified polypeptides were directly lyophilized and used in this study.

Dephosphorylation of pp12 protein. (i) Alkaline hydrolysis. The [^{32}P]-labeled pp12 protein (23 to 132 μg) was incubated at 38°C in a 50-μl volume of 0.2 M NaOH for various lengths of time (2 to 60 min). The reaction was stopped by neutralization of the mixture with 4 N HCl. The [^{32}P]-labeled pp12 protein remaining was determined by adding 400 μg of albumin and 1.5 ml of 10% trichloroacetic acid to the protein solution, incubating at 4°C for 5 min, and collecting the acid-insoluble protein on glass fiber filters. The filters were washed with 5% trichloroacetic acid, dried, and counted in a liquid scintillation counter. The amount of phosphate released from the pp12 protein was calculated from the amount of acid-insoluble ^{32}P label before and after treatment.

(ii) Enzymatic hydrolysis. RSV (Pr-C) pp12 (177 μg) was incubated at 38°C in a 0.2-ml volume of 40 mM Tris-hydrochloride, pH 8, containing BAP (8.9 U) for various lengths of time (2 to 27 h). The reaction was stopped by separating BAP from the pp12 protein by chromatography on a 15-ml Sephadex G-100 gel filtration column equilibrated with 0.5 M ammonium carbonate, pH 8.5. Fractions (0.5 ml) were collected and monitored for [^{32}P] label and absorbancy at 280 nm. Those fractions containing the pp12 protein were lyophilized. The percent removal of phosphate was determined as above. Samples of the treated proteins (6 to 26 μg) were analyzed by 0.1% sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis in 0.1 M Tris-bicine buffer, pH 8.2, to ensure that the protein was intact after phosphate hydrolysis.

Chemical modification with TNBS. RSV (Pr-C) pp12 (1.3 μg) was incubated for 30 min at room temperature in a 7-μl reaction volume of 70 mM sodium carbonate, pH 8.5, containing 0.1 to 0.5 μg of TNBS (9). The reaction was stopped by diluting the reaction mixture with an equal volume of 10 mM NaCl–10 mM Tris-hydrochloride (pH 7.5)–0.1 mM dithioerythritol and placing the tubes in ice. Samples of the reaction mixture containing 0.16 μg of pp12 protein were analyzed for binding of ^{32}P-labeled 60S RSV (Pr-C) RNA (0.41 nmol; 170 cpm/pmol) or [^{3}H]-labeled reovirus RNA (224 pmol; 16.6 cpm/pmol) as described below. The extent of reaction of TNBS with the pp12 protein was determined by measuring the absorbancy at 340 nm as described by Habeeb (9).

Chemical modification with phenylglyoxal. RSV (Pr-C) pp12 (12.4 μg) was incubated in a 50-μl volume of 0.12 M sodium carbonate (pH 7.5)–20 mM phenylglyoxal at room temperature for various lengths of time, and 5-μl samples were withdrawn as indicated. These were diluted with 9 μl of 10 mM NaCl–10 mM Tris-hydrochloride (pH 7.5)–0.1 mM dithioerythritol and placed in ice. Samples of the reaction mixture containing 0.17 μg of pp12 protein were analyzed for binding to RNA as described below.

Nitrocellulose filter assay for measuring binding of polynucleotides to pp12 protein. The nitrocellulose filter assay was as described by Hizi et al. (10). Various concentrations of pp12 protein were mixed with labeled RNA or DNA in a 30-μl volume of 10 mM Tris-hydrochloride (pH 7.5)–10 mM NaCl–0.1 mM dithioerythritol and incubated at 4°C for 10 min. The binding mixture was then diluted with 1 ml of the

above-described buffer and immediately passed through presoaked nitrocellulose filters. The filters were washed twice with 2.5 ml of the above-described buffer and dried, and the radioactivity retained on the filter was determined in a liquid scintillation counter. When the binding reactions were carried out at different pH values, 10 mM sodium citrate (pH 4.5), 10 mM sodium acetate (pH 5.1, 5.5, 5.8, and 6.1), or 10 mM sodium cacadylate (pH 6.5 and 7.0) were substituted for the 10 mM Tris-hydrochloride (pH 7.5) in the above-described buffer. Scatchard plots of the binding data were used to calculate the apparent binding constants (K_{app}) of the pp12 protein for the various polynucleotides.

Proton NMR studies of pp12 protein. Proton nuclear magnetic resonance (NMR) spectra were obtained at 270 MHz in a 5-mm probe by use of a Bruker WH 180/270 Fourier transform NMR spectrometer equipped with a Nicolet 1180 computer. The probe temperature for all the measurements was 21°C. Data (2,000 scans per spectrum) were gathered, using the first 8,192 data points in the quadrature mode, a 90° pulse (7 μs), and a total delay of 1.7 s. Data were transformed after zero filling with the full 32,768 data points.

Samples of ca. 10 mg of pp12 protein were lyophilized several times from deuterated water (D_2O), and a trace amount of 3-trimethylsilyl propionate was added as an internal reference at 0.0 ppm. Samples (0.5 ml) were placed in 5-mm sample tubes from Wilmad Glass Co. The pH was adjusted with 0.1 M DCl or 0.1 M NaOD and checked before and after obtaining the NMR spectrum. The pH reading was not corrected for deuterated water. Theoretical pH curves were fitted to the pH-dependent ^1H chemical shifts of pp12 histidyl protons as described by Dwek (6).

RESULTS

Involvement of pp12 amino acid residues in RNA binding. The nature of the amino acid residues involved in the interaction of pp12 with RNA had not been investigated previously. However, the positively charged arginine and lysine residues were considered strong candidates for interaction with the phosphate backbone of RNA (or DNA), especially since these residues are found in clusters throughout the polypeptide chain of the pp12 protein. This hypothesis was tested by modifying chemically each type of residue and determining if the affinity of the modified pp12 for RNA was altered.

The involvement of 1 or more of the 11 positively charged arginine residues of RSV (Pr-C) pp12 in RNA binding was tested by modification of these residues with the arginine-specific reagent phenylglyoxal (4, 18). Modification of arginine residues completely abolished RNA binding (Table 1). Incubation of pp12 under the conditions described above but without phenylglyoxal had no effect on RNA-binding activity (data not shown). The involvement of the NH_2-terminus and the five positively charged lysine residues of pp12 in binding to RNA was examined by studying the effect of modification of these residues with TNBS as described by Habeeb (9). When 45% of the amino groups were modified as determined by measuring the absorbancy at 340 nm (data not shown), pp12 no longer bound RNA to the nitrocellulose filters (0.4% TNBS, Fig. 1). In the primary sequence of RSV (Pr-C) pp12, the five lysine residues were found in two clusters: at residues 36, 37, and 38 and at residues 57 and 61. It is interesting to note, therefore, that when about half of the amino groups of the protein were modified, the RNA-binding activity of the protein was blocked.

TABLE 1. Effect of phenylglyoxal treatment on the binding of RSV (Pr-C) pp12 to RNA

Amt of incubation with phenylglyoxal[a] (min)	% RNA retained on nitrocellulose filters	
	60S RSV (Pr-C)	Reovirus
0	100[b]	100[c]
10	87	76
20	33	6
30	2	2

[a] RSV (Pr-C) pp12 (9.8 μg) was treated with phenylglyoxal (1 μmol) at room temperature for various lengths of time as described in the text. A sample containing 0.14 μg of RSV (Pr-C) pp12 was used to measure the retention of ^{32}P-labeled 60S RSV (Pr-C) RNA (421 pmol; 170 cpm/pmol of nucleotide) or ^3H-labeled reovirus RNA (16.6 cpm/pmol of nucleotide; 330 pmol of nucleotide) to nitrocellulose filters at pH 7.5 as described in the text.

[b] The retention of 244 pmol of 60S RSV (Pr-C) RNA to the nitrocellulose filters is 100% binding.

[c] The retention of 231 pmol of reovirus RNA to the nitrocellulose filters is 100% binding.

Effect of pH on binding of 60S RSV (Pr-C) RNA to pp12 protein. The results from the above chemical modification studies suggest that both arginine and lysine residues are involved in the binding of pp12 to RNA. To obtain an indication of the involvement of other charged amino acid residues in the binding process, we investigated the pH dependence of the interaction. The binding of the pp12 protein to viral RNA was found to be sensitive to pH values between 4.5 and 7.5, suggesting that amino acid residues other than arginine and lysine are involved in binding. The retention of ^{32}P-labeled 60S RSV (Pr-C) RNA to nitrocellulose filters in the presence of a constant amount of AMV pp12 protein at pH 4.5 and 7.5 is shown in Fig. 2. The 50% saturation value for binding RNA at pH 7.5 occurred after less than 45 pmol of RNA nucleotide was added, whereas at pH 4.5 the 50% saturation value

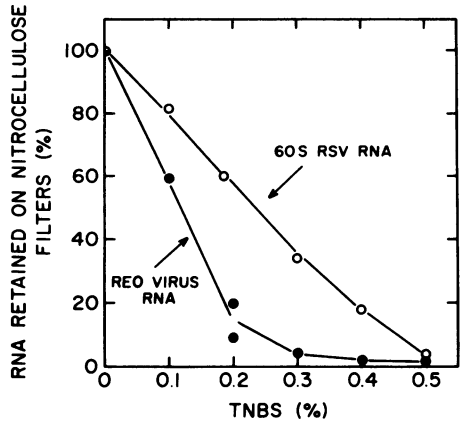

FIG. 1. Effect of treating RSV (Pr-C) pp12 with TNBS upon its binding to RNA. Increasing amounts of TNBS (0.1 to 0.5 μg) were incubated with RSV (Pr-C) pp12 (1 μg) as described in the text. The treated protein was assayed for binding to ^{32}P-labeled single-stranded 60S RSV (Pr-C) RNA or ^{3}H-labeled double-stranded reovirus RNA as described in Table 1, footnote *a*. Symbols: ○, RSV (Pr-C) pp12 binding to 60S RSV (Pr-C) RNA; ●, RSV (Pr-C) pp12 binding to reovirus RNA.

shifted to a concentration greater than 200 pmol. When these data were plotted according to Scatchard, a linear plot was obtained from which a K_{app} for AMV pp12 binding to 60S RSV (Pr-C) RNA at pH 7.5 was calculated to be 1.1×10^{11} M^{-1}, as previously reported (14). The K_{app} for binding RNA at pH 4.5, on the other hand, was calculated to be 3.4×10^{9} M^{-1}, a value decreased more than 32-fold from that at pH 7.5.

To more accurately determine the effect of pH on the binding of AMV pp12 to 60S viral RNA, the K_{app} was determined as a function of pH between 4.5 and 7.5 (Fig. 3, closed circles). We observed a large decrease in pp12-binding affinity for viral RNA when the pH was dropped below 6, with the midpoint of this change occurring at pH 5.5. A similar pH-dependent decrease in the K_{app} for binding 60S RSV (Pr-C) RNA was observed for the RSV (Pr-C) pp12 protein (Fig. 3, open circles).

Effect of pH on binding of RNA to cyanogen bromide peptides of RSV (Pr-C) pp12. The pH affected the ability of the pp12 protein to bind to RNA (Fig. 3). The question arises as to whether this is a property of the protein itself or of the RNA to which it binds. It was previously demonstrated that cyanogen bromide treatment of RSV (Pr-C) pp12 protein produces a polypeptide containing the 53 NH_2-terminal residues and a polypeptide containing the remaining 36 COOH-terminal amino acid residues, both of which bind to viral RNA (Misono et al., Fed. Proc. **39**:1611, 1980). The two cyanogen bromide peptides of RSV (Pr-C) pp12 were prepared as described above, and their affinity for binding to 60S RSV (Pr-C) RNA was determined as a function of pH (Fig. 3, open and closed squares). In contrast to the intact pp12 protein, neither peptide exhibited pH-dependent binding to viral RNA. The K_{app} for binding 60S RSV (Pr-C) RNA to the cyanogen bromide peptides was about 2×10^{9} M^{-1}, a value approximately equal to the K_{app} of the intact protein at pH 5.5. These results indicate that the change in the K_{app} for binding to 60S RSV (Pr-C) RNA as a function of pH is an intrinsic property of the pp12 protein and not of the RNA and that the intact pp12 protein is required for the change. [It should be pointed out that the K_{app}s for binding RNA to the cyanogen bromide peptides of pp12 are still higher than the K_{app} of 1.5×10^{9} M^{-1} observed for degraded RSV (Pr-C) pp12 protein.]

Specificity of pp12-polynucleotide interactions. The effect of pH on the binding properties of the pp12 protein to specific polynucleotides was further studied by the nitrocellulose filter binding assay. The K_{app}s for binding single- and double-stranded RNA and DNA were determined at pH 7.5 and 4.5 (Table 2). At pH 7.5, the pp12 protein preferentially bound to single-stranded polynucleotides, either RNA or DNA, in agreement with the results of Smith and Bailey (21) for AMV pp12 binding to double- and single-stranded salmon sperm DNA at pH 8.1. For instance, the binding of pp12 to reovirus

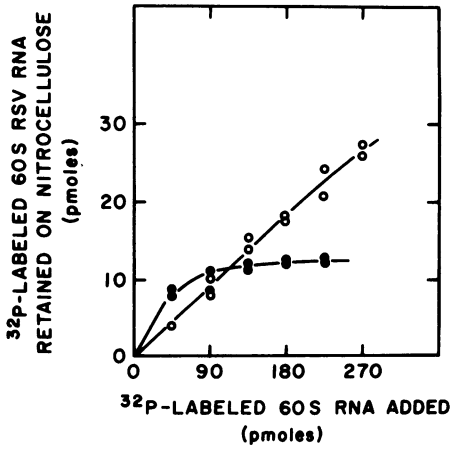

FIG. 2. Binding of ^{32}P-labeled 60S RSV (Pr-C) RNA to AMV pp12 at pH 4.5 and 7.5. Increasing amounts of ^{32}P-labeled 60S RSV (Pr-C) RNA (40 to 386 pmol of RNA nucleotide, 252 cpm/pmol) were incubated with AMV pp12 protein (0.08 μg) at either pH 7.5 or pH 4.5, and the protein-RNA complex was detected by its retention on nitrocellulose filters as described in the text. Symbols: ●, binding of pp12 protein to 60S RSV (Pr-C) RNA at pH 7.5; ○, pp12 protein binding to RSV (Pr-C) RNA at pH 4.5.

mRNA is eightfold stronger than the binding of pp12 to double-stranded reovirus RNA. Similarly, the K_{app} for binding to single-stranded pBR322 DNA was 14-fold stronger than that for binding to double-stranded DNA. Selective binding to single-stranded polynucleotides was lost when the pH was decreased to 4.5. These binding studies demonstrate that the pp12 protein binds to polynucleotides in two ways: (i) to double-stranded polynucleotides in a nonspecific, pH-independent, low-affinity manner and (ii) to single-stranded polynucleotides in a specific, pH-dependent, high-affinity manner.

Further information on the two types of pp12 interactions with polynucleotides was obtained from extension of the chemical modification studies. A difference was noted in the extent of modification of arginine residues associated with complete loss of binding to 60S RSV (Pr-C) RNA relative to loss of binding to reovirus double-stranded RNA (Table 1), suggesting that binding to the latter involves one or more rapidly reacting arginyl residues that are not associated with loss of binding to 60S viral RNA. Likewise, when ca. 21% of the amino groups were reacted with TNBS (0.2% TNBS; Fig. 1), 90% of the binding to double-stranded reovirus RNA was abolished, whereas only 40% of the binding to 60S RSV (Pr-C) RNA was blocked. Thus, binding to double-stranded RNA by the pp12 protein was more sensitive to modification of rapidly reacting amino groups than was binding to single-stranded RNA. The results of the combined chemical modification studies suggest that both amino groups and arginine residues are important for the binding of RNA and that those groups which bind double-stranded RNA are apparently more exposed to chemical reagents than those which are involved in binding single-stranded RNA. This difference in pp12 binding to RNA forms is also reflected in Table 2, which shows that binding to double-stranded RNA occurs at a lower K_{app} than does binding to single-stranded RNA.

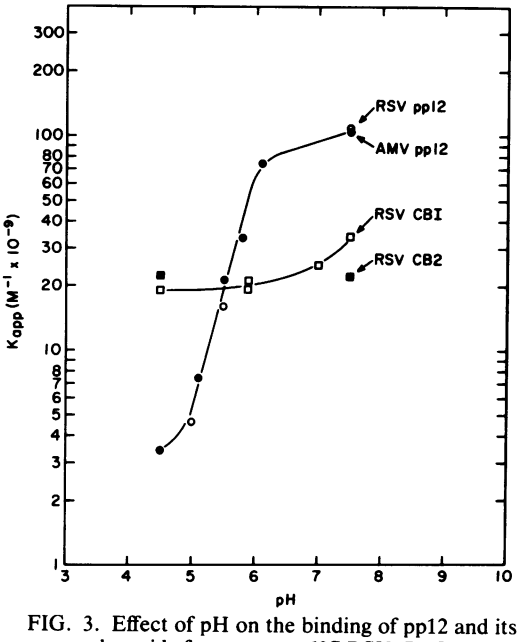

FIG. 3. Effect of pH on the binding of pp12 and its cyanogen bromide fragments to 60S RSV (Pr-C) RNA. The K_{app} was calculated from Scatchard plots for the binding of AMV or RSV (Pr-C) pp12 proteins (each 0.08 μg), the amino-terminal cyanogen bromide peptide of RSV (Pr-C) pp12 (1.6 μg), or the carboxyl-terminal cyanogen bromide peptide of RSV (Pr-C) pp12 (3.1 μg) to various amounts of ^{32}P-labeled 60S RSV (Pr-C) RNA (252 cpm/pmol of nucleotide) as described in the text. Symbols: K_{app} for binding 60S RSV (Pr-C) RNA by AMV pp12, ●; by RSV (Pr-C) pp12, ○; by the amino-terminal cyanogen bromide peptide of RSV (Pr-C) pp12, □; by the carboxyl-terminal cyanogen bromide peptide of RSV (Pr-C) pp12, ■.

TABLE 2. pH dependence of the binding of pp12 to single- and double-stranded polynucleotides

Polynucleotide added	K_{app}[a] ($M^{-1} \times 10^{-9}$) at:	
	pH 7.5	pH 4.5
60S RSV (Pr-C) RNA (ss)[b]	110	3.4
Reovirus RNA (ds)[b]	0.74[c] (0.2[d])	0.94[c]
Reovirus mRNA (ss)	6.0[c] (5.4[d])	0.99[c] (0.4[d])
pBR322 DNA (denatured[e]) (ss)	9.2	0.48
pBR322 DNA (RF III[f]) (ds)	0.66	0.4

[a] K_{app} values were calculated from Scatchard plots for the binding of various RNAs or DNAs as described in the legend to Fig. 2.

[b] ss, Single-stranded. ds, Double-stranded.

[c] K_{app} values were calculated assuming the average size of the 10 reovirus RNAs to be 2,021 nucleotides in length.

[d] The pp12 protein was treated with 30 mM *N*-ethylmaleimide before the K_{app} for binding RNA was determined.

[e] [^{3}H]pBR322 DNA (RF III, 2 cpm/pmol of nucleotide) was denatured by boiling for 5 min and then placed in ice.

[f] [^{3}H]pBR322 DNA (RF I, 2 cpm/pmol of nucleotide) was digested with BamHI as described in the text to prepare RF III DNA.

The possible involvement of one or more of the six cysteine residues of RSV (Pr-C) pp12 in binding RNA was examined by treatment of the protein with 30 mM *N*-ethylmaleimide, a concentration sufficient to modify all six cysteine residues. No change was observed in the K_{app} at pH 7.5 or 4.5 for binding of the modified protein to reovirus RNA (Table 2, values in parentheses). In contrast, the DNA polymerase and RNase H activities of reverse transcriptase were

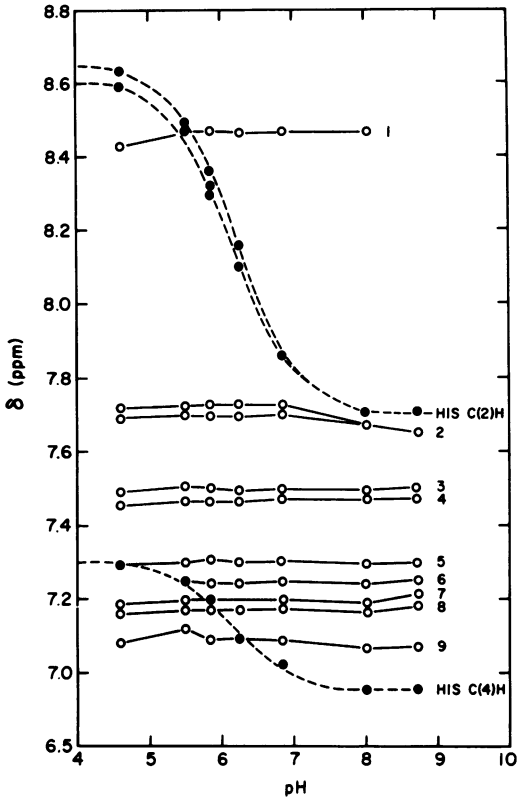

FIG. 4. pH dependence of the chemical shifts of the 270-MHz proton NMR resonances from aromatic residues of AMV pp12. The curves (---) drawn for the histidine (HIS) C(2)H and C(4)H protons are theoretical curves; the parameters for the two C(2)H protons are as follows: pK_a, 6.18; δHX, 8.6 ppm, and $δX^-$, 7.70 ppm; and pK_a, 6.22; δHX, 8.64 ppm, and $δX^-$, 7.70 ppm. The parameters for both C(4)H protons are as follows: pK_a, 6.2, δHX 7.30 ppm, and $δX^-$ 6.95 ppm. (●) Histidine titers. The remaining resonances, numbered 1 to 9 (○), derive from as yet unassigned protons from the phenylalanine, tryptophan, and two tyrosine residues of the AMV pp12 protein.

inactivated by a similar treatment. We conclude, therefore, that none of the cysteine residues of RSV (Pr-C) pp12 are located in the RNA-binding site or associated with the pH dependence of RNA binding.

Proton NMR analysis of pp12 protein. The data presented in Fig. 3 suggest that one or more amino acid residues of pp12 which have a pK_a value of about 5.5 may be responsible for the transition from the high-affinity to the low-affinity RNA-binding state. Only a few amino acid residues have side chains with pK_a values near this pH: histidine (pK_a, 3 to 8.5 [11]); phosphoserine (pK_a, 5.65 for the free amino acid); glutamic and aspartic acids (pK_a, 3.4 to 4.6); and the COOH-terminus of the protein (pK_a, 3.6) (1, 17). One can observe the protons on the amino acid side chains of histidine, tyrosine, tryptophan, and phenylalanine in proteins by using proton NMR. The pH dependence of the chemical shifts for these residues in AMV pp12 is shown in Fig. 4. As expected, the proton resonances of the tyrosine, tryptophan, and phenylalanine residues were largely independent of pH values below 9 (Fig. 4, lines 1 to 9). In contrast, the protons of the two histidine residues in the pp12 protein titrate with pK_a values of 6.18 and 6.22 for the C(2)H protons and 6.2 for both C(4)H protons (Fig. 4, closed circles). These pK_a values are close to the average values found for histidyl protons in a variety of other proteins (11). The fact that the histidyl protons titrate at a pH higher than the pH-dependent change in binding to 60S RSV (Pr-C) RNA suggests that neither histidine in pp12 is directly involved in the pH-dependent change in RNA binding.

Of the remaining amino acid residues in pp12, only phosphoserine had a pK_a value close to that of the RNA-binding transition. This fact suggests that one or more of the phosphoserine residues known to be present in the pp12 protein (13) may be involved in modulating its RNA-binding activity.

Dephosphorylation of pp12 protein and its effects on RNA binding. To determine if removal of the phosphate group from serine residues on the pp12 protein affects its ability to bind to viral RNA, we carried out the following experiments. The pp12 protein was purified from RSV (Pr-C) grown in the presence of ^{32}P. When this protein was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5, lane 7), all of the ^{32}P label was found to comigrate with the pp12 protein, as detected by Coomassie blue staining (lane 6). Thus, the ^{32}P label could be used to monitor the amount of phosphate associated with the protein. The pp12 protein was treated with alkali or BAP; a sample of the protein was acid precipitated to determine the amount of phosphate released, a second sample was analyzed by SDS-polyacrylamide gel electrophoresis, and a third sample was used to determine the K_{app} for binding viral RNA as described above.

Incubation of the RSV (Pr-C) pp12 protein with 0.2 M NaOH for 1 h or with BAP at pH 8 for 27 h resulted in the release from the protein of 70 and 74% of the phosphate label, respectively. Neither procedure resulted in cleavage of the pp12 protein, as analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). The majority of the Coomassie blue-staining material was detected on the gel as a single band in the location of pp12. Similar results were obtained for the AMV and PR-E 95C pp12 proteins after alkali treatment (Fig. 5). The percent release of phosphate was estimated by the release of ^{32}P

from the RSV (Pr-C) pp12 protein added as a carrier for the procedures involving the AMV or PR-E 95C pp12 proteins.

The K_{app} for binding 60S RSV (Pr-C) RNA at pH 7.5 was determined for each of these dephosphorylated proteins as described above. The K_{app} for the pp12 proteins dephosphorylated with alkali treatment ranged between 1.2×10^9 to 1.8×10^9 M^{-1} or about 100-fold lower than the K_{app} for the untreated protein at this pH. A typical Scatchard plot for binding of the alkali-treated RSV (Pr-C) pp12 protein to viral RNA is shown in Fig. 6A. The K_{app} for the RSV (Pr-C) pp12 protein dephosphorylated by incubation with BAP was calculated from a Scatchard plot similar to that shown in Fig. 6A (data not shown) and thus was the same as for the protein treated with alkali.

Incubation for shorter periods by either procedure resulted in the release of less of the phosphate from the ^{32}P-labeled RSV (Pr-C) pp12 protein. For instance, incubation of the RSV (Pr-C) pp12 protein with BAP for 5 h released only 21% of the phosphate label from the protein. The binding to 60S RSV (Pr-C) RNA by this partially dephosphorylated RSV (Pr-C) pp12 protein was determined, and these data are shown in a Scatchard plot in Fig. 6B. In contrast to what is observed for Scatchard plots for native pp12 (14) or for pp12 protein with 70% of the phosphate released (Fig. 6A), a biphasic curve was obtained. This indicates that there are two populations of pp12 proteins binding to the RNA. K_{app} values of 2.2×10^{11} M^{-1} and 3×10^9 M^{-1} were calculated from this plot. These K_{app} values correspond to the values obtained for the native protein and the pp12 protein with 70% of the phosphate removed, respectively. These data strongly suggest that at a neutral pH the RNA-binding affinity of the pp12 protein is determined by whether or not the protein is phosphorylated.

By estimating the relative contribution to the binding of RNA by the high-affinity (phosphorylated) and low-affinity (dephosphorylated) pp12 proteins in the preparation with 21% of the phosphate released, an average K_{app} of 1.65×10^{10} M^{-1} was calculated. A similar analysis was

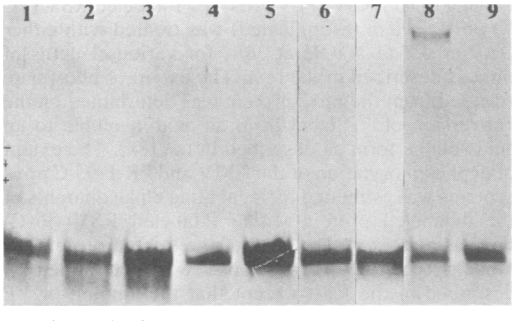

FIG. 5. SDS-polyacrylamide gel electrophoretic analysis of enzymatically and chemically dephosphorylated avian retrovirus pp12. Avian sarcoma virus pp12 (as indicated) was treated with either BAP or 0.2 M NaOH at 38°C for various lengths of time as described in the text. Samples of each reaction mixture containing 8 to 8.5 μg of pp12 were analyzed on a 10% polyacrylamide gel containing 0.1% SDS–0.1 M Tris-bicine at pH 8.2. Electrophoresis was carried out at 26 mA for 2.5 h, and protein was detected by staining with Coomassie blue as described in the text. pp12 protein treated with 0.2 M NaOH for 90 min: lane 1, RSV (Pr-C) pp12 (24.5 μg); lane 2, AMV pp12 (28 μg); lane 3, PR-E 95C pp12 (26 μg). Untreated pp12: lane 4, AMV pp12 (23 μg); lane 5, PR-E 95C pp12 (24 μg); lane 6, RSV (Pr-C) pp12 (24 μg); lane 7, an autoradiogram of the ^{32}P-labeled RSV (Pr-C) pp12 protein. Lane 8, RSV (Pr-C) pp12 (8.5 μg) treated with BAP for 27 h; lane 9, RSV (Pr-C) pp12 (17 μg). Migration of the protein was from the negative pole to the positive pole.

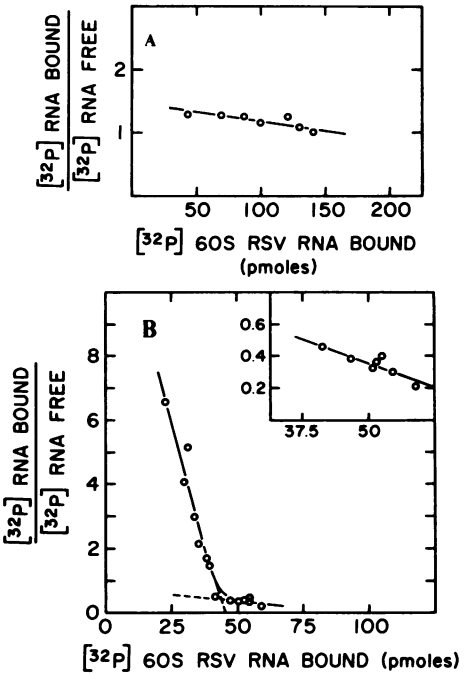

FIG. 6. Scatchard plot of the data for 60S RSV (Pr-C) RNA binding to pp12 protein dephosphorylated by alkali or BAP treatment. AMV pp12 was treated with 0.2 M NaOH for 60 min as described in the text, releasing 70% of its associated phosphate. RSV (Pr-C) pp12 was treated with BAP for 5 h at 38°C as described in the text, releasing 21% of its associated phosphate. (A) Scatchard plot of the data for binding of dephosphorylated AMV pp12 to 60S RSV (Pr-C) RNA. (B) Scatchard plot of the data for binding of dephosphorylated RSV (Pr-C) pp12 to 60S RSV (Pr-C) RNA. The insert represents an expanded scale of the Scatchard plot between 37 and 70 pmol of RNA bound.

carried out for pp12 protein from which various amounts of phosphate were released by treatment with either alkali or BAP. The resulting K_{app} values are plotted in Fig. 7 as a function of the extent of removal of phosphate from the pp12 protein. The average K_{app} for binding RNA appeared to decay exponentially as phosphate was released from the pp12 protein. Furthermore, either method of dephosphorylation of RSV (Pr-C) pp12 resulted in a similar decrease in the average K_{app} for binding RNA (Fig. 7). These results, taken together with those in Fig. 3 and 6, indicate that the decrease which we measured in the K_{app} for binding RNA is the result of dephosphorylation of the protein. The shift from the high-affinity to the low-affinity binding state at pH 7.5 was apparently complete with 50 to 60% of the labeled phosphate was released from the pp12 protein. This suggests that there may be two populations of phosphoserine residues on the pp12 protein, one of which is responsible for the change in RNA-binding activity.

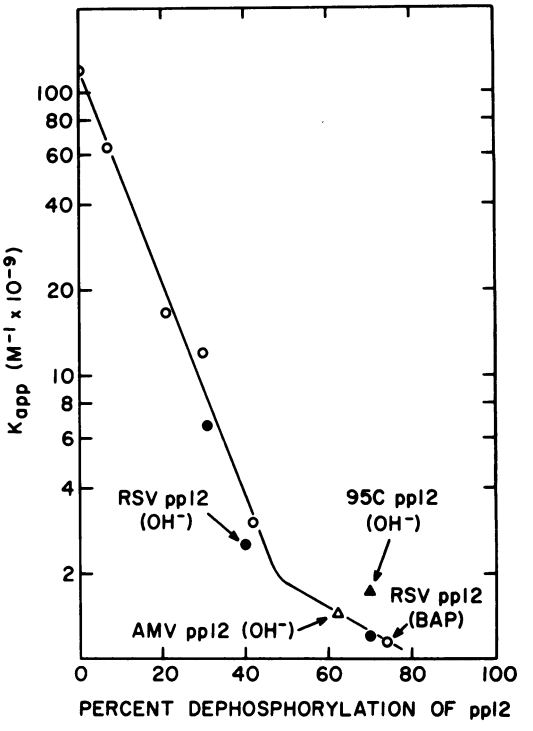

FIG. 7. Effect of enzymatic and alkaline dephosphorylation of the avian retrovirus pp12 protein on its binding to 60S RSV (Pr-C) RNA. ^{32}P-labeled RSV (Pr-C) pp12 protein (as indicated) was treated with either BAP or 0.2 M NaOH at 38°C for various lengths of time as described in the text. The extent of phosphate released from the pp12 protein was determined by the conversion of ^{32}P label from an acid-insoluble to an acid-soluble form as described in the text. The extent of dephosphorylation of the AMV and PR-E 95-C pp12 proteins was estimated by incubating equal amounts of the unlabeled protein with ^{32}P-labeled RSV (Pr-C) pp12 as a marker. The K_{app} of the treated protein for binding 60S RSV (Pr-C) RNA was determined as described in the text, except that the amount of ^{32}P label remaining with the protein, which was less than 5% of the total ^{32}P labeled, was subtracted from the amount of ^{32}P label retained on the nitrocellulose filter when the RNA was present. The pp12 protein was separated from the alkaline phosphatase before the RNA-binding determination as described in the text. Symbols: ○, RSV (Pr-C) pp12 treated with BAP; ●, RSV (Pr-C) pp12 treated with 0.2 M NaOH; △, AMV pp12 treated with 0.2 M NaOH; ▲, PR-E 95-C pp12 treated with 0.2 M NaOH.

DISCUSSION

This report provides evidence that retrovirus pp12 proteins [AMV, RSV (Pr-C), and PR-E 95C] have a pH-dependent affinity for single-stranded polynucleotides. Binding to double-stranded polynucleotides is pH independent and occurs only with low affinity. Arginine and lysine residues, but not cysteine residues, appear to be involved in both binding processes. The conversion of the pp12 protein from the high-affinity to the low-affinity binding state can be mediated either by direct removal of the phosphate group from the protein or by protonation of a residue presumed to be phosphoserine. (Other experiments in progress have shown that the RNA-binding activity of the chemically dephosphorylated pp12 protein can be completely restored by its phosphorylation, catalyzed by a protease-activated kinase I [23] purified from rabbit reticulocytes [L. Collins, J. Traugh, and J. Leis, manuscript in preparation].) Since decreasing the pH causes a decrease in the affinity of the pp12 protein for RNA, a conformational change in the protein rather than electrostatic repulsion may be responsible for the change in K_{app}. This interpretation would be consistent with the finding that the pp12 protein must be intact to achieve the pH-dependent high-affinity RNA-binding state, since this finding was not observed with the two cyanogen bromide peptides either alone or mixed together. In addition, preliminary studies of the UV difference spectrum of the AMV pp12 protein at pH 3.8 and 7.1 are suggestive of a conformational change in the protein.

The characteristics of the phosphorylated residue(s) of pp12 are incompletely understood at present. All of the amino acids that are phosphorylated in pp12 are phosphoserine residues, since we do not detect phosphothreonine or phosphotyrosine residues, in agreement with the results of Lai (13). As yet we do not know which of the serine residues of the protein are phosphorylated, although we have shown that when ^{32}P-labeled pp12 is digested with cyanogen bro-

mide, all of the ^{32}P label is associated with the amino-terminal fragment (T. Vanaman and J. Leis, unpublished data). Thus, one or more of the four serine residues in the amino-terminal cyanogen bromide peptide of the pp12 protein are phosphorylated. Further studies of this aspect of the pp12 protein are in progress.

Based on the data in this report, we would propose a biological model for packaging and uncoating of viral RNA that involves the two binding states of the pp12 protein. The pp12 protein, after being translated as part of the Pr 76 *gag* precursor polypeptide, is phosphorylated. The phosphoprotein then binds to the single-stranded regions of the 35S viral RNA and, in conjunction with the pp19 protein and reverse transcriptase, forms a ribonucleoprotein complex that becomes the nucleocapsid of the virus. (The proteolytic processing of the Pr 76 *gag* precursor could occur either before or after these components bind to RNA.) During the eclipse phase of the virus, the pp12 protein is dephosphorylated by action of a protein phosphatase. There are at least two such enzymes present in virions which have been partially purified (J. Leis, unpublished data). The pp12 protein, now in its low-affinity RNA-binding form, presumably can be displaced from the RNA by reverse transcriptase as it transcribes the RNA. The possibility that the pp12 protein stimulates reverse transcription of viral RNA in the presence of a protein phosphatase is being investigated.

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