BINDING SITES FOR TYPE C VIRAL PHOSPHOPROTEIN ON THE VIRAL RNA GENOME

Arup Sen, Doris Larson, and George J. Todaro

Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Md.

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SUMMARY: The distribution of binding sites for R-MuLV p12 phosphoprotein on the viral genome has been examined. Ribonucleoprotein complexes formed using 3'-poly A-containing viral RNA fragments of varying lengths and *in vitro* radioiodinated p12 protein have been analyzed by sedimentation velocity and buoyant density gradients. Binding sites for 2-3 molecules of p12 protein can be detected within the first 400 nucleotides from the 3'-poly A segment. The possible presence of binding sites near the middle of the genome (~2500 nucleotides from the 3'-end) and very close to the 5'-terminus (within the terminal 100-200 nucleotides) is also indicated.

INTRODUCTION: The major structural phosphoprotein of type C viruses specifically binds to the homologous viral 70S genome or its 35S subunits (1,2). Our previous studies with the infectious rodent viruses, the endogenous primate and cat viruses and the avian viruses indicated that only a few molecules (8-10 per 70S genome and 4-5 per 35S subunit) of the protein can bind to the homologous viral RNA molecules (1,3,4). We now present data to define the regions on the viral RNA genome where the binding sites are located. We have used poly A-containing viral RNA fragments of different sizes and estimated the stoichiometric ratio of protein bound to RNA, at protein saturation, for each of these size classes. The data indicate that the binding sites are arranged in a nonrandom, clustered pattern with one region close to the 3'-poly A terminus of the genomic RNA.

MATERIALS AND METHODS: All cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum.

Rauscher murine leukemia virus (R-MuLV) grown in NIH/3T3 cells (5) were shifted to phosphate-free medium (GIBCO, Grand Island, N. Y.) supplemented with 5% dialyzed calf serum (6 hr at 37°C) and labeled with carrier-free [^{32}P]phosphoric acid (New England Nuclear, Boston, Ma.) (100 μCi/ml) for 12 hr. Culture fluids were collected every 4 hr and stored on ice for 30 hr. Labeled virus was harvested and purified by banding in a sucrose gradient to 1.16 gm/cc

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as described previously (3). Unlabeled virus for viral RNA purification was obtained in a similar manner from cells maintained in normal medium. Virus for protein purification was obtained from culture supernatants harvested at 12 hr intervals and stored at -20°C until processing.

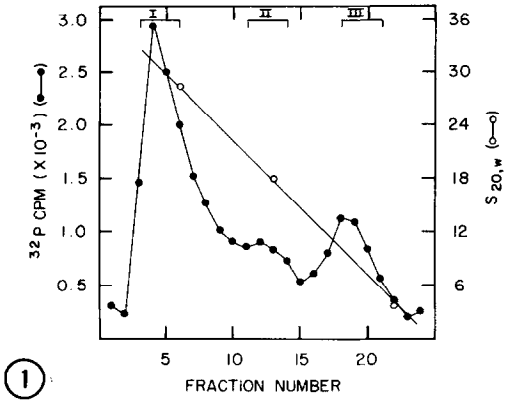
In order to obtain viral RNA, radiolabeled or unlabeled R-MuLV was disrupted by sodium dodecyl sulfate, treated with proteinase K (E. Merck, Darmstadt, Germany) and extracted with chloroform-isoamyl alcohol mixture (24:1 v/v); viral 70S RNA was then purified by sucrose velocity sedimentation as described previously (3).

Viral p12 protein was purified by polyethylene glycol extraction and Sephadex G-75 gel filtration as described (3). The purified protein was stored at -80°C in 10 mM Na-phosphate buffer (pH 7.5) containing 25 mM NaCl. Ten µg of protein were iodinated by a minor modification of the chloramine T method (6) to a specific activity of $\sim 2 \times 10^6$ cpm/µg and stored on ice with 50 µg/ml bovine serum albumin carrier protein until use.

Binding assays were performed in 0.01 M Tris-HCl buffer (pH 7.2) containing 50 mM NaCl and 2 mM EDTA and stabilized by formaldehyde crosslinking as described previously (3) and illustrated in the text below. For the analysis of nucleoprotein complexes by $CsSO_4$ buoyant density gradient, incubation mixture in a volume of 75 µl was added to 5.1 ml of $CsSO_4$ at a density of 1.56 gm/cc. The mixture was centrifuged to equilibrium in a Spinco SW50.1 rotor at 20°C for 60 hr at 31,000 rpm. Fractions were collected from the bottom; CCl_3COOH precipitable radioactivity was measured; refractive indices of sample fractions were measured for density calibration of the gradient.

Protein was measured by the method of Lowry et al. (7) and by absorbance at 280 nm. The unlabeled viral RNA was measured by absorbance at 260 nm (40 µg RNA = 1.0 A_{260}).

RESULTS AND DISCUSSION: The neutral sucrose sedimentation profile of ^{32}P-labeled 70S viral RNA, heat denatured in the presence of formamide, is shown in Figure 1. In a virus preparation obtained from culture supernatant harvested every 3-4 hr, the majority of the viral RNA migrated at 30-35S. However, detectable amounts of an intermediate (~20S) and a small size class RNA (<10S) were also recovered. The three size classes were pooled separately (as indicated by brackets in Fig. 1). Each pool was adjusted to 0.5 M NaCl and passed over an oligo dT_{12-18}-cellulose column (Collaborative Research, Waltham, Ma.) equilibrated with TE buffer containing 0.5 M NaCl. Unbound viral RNA was rinsed with the column buffer; bound RNA was then eluted with TE buffer without any salt. This RNA was readjusted to 0.5 M NaCl and oligo dT-cellulose selection was repeated. The final poly A-containing viral RNA was recovered in 300-400 µl of TE buffer. The unbound viral RNA from each



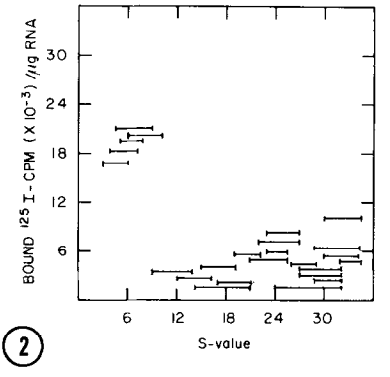


Figure 1: Preparative sucrose velocity sedimentation gradient of denatured viral RNA. ^{32}P-labeled 70S viral RNA extracted from rapid harvest virus was purified by sucrose velocity gradient and concentrated by ethanol precipitation (3). The viral RNA was resuspended in TE buffer (0.01 M Tris [pH 7.2] and 0.002 M ethylenediaminetetraacetic acid [EDTA]), heat denatured at 65°C for 90 s in the presence of 3% freshly deionized formamide, and centrifuged through a linear 5-20% sucrose gradient in TE buffer with 0.01 M NaCl at 20°C in a Spinco SW41 rotor (4.5 hr at 38,000 rpm). Fractions were collected and acid precipitable ^{32}P-radioactivity was measured in an aliquot of each fraction. ^{3}H-labeled standard RNA markers of known sedimentation values were centrifuged in a parallel gradient.

Figure 2: The extent of ^{125}I-p12 protein binding to poly A-containing viral RNA molecules of different size classes. Unlabeled, denatured viral RNA molecules of different sizes were prepared in a manner analogous to that illustrated in Fig. 1. Poly A-containing fraction from each class was obtained by oligo dT-cellulose chromatography and used to measure their ability to bind ^{125}I-p12 protein. The figure represents the amount of ^{125}I-p12 protein bound per µg of RNA from several different size classes obtained from different preparations. The extent of binding was determined as illustrated in the text.

size class was concentrated by ethanol precipitation and resuspended in TE buffer.

Poly A-containing unlabeled viral RNA molecules of different size classes at comparable molar concentrations were adjusted to 0.05 M with NaCl in 50 µl incubation volume and an approximately 1000-fold molar excess of ^{125}I-labeled p12 protein was added to each. Binding incubation was carried out at 22°C for 1.5 hr, the products were stabilized by formaldehyde crosslinking (1.8% final, 6-8 hr at 4°C) and analyzed by sedimentation velocity as described previously (3). The lengths of centrifugation time were varied appropriately to obtain separation of nucleoprotein complexes from the unbound protein. For each size

class, the ^{125}I-protein counts sedimenting at the RNA size region of the gradient were added and the protein counts in that region of a parallel gradient of a control incubation of protein without any RNA or with a heterologous RNA were subtracted as background. The RNA molecules containing poly A always bound stoichiometrically more p12 protein than did the RNA molecules without poly A. This suggested that there may be binding sites in close proximity to the poly A segment. In order to test this, parallel incubations were performed using the R-MuLV p12 protein and small poly A-containing viral RNAs from R-MuLV, from the endogenous cat virus (RD-114), and from the avian virus (RSV). Binding of the R-MuLV p12 protein was detected only to the homologous RNA, indicating that the poly A segment itself did not contribute to the higher extent of p12 protein binding.

In Fig. 2 the weight ratio of protein to RNA is presented as a function of the length of RNA fragments from the 3'-poly A stretch. A high level of binding can be seen between the 5-9S size class (200-400 bases) from the 3'-end; beyond this stretch there is a reduction in the weight ratio of protein to RNA while the molar ratio remains constant. The weight ratio appears to go up in the size range of 18-23S from the 3'-end, suggesting another binding region near the middle of the genomic subunit. There may also be an increase in the bound protein to RNA weight ratio with near full-length subunit; however, this was not reproducibly obtained. In the avian sarcoma viruses and murine leukemia viruses the 35S RNA subunit has been shown to possess common oligo nucleotides repeated at the 5'- and the 3'-ends (8-11). With our methods, however, we cannot unequivocally prove the presence of all the nucleotides at the 5'-end in the near 35S size RNA molecules prepared by sedimentation velocity gradient. Consequently, we cannot determine, by these techniques, whether there also exists a specific binding site close to the 5'-end of the genome.

An estimate of protein/RNA weight ratio can be obtained by cesium sulfate equilibrium density gradient analyses of the ribonucleoprotein complexes.

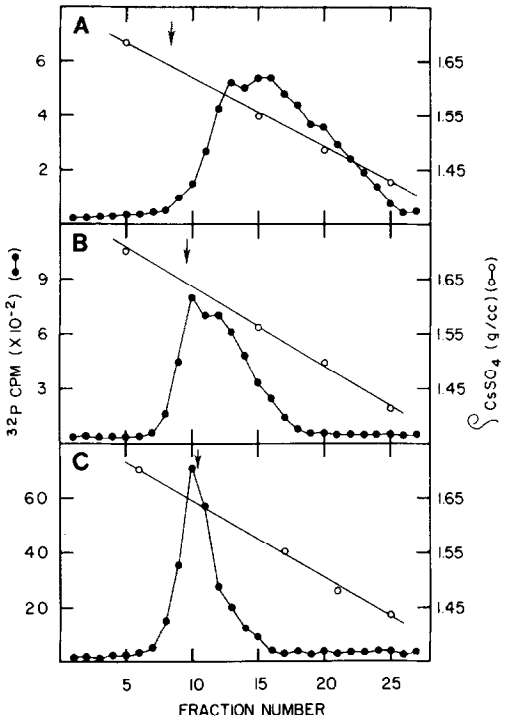


Figure 3: Buoyant $CsSO_4$ density gradient analysis of nucleoprotein complexes formed with ^{32}P-labeled, poly A-containing viral RNAs of different size classes. Poly A-containing ^{32}P-labeled viral RNA fragments were obtained by velocity sedimentation and oligo dT-cellulose chromatography. A vast molar excess of unlabeled p12 protein was added to comparable molar amounts of each size class of RNA. The in vitro complexes were formaldehyde crosslinked and subjected to equilibrium density gradient centrifugation as described in the Materials and Methods. The position of the arrow indicates the peak of pure viral RNA in a parallel gradient.

Panel A: 5-9S size poly A-containing viral RNA
Panel B: 18-23S size poly A-containing viral RNA
Panel C: >30S size poly A-containing viral RNA

^{32}P-labeled poly A-containing viral RNA fragments of three major size classes, (i) short (5-9S), (ii) intermediate (18-23S), and (iii) long (>30S), were incubated with excess of unlabeled p12 protein and after formaldehyde cross-linking the products were analyzed by $CsSO_4$ buoyant density centrifugation. The results are presented in Fig. 3. As seen in panel C the large size RNA did not show a detectable shift in density from pure RNA. The short size class (5-9S) showed a considerable shift in density (about 0.10 gm/cc)

(panel A) while the intermediate size class showed a variable amount of skewing of the [^{32}P]radioactivity profile toward the lighter density region (panel B). Assuming pure type C viral RNA to have a density of 1.64 gm/cc in cesium sulfate (as observed in the control RNA equilibrium gradients) and pure protein to be 1.30-1.32 gm/cc, a shift of 0.10 gm/cc density for a 5-9S size RNA (i.e., ave. ∿250 nucleotides) indicates a p12 protein/RNA molar ratio of ∿2.8 for the shortest size class (for the details of calculation see Sen, Sherr and Todaro, 1976 [1]). This would indicate that two or possibly three molecules of p12 protein bind within the first 400 nucleotides from the 3'-poly A end. The skewed ^{32}P profile to the lighter density region and an increase in the p12/RNA weight ratio with the intermediate size (18-23S) RNA molecules might reflect the presence of binding sites in the middle of the genome.

The specific binding of the p12 protein to the homologous viral RNA *in vitro* (1-4), the association of a limited number of the p12 molecules with the genome *in situ* (3) and the regulation of the extent of binding by the level of phosphorylation of the protein (12) suggested a regulatory role of this nucleoprotein interaction in the viral life cycle. An understanding of the manner in which the binding sites are distributed on the viral genome would allow localization of candidate regulatory sequences on the viral RNA. A physical map order of the different viral genes on the linear infectious viral genome has been established (13-16). The funtion of the "common" sequence region which extends approximately 1000 bases from the 3'-poly A end (17,18) is unknown. Our data indicate the presence of specific phosphoprotein binding sequences in this region. No detectable difference in the extent of binding of avian p19 protein to viral RNA purified from nondefective and transformation defective (td) strains of avian type C viruses has been found, suggesting no binding sites in the *sarc* region (Sen, unpublished data). The binding sequences in the different parts of the viral genome might represent the same base sequence and/or possess some common secondary structure. Purified ribonucleoprotein complexes formed with poly A-containing RNA fragments of different

size classes can also be generated *in situ* by UV-irradiation of intact virion particles (3). These complexes contain only p12 protein molecules and preliminary mapping data obtained with such complexes agree with the *in vitro* data described above (Sen, unpublished observation). With the availability of approaches to generate significant quantities of subgenomic ribonucleoprotein complexes, either reconstructed *in vitro* or formed *in situ*, it will be possible to purify the ribonuclease resistant fragments and characterize specific binding sequences on the viral genome.

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