Binding of Human Immunodeficiency Virus Type 1 (HIV-1) RNA to Recombinant HIV-1 *gag* Polyprotein

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We have expressed the human immunodeficiency virus type 1 (HIV-1) *gag* polyprotein (Pr55^{gag}) in bacteria under the control of the T7 phage gene 10 promoter. When the gene encoding the viral protease is included in *cis*, in the –1 reading frame, the expected proteolytic cleavage products MA and CA are produced. Disruption of the protease-coding sequence prevents proteolytic processing, and full-length polyprotein is produced. Pr55^{gag}, separated from bacterial proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immobilized on nitrocellulose membranes, binds RNA containing sequences from the 5' end of the HIV-1 genome. This binding is tolerant of a wide range of pH and temperature but has distinct salt preferences. Conditions were identified which prevented nonspecific binding of RNA to bacterial proteins but still allowed binding to Pr55^{gag}. Under these conditions, irrelevant RNA probes lacking HIV-1 sequences bound Pr55^{gag} less efficiently. Quantitation of binding to Pr55^{gag} by HIV-1 RNA probes with deletion mutations demonstrated that there are two regions lying within the HIV-1 *gag* gene which independently promote binding of RNA to Pr55^{gag}.

Assembly of infectious retroviral particles requires the selective encapsidation of unspliced, viral genomic RNA from a pool of cellular and viral RNAs. Host RNA synthesis is not suppressed by retroviral infection, and exogenous viral RNA constitutes only about 1% of mRNA isolated from cells infected by avian or murine retroviruses. Nevertheless, little heterologous RNA is encapsidated (4). In addition, though spliced and unspliced forms of viral RNA may be present in the cytoplasm in roughly equal amounts, at least 95% of the packaged RNA is unspliced genomic RNA (33).

Subgenomic viral RNAs share 5' and 3' termini with genomic RNA, suggesting that *cis*-acting encapsidation signals are encoded at least in part by sequences which are removed by splicing. Analysis of the phenotypic effects of deletion mutations in avian, murine, and human retroviral sequences has demonstrated that the major packaging signal is found near the 5' end of the RNA, but 3' to the splice donor site (2, 10, 39, 45, 70); deletion of this packaging region decreased packaging of the altered RNA to 0.1 to 10% of wild-type levels. The fact that viral RNAs containing these deletions are still packaged more efficiently than heterologous RNAs (20, 33, 39, 44) suggests that there may be additional packaging signals. One such signal in Moloney murine leukemia virus (M-MuLV) was identified within the *gag* coding region because of its stimulatory effect on packaging vectors and on the packaging of heterologous RNAs (1, 3, 6). Another signal was identified at the 5' end of U5 (49), though this region is only needed in the context of whole viral RNA.

Rous sarcoma virus (RSV) is unusual in that the major encapsidation sequence is present in both spliced and unspliced RNAs (33, 34), suggesting that other regions of the RSV genome contain *cis*-acting packaging signals. Attempts to identify them have been only moderately successful. One group reported that deletions in the region encoding the RSV *gag* matrix protein (140 bp downstream of the splice donor) block packaging (54), though others were not able to confirm

the importance of this sequence (51). RSV packaging may require at least one copy of a 115-bp repeat from the 3' end of the genome (64), but this sequence is also present in both genomic and subgenomic RSV RNAs.

The primary translation product of the *gag* gene is a polyprotein, which is the only *trans*-acting factor thought to be required for packaging. Virion particles assemble without *env* glycoprotein (41, 55), reverse transcriptase (19, 55, 60), viral protease (14, 26), or genomic RNA (40, 42), whereas mutations in *gag* often prevent assembly (26, 28, 61). Particles can be released when the only viral translation products are *gag* encoded (23, 55, 63) and have been shown in one case to package viral genomic RNA (63). The *gag* polyprotein is cleaved into the mature viral proteins by the viral protease at the time of particle budding (71), but it is probably the *gag* precursor polyprotein and not any of its cleavage products which directs the specific packaging of genomic RNA; virions produced without cleavage specifically package viral RNAs (14).

Purified *gag* polyprotein from M-MuLV virions has been shown to bind nucleic acids (32, 56), though no attempts were made to demonstrate binding specificity. Of the polyprotein cleavage products the nucleocapsid (NC) and matrix (MA) proteins have been found to bind nucleic acids, though with no specificity for viral sequences (16, 50, 59, 65). The NC proteins of all retroviruses possess a conserved motif known as the Cys-His box (12), which is thought to be analogous to the zinc finger motif of many DNA-binding proteins (7). Mutations which disrupt the Cys-His motif disrupt packaging of RNA in virions (18, 24, 25, 31, 47), and there are specific NC mutants with a suggestion of altered binding specificity (24, 47).

To study the interaction of *gag* protein and RNA, we have expressed the human immunodeficiency virus type 1 (HIV-1) *gag* polyprotein (Pr55^{gag}) in *Escherichia coli*. When immobilized on nitrocellulose, this protein binds RNA probes with specificity for RNAs containing HIV-1 sequences at the 5' end of the genome. Employing this blotting protocol, we have evaluated the ability of Pr55^{gag} to bind different HIV-1 RNA probes.

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MATERIALS AND METHODS

Cloned DNAs. Retrovirus sequence numbering is with respect to the 5' edge of the 5' long terminal repeat of the DNA provirus. pHXBC2 (21) contains an infectious clone of HIV-1 provirus human T-cell leukemia virus type IIIB and was a gift of William A. Haseltine (Harvard Medical School). pNCA contains a proviral copy of M-MuLV (11). pGEMEX-1 (Promega, Madison, Wis.) is a commercial vector containing the T7 gene 10 promoter (66) upstream from an *NdeI* site at the gene 10 start codon. pSP72 (Promega) is a commercial vector with a polylinker between the T7 and SP6 promoters. M13mp18 (73) was used as a vector for oligonucleotide-directed mutagenesis.

Bacterial cultures. Plasmid DNAs were cloned in *E. coli* DH5 α by standard methods (57). Plasmids were isolated either from 2-ml cultures by the boiling lysis method or from 250-ml cultures by the Triton lysis method with subsequent purification by equilibrium density gradient centrifugation in cesium chloride (57). JM109 was used to produce infectious single-stranded DNA with M13. CJ236 (*dut ung F'*) was used for mutagenesis by the method of Kunkel (37). JB-DE3, a *lon* mutant strain containing the T7 polymerase gene under the control of the *lac* mutant UV5 promoter, was used to express protein from plasmids containing the T7 promoter; this strain was a gift of George Gaitanaris (Columbia University).

Cloning and mutagenesis. An expression construct for the production of native HIV-1 *gag* protein was engineered from pHXBC2 and pGEMEX-1 DNAs by employing standard techniques (57). A *SacI-PstI* fragment from pHXBC2 (nucleotides 679 to 1416) was subcloned into M13mp18 and mutagenized with an oligonucleotide of sequence 5'-CGCTCTCGCACCCATATGTCCTTCTAGC-3'. The resulting mutation created an *NdeI* site that includes the *gag* initiation codon. pGEMEX-1 was modified to remove a redundant *NdeI* site; the parent plasmid was digested with *AatII* and *BspMII*, overhangs were removed or filled with the Klenow fragment of DNA polymerase, and the blunt ends were ligated with T4 DNA ligase. The resulting expression vector was prepared for the HIV-1 *gag* gene insert by digestion with *NdeI* and *NsiI*. The mutant HXBC2 sequence from the *NdeI* site at the *gag* initiation codon (nucleotide 789) to the *NsiI* site in the middle of *pol* (nucleotide 2924) was inserted into the expression vector to create pT7HG(pro⁺). pT7HG(pro⁻) contained a deletion in the viral protease coding sequence, created by digesting with *BclI* and *MscI*, treating with Klenow fragment, and then cyclizing with T4 DNA ligase.

pSP72 was used as the parent plasmid for construction of DNA templates for the in vitro synthesis of RNA. pHRBP contains a *BglII-PvuII* fragment of HXBC2 (nucleotides 473 to 1144) inserted into the polylinker of pSP72. pMRSP contains a *SacI-PvuII* fragment from pNCA (nucleotides 419 to 1886). Truncated forms of pHRBP were engineered by digestion with selected restriction enzymes, treatment with Klenow fragment, and then ligation with T4 DNA ligase.

Expression of protein in bacteria. Cultures (2 ml) from isolated colonies of strain JB-DE3 carrying the selected expression vector were grown overnight at 37°C in NZCYM/ampicillin (57). Cultures were diluted 1:10 in NZCYM/ampicillin and grown at 37°C for 1 h prior to induction of the T7 polymerase with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Standard induction with IPTG was for 3 h at 37°C. Following induction, cultures (1.5 ml) were centrifuged and the bacterial pellet was boiled in 100 μ l of sodium dodecyl sulfate (SDS) loading buffer containing 2% SDS, 100

mM dithiothreitol (DTT), 10% glycerol, 50 mM Tris-Cl (pH 6.8), and 0.1% bromophenol blue. Proteins from whole bacterial lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (38) and either stained with Coomassie blue or detected immunologically by Western immunoblot (9). In the latter case, proteins were transferred onto nitrocellulose membranes (BA85; pore size, 0.45 μ m; Schleicher & Schuell, Inc., Keene, N.H.) with a Bio-Rad Transblot apparatus in which a 0.19 M glycine-0.025 M Tris base was used at 350 mA for 2 h. Blots were probed with monoclonal antibodies against the HIV-1 MA protein, p17 (no. 9282; Biotech Research Laboratories, Billerica, Mass.), against the CA protein, p24 (no. 9283, Biotech Research Laboratories), or against the HIV-1 *gag* carboxy-terminal protein, p6 (68) (a gift from M. G. Sarngadharan and Fulvia Di Marzo Veronese, Advanced Bioscience Laboratories, Inc., Kensington, Md.). Antibody binding was revealed with an alkaline phosphatase reaction employing a Vectastain Elite kit (Vector Laboratories, Burlingame, Calif.).

In vitro synthesis of RNA. Plasmid DNAs were purified on cesium gradients and then linearized to serve as templates for RNA synthesis. Radiolabeled RNAs were synthesized with either T7 or SP6 RNA polymerase (Boehringer Mannheim, Indianapolis, Ind.) in 20- μ l volumes for 60 min at 37°C (57). Reactions contained 1 μ g of linear plasmid DNA; 40 mM Tris-Cl (pH 7.5); 6 mM MgCl₂; 2 mM spermidine; 10 mM NaCl; 10 mM DTT; 50 U of human placental RNase inhibitor (Boehringer Mannheim); 0.5 mM (each) ATP, GTP, and UTP; 12 μ M CTP; and 50 μ Ci of [α -³²P]CTP (800 Ci/mmol). The reaction products were treated with 1 U of DNase I (Promega) to remove the template, extracted twice with a 1:1 mixture of phenol and chloroform and once with chloroform, and then precipitated twice in ethanol. The integrity of the RNA products was assessed by electrophoresis on acrylamide gels under denaturing conditions with 8.3 M urea.

Northwestern blot. Following a standard induction, bacterial pellets were heated to 100°C in SDS loading buffer and the proteins were separated by SDS-PAGE and electroblotted to nitrocellulose membranes. The nitrocellulose membranes were incubated for 12 h at 4°C in NW buffer (30 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.5], 200 mM KCl, 10 μ M ZnCl₂, 2 mM DTT, and 500 μ g of heparin per ml) or in modified buffers as indicated. Uniformly labeled RNA probes were incubated with blots at 22°C for 1 h. Probe concentrations were in the linear range for binding. Typically, between 5×10^5 and 3×10^6 cpm (roughly 3×10^{-2} pmol or 6 ng of RNA) of the 671-nucleotide probe, synthesized by using *PvuII*-linearized pHRBP as a template, was added to each strip. Blots were washed twice (10 min per wash) in NW buffer, and binding of RNA probe was demonstrated by autoradiography. The amount of probe bound was determined with a Betascope model 603 Blot Analyzer (Betagen Corp., Waltham, Mass.). In a typical experiment, 300 cpm (9×10^{-6} pmol or 1.8 pg) of probe synthesized by using *PvuII*-linearized pHRBP was bound per lane.

RESULTS

Expression of recombinant HIV-1 *gag* protein. To place the HIV-1 *gag* sequences downstream of the T7 phage gene 10 promoter, a mutation was first introduced at the beginning of the *gag* coding region by oligonucleotide-directed mutagenesis (Fig. 1). This created a new restriction site (*NdeI*) which allowed us to insert the *gag* start codon in the proper

FIG. 1. Construction of HIV-1 *gag* expression plasmids. An *Nde*I restriction site was engineered at the beginning of the HXB2C *gag* coding region to permit transfer of the complete intact *gag* sequence. The *Nde*I-*Nsi*I fragment shown was subcloned into a modified pGEMEX vector to create pT7HG(pro⁺). pT7HG(pro⁻) was created by deleting a *Bcl*I-*Msc*I fragment from the protease coding region. LTR, long terminal repeat.

position with respect to the gene 10 Shine-Dalgarno sequence. Our initial expression construct [pT7HG(pro⁺)] contained the complete *gag* coding sequence and a portion of the *pol* gene, including the protease and part of the reverse transcriptase coding sequences (Fig. 1).

Protein expression in IPTG-induced bacteria transformed with pT7HG(pro⁺) was evaluated by SDS-PAGE of bacterial lysates (Fig. 2). Coomassie-stained gels showed only subtle changes in expression compared with untransformed cells, cells transformed with vector alone, or uninduced cells. When proteins were blotted to nitrocellulose membranes and probed with monoclonal anti-p24 antibody (Fig. 2B), however, a single band was observed at 41 kDa and a doublet was observed at 25/24 kDa. These bands are of the size expected to result from partial and complete cleavage of Pr55^{gag} by the viral protease; p41 is an incomplete cleavage product containing the MA and CA proteins, and p24 and p25 are variant forms of the CA protein differing at their carboxy termini. Similarly, bands of 41 and 17 kDa were

seen when monoclonal anti-p17 antibody was used for immunoblotting (data not shown), which is consistent with formation of cleaved *gag* proteins. We concluded that induction with pT7HG(pro⁺) produced an active viral protease which precluded the stable production of Pr55^{gag}. To confirm this idea, and to generate unprocessed Pr55^{gag}, we constructed pT7HG(pro⁻) (Fig. 1), which contains a deletion in the viral protease coding region. Induction of protein from bacteria transformed with this construct produced a protein of 55 kDa which was identifiable on Coomassie-stained gels (Fig. 2A). Two faster-migrating bands were consistently seen at 49 and 43 kDa; no proteins related to the normal cleavage products were formed. All three proteins were recognized by monoclonal antibodies to the matrix protein, p17, and to the capsid protein, p24, but only the largest was recognized by a monoclonal antibody to p6, the carboxy-terminal fragment of Pr55^{gag} (Fig. 2C); this suggests that p49 and p43 are C-terminal degradation products. The relative abundance of the three major proteins was remarkably constant in all experiments.

Pr55^{gag} preferentially binds RNAs containing HIV-1 sequences. We examined the ability of Pr55^{gag} to bind RNA synthesized in vitro by using plasmid pHRBP linearized with *Pvu*II as a template (probe BP; see below). The HIV-1 sequence in this construct begins near the RNA cap site, includes the region between the splice donor (nucleotide 742) and the *gag* gene (nucleotide 789), and contains the majority of the *gag* MA coding sequence. Our synthetic RNA probe had a specific activity of 1.5×10^8 cpm/ μ g and migrated as a discrete band at the expected position on denaturing acrylamide gels (data not shown).

In initial experiments we performed the binding in buffer containing EDTA and used bovine serum albumin (BSA) to block nonspecific binding. Several proteins unrelated to the expression construct—presumably bacterial nucleic acid-binding proteins—bound the RNA probe (Fig. 3A). Following induction of cells carrying pT7HG(pro⁺), a new protein of about 15 kDa was found to bind probe. One of the expected protease cleavage products of about this size would contain the NC protein. Following induction of cells carrying pT7HG(pro⁻), three proteins with mobility identical to that of Pr55^{gag} and its C-terminal degradation products bound probe strongly. Lysates retained activity for several weeks when stored at -70°C .

To eliminate binding of HIV-1 RNA to nonspecific bacterial proteins we made the binding conditions more stringent. Replacement of EDTA with ZnCl_2 and/or MgCl_2 eliminated binding to high-molecular-weight bacterial proteins (Fig. 3B). When BSA was replaced with heparin, binding by the bacterial proteins was greatly decreased while binding to Pr55^{gag} was not diminished. Under optimal conditions the binding to Pr55^{gag} and the two smaller proteins was the only binding detected. The amount of specific binding seen was linear with respect to the amount of lysate loaded on the gel. We observed specific binding within 30 s of incubation with 10^6 cpm (6 ng) of RNA probe, and maximum levels of binding were reached within 5 to 10 min. When blots were washed free of unbound probe and placed in fresh buffer, we saw no loss of signal after incubations of up to 4 h (data not shown).

Effects of monovalent cations on RNA binding to Pr55^{gag}. In pilot experiments, little binding activity was observed when KCl in the binding buffer was replaced with NaCl. Because of this observation we systematically examined the effects on binding of various concentrations of different monovalent cations (Fig. 4). No binding activity was seen if there was no

FIG. 2. Expression of HIV-1 *gag* protein. Following standard induction with IPTG (3 h except where indicated), bacteria were pelleted and boiled in SDS, and the proteins were separated by electrophoresis on SDS-polyacrylamide gels. (A) Coomassie stain of protein gel. Lane 1, no plasmid; lane 2, pT7HG(pro⁺); lanes 3 to 6, pT7HG(pro⁻), 0, 0.5, 1.0, and 3.0 h, respectively, postinduction. (B) Western blot with monoclonal anti-CA (p24) antibody. Lane 1, no plasmid; lane 2, vector alone without HIV-1 insert; lane 3, pT7HG(pro⁺); lane 4, pT7HG(pro⁻). (C) Western blot against pT7HG(pro⁻) with three different monoclonal antibodies. Lane 1, monoclonal anti-CA (p24) antibody; lane 2, monoclonal anti-MA (p17) antibody; lane 3, monoclonal anti-p6 antibody. Molecular mass markers are shown along the left side of panel A. Positions of the *gag* polyprotein precursor, p55, and of the viral protease cleavage products, p41 and p24/p25, are shown.

FIG. 3. Binding of HIV-1 RNA to recombinant HIV-1 *gag* protein. Following induction with IPTG, bacterial pellets were boiled in SDS, and proteins were separated by electrophoresis on SDS-polyacrylamide gels and blotted to nitrocellulose. Blots were probed with RNA synthesized in vitro from pHRBP DNA linearized with *Pvu*II. (A) Buffer conditions: 30 mM HEPES (pH 7.5), 100 mM K glutamate, 2 mM EDTA, 2 mM DTT, and 1 mg of BSA per ml. (B) Buffer conditions: 30 mM HEPES (pH 7.5), 100 mM K glutamate, 5 mM MgCl₂, 10 μ M ZnCl₂, 2 mM DTT, and 500 μ g of heparin per ml. Lanes 1, vector alone; lanes 2, pT7HG(pro⁺); lanes 3, pT7HG(pro⁻). The position of the *gag* precursor protein, p55, is shown.

monovalent cation present in the buffer. The minimal concentration of monovalent cation necessary for binding decreased with the increasing atomic number of the salt. Maximum binding was observed in the range of physiological salt concentrations, and higher concentrations of salt inhibited binding. Our standard NW buffer for all subsequent experiments included 200 mM KCl.

Effects of pH and temperature on RNA binding to Pr55^{gag}. We examined the effect of pH on HIV-1 RNA binding to Pr55^{gag} with several buffers (Fig. 5). The pH of all buffers was confirmed at the conclusion of each binding experiment. Strong binding was observed in a range of pHs from 5.5 to 9.5. This behavior was distinct from that of an isolated NC protein, which was reported as binding only in a narrow pH range (46). Binding was compared at 4, 22, and 37°C, and similar results were obtained at all three temperatures (data not shown). We performed subsequent binding experiments at 22°C.

Mapping of HIV-1 RNA for binding to Pr55^{gag}. To test the binding for sequence specificity, we compared the binding of our HIV-1 RNA probe with that of heterologous RNA probes. We also attempted to map the sequences necessary for binding by testing HIV-1 RNA probes with deletion mutations. For these experiments we used lysates from cells transformed with pT7HG(pro⁻). SDS-PAGE preparative gels were run and blotted to nitrocellulose; the filter was cut into identical vertical strips and incubated in standard NW buffer with different RNA probes. All probes were synthesized in vitro with a specific activity of 1.5×10^8 cpm/ μ g and assayed on denaturing polyacrylamide gels to evaluate their integrity. Each probe was tested at several concentrations to ensure that the amount of probe was always within the linear range for binding.

In the first series of experiments, we added the same microgram amount of RNA (i.e., the same amount of radioactivity) of each probe per ml and measured the radioactivity

FIG. 4. Effects of different monovalent cations on binding of HIV-1 RNA to recombinant HIV-1 *gag* protein. Blots were prepared with protein from bacteria carrying pT7HG(pro⁻) as described in the legend to Fig. 3 and probed with BP RNA synthesized in vitro. Binding buffer contained 30 mM HEPES, 500 μ g of heparin per ml, 10 μ M ZnCl₂, and 2 mM DTT, with the following salts: LiCl (A), NaCl (B), KCl (C), RuCl (D), and CsCl (E). Salt concentrations (millimolar) are listed at the bottom.

(in counts per minute) bound to Pr55^{gag} (Fig. 6). Results for the amount of each probe bound are reported as a percentage of the counts of the parental probe bound. The RNA-binding activity was highly specific for the HIV-1 RNA. We found that neither antisense RNA from pHRBP DNA nor RNA from pMRSP DNA, which contains sequences sufficient for M-MuLV packaging, bound significantly to Pr55^{gag}. When RNAs truncated at the 3' end were tested, binding activity was lost; the binding required sequences 3' to the *Bss*HII

site. Subsequent studies with other truncated RNAs identified two regions, each less than 200 nucleotides long, with strong independent binding activity: one lies between nucleotides 789 and 957, and the other lies between nucleotides 957 and 1144 (Fig. 6).

Previous studies of HIV-1 have shown that the sequence between the splice donor and the start of the *gag* gene are important for packaging (2, 10, 39). We found that RNA lacking this region (probe BP- Δ BsN) retained binding to 70% of the levels of the parent construct (Fig. 6). When conditions were made more stringent by adding increasing concentrations of NaCl to our standard NW buffer, this difference was gradually accentuated (Table 1). For example, with the addition of 200 mM NaCl, BP- Δ BsN RNA bound at 20% of the levels of the parental BP RNA. The relative binding of different RNAs was highly dependent on the exact buffer conditions employed.

In a second series of experiments we compared the binding of different RNAs to Pr55^{gag} when equal moles, rather than micrograms, of RNA per milliliter were added (Fig. 7). Results for the amount of each probe bound are reported as a percentage of the moles of the parental probe bound. Again, neither antisense RNA from pHRBP DNA nor RNA from pMRSP DNA bound significantly to Pr55^{gag}. Independent binding activity was again seen with RNAs retaining sequences between nucleotides 789 and 957 or between nucleotides 957 and 1144. The binding activity of BP- Δ BsN RNA was similar in these experiments as when equal micrograms of RNA were added (70 to 80% of parental RNA). The relative binding efficiency of these RNAs was found to be similar at 37°C as at the standard 22°C (data not shown). We conclude that whether probes were normalized for mass or for number of moles, and under a variety of experimental conditions, specific binding of HIV-1 RNA to Pr55^{gag} was observed.

Competition experiments. We attempted to block the binding of labeled BP RNA by preincubating blots with unlabeled RNAs from 1 to 24 h prior to the addition of labeled probe (data not shown). In these experiments we added 10⁶ cpm of

FIG. 5. Effects of pH on binding of HIV-1 RNA to recombinant HIV-1 *gag* protein. Blots were prepared with protein from bacteria carrying pT7HG(pro⁻) as described in the legend to Fig. 3 and probed with BP RNA. Blots were probed in solutions containing 500 μ g of heparin per ml, 200 mM KCl, 10 μ M ZnCl₂, and 2 mM DTT, with the following buffers: 20 mM glutamic acid (A), 10 mM Tris-acetate (B), and 10 mM HEPES-KOH (C). The pH of each lane is listed across the bottom.

FIG. 6. Binding of HIV-1 RNA fragments to Pr55^{gag} with RNA probes at the same concentration in micrograms per milliliter. Replicate blots were prepared with protein from bacteria carrying pT7HG(pro⁻) as described in the legend to Fig. 3. Blots were incubated for 24 h in 30 mM HEPES (pH 7.5), 500 μg of heparin per ml, 200 mM KCl, 10 μM ZnCl₂, and 2 mM DTT. Blots were probed for 1 h at 22°C with uniformly labeled T7 or SP6 RNA synthesized in vitro from linearized plasmids of the sequences indicated in the figure. Blots were washed twice for 10 min with the buffer described above, and radioactivity was measured with a Betascope Blot Analyzer. Results for the amount of each construct bound are reported as a percentage of the counts per minute of the parent construct bound.

BP probe, or about 6 ng of labeled RNA. Approximately 100 μg of yeast total RNA, yeast tRNA, or bovine total RNA was required to inhibit subsequent binding of probe to 20% of unblocked levels. One microgram of unlabeled BP RNA was sufficient to block binding down to about 20% of unblocked levels. RNAs synthesized from pMRSP DNA or from pHRBP DNA linearized at BssHII were between 2 and 10 times less effective than BP RNA in blocking subsequent binding. The trends that we observed in these competition experiments were consistent with our other binding data, but the magnitude of these effects was not sufficient to allow us to reliably study binding kinetics.

TABLE 1. Effects of salt concentration on binding of HIV-1 RNAs to Pr55^{gag}

NaCl (mM)	Radioactivity bound ^b (cpm) to probe:		Bound BPΔBSN/ bound BP
	BP	BPΔBSN	
0	1,079	729	0.68
50	910	558	0.61
100	537	255	0.47
200	243	52	0.21
300	19	2	0.11

^a Lysates from bacteria carrying pT7HG(pro⁻) were subjected to SDS-PAGE and blotted to nitrocellulose. Blots were incubated in standard NW buffer (see Materials and Methods), except that NaCl was added as indicated. Blots were probed for 1 h at 22°C.

^b Radioactivity bound to Pr55^{gag} was determined with a Betascope model 603 Blot Analyzer.

DISCUSSION

Expression of Pr55^{gag}. Pr55^{gag} expressed in bacteria appears to be a relatively stable protein, as has been found after expression in vitro (36), in yeasts (30, 35, 69), in insect cells (23, 43), and in vaccinia virus systems (22). In accord with the results of other laboratories, we reproducibly observed faster migrating species in addition to Pr55^{gag}. These shorter protein products appear to be C-terminal degradation products, since monoclonal anti-p6 antibody reacts only with p55 and not with p49 or p43. The host bacterium that we used for protein expression was deficient for the *lon* protease, suggesting that a different system must be responsible for the production of these proteins. The C terminus of Pr55^{gag} is relatively hydrophobic, and it has been reported that hydrophobic C termini render bacterially expressed proteins susceptible to an as-yet-undefined protease (52). Alternatively, p49 and p43 could result from the premature termination of translation. On the basis of the electrophoretic mobility of these proteins, we would predict that p49 lacks the p6 protein and that p43 lacks a portion of NC as well as all of p6.

Active viral protease is probably being produced in our system because the proteins produced after induction of pT7HGpro⁺, as detected with the appropriate monoclonal antibodies, are the expected sizes of the viral protease cleavage products. Disruption of the protease coding sequence by linker insertion mutagenesis (data not shown) or by deletion mutagenesis [pT7HG(pro⁻)] prevents the formation of these smaller proteins and allows us to express full-length Pr55^{gag}. The expression of active viral protease is surprising, since the protease coding sequence in HIV-1 is in

FIG. 7. Binding of HIV-1 RNA fragments to Pr55^{gag} with RNA probes at the same moles of RNA per milliliter. Blots were prepared and treated as described in the legend to Fig. 6. Results for the amount of each construct bound are reported as a percentage of the moles of the parent construct bound.

the *pol* reading frame, the -1 frame with respect to *gag*, and normally translation of the HIV-1 protease requires a ribosomal frameshift (29). There are several examples of frameshifting in prokaryotic systems (8, 13, 17, 62) and the frameshift signals from HIV-1 and mouse mammary tumor virus have been shown to work in bacterial systems (72). We suggest that viral protease is being translated via a frameshift mechanism in our system, yielding levels of protease sufficient for essentially complete processing of Pr55^{gag}. Alternatively, it may be that the protease region is expressed separately via an independent translation mechanism. The nearest start site 5' to the protease coding sequence in the *pol* reading frame is at nucleotide 2033.

Unlike Pr55^{gag} produced in eukaryotic systems (22, 23, 26, 30), our product is presumably not myristylated; *E. coli* lacks the enzyme necessary for this cotranslational modification. Nevertheless, the protein apparently assumes a functionally relevant structure and is cleaved by the viral protease in the same fashion as is the native viral protein. Though the viral protease will hydrolyze short, synthetic peptides corresponding to substrate cleavage sites (15, 58), there is reason to believe that cleavage of full-length polyprotein requires native conformation. One group has reported that protease would not cleave denatured substrate (27), and we saw no protease processing when we expressed p55 as a *trpE* fusion protein with the identical protease coding sequence as used for pT7HG(pro⁺) (data not shown). Furthermore, we have found that selected linker insertion mutations in the Pr55^{gag} coding sequence can disrupt proteolytic cleavages at sites remote from the mutation (data not shown). We presume that these mutations disrupt the conformation of the substrate. The viral protease may recognize primary sequence without concern for complex structure, but the conformation of the substrate may determine whether or not the sequence is accessible to the enzyme (67).

Binding of HIV-1 RNA to Pr55^{gag}. We have shown that Pr55^{gag} isolated from whole bacterial lysate by SDS-PAGE

and immobilized on nitrocellulose will bind RNA containing 5' HIV-1 sequences. In initial experiments we saw binding of probe to bacterial proteins, but under more stringent conditions we were able to eliminate that binding while retaining binding to Pr55^{gag}. Our assay is convenient in that there is no requirement for protein purification or for denaturation and renaturation steps; the protein appears to become competent to bind after simple removal of the SDS during an overnight incubation. We suspect that conformation is important for this binding activity because we had difficulty demonstrating binding with a closely related *trpE*-p55 fusion construct. The binding is tolerant of a reasonably wide pH range, in contrast to the narrow range seen for isolated NC protein (46). There is a requirement for salt with maximum binding in the range of physiological salt concentrations. Why binding improves with increasing atomic number for monovalent cations is not clear; we cannot say if the effect is on the RNA or the protein. A similar trend is seen with binding of *lac* repressor protein and operator DNA (5).

In competition experiments, preincubation of the blotted protein with unlabeled RNA demonstrated partial inhibition of subsequent labeled RNA binding. In a typical experiment we added 10^6 cpm of specific probe, which is roughly 3×10^{-2} pmol or 6 ng of RNA; of this added probe, only a very small fraction, roughly 10^{-5} pmol or 2 pg, actually bound to Pr55^{gag}. Preincubation of the blots with microgram quantities of unlabeled, specific RNA was partially effective in blocking subsequent binding. It is possible that the SDS-denatured protein may be continually renaturing during the binding reaction, thus making complete inhibition difficult. We hope to eventually determine accurate kinetic data of binding in solution with purified preparations of Pr55^{gag}.

Two C-terminally truncated *gag* proteins, as well as the intact Pr55^{gag}, bound RNA. From the estimated sizes of these species, we believe that only approximately half of the NC domain, and thus only one of the two Cys-His boxes in the HIV-1 NC, is retained in the smallest protein. These

results are in agreement with other suggestions that the N-terminal box is the more important one for NC RNA-binding activity (18, 25, 48). In preliminary work we have found that the ability of Pr55^{gag} to bind nucleic acids is dependent on the sequences in this Cys-His box; mutations which disrupt this region prevent Pr55^{gag} from binding (data not shown). In light of this and others' findings that isolated NC does not bind with specificity (16, 50, 59) we believe that the NC domain is necessary but not sufficient for Pr55 binding specificity. We plan mutagenesis studies of Pr55^{gag} to try to identify other regions of the protein which are necessary—perhaps in cooperation with NC—for specific binding.

Sequence specificity of Pr55^{gag} binding activity. The binding activity detected in these experiments showed significant specificity for RNA sequences present at the 5' end of the HIV-1 genome. This is the first observation of a sequence-specific interaction between retroviral RNA and virion capsid proteins. Binding sites are not present at a high frequency on heterologous RNAs, not even on other retroviral RNAs; RNA containing the M-MuLV packaging region (the ψ region) bound very poorly. The binding shows a specificity that is at least consistent with the expected specificity of the protein acting in vivo to package virion RNA. We have identified two regions within the HIV-1 RNA which are independently sufficient to permit binding to Pr55^{gag}, and their positions in the *gag* gene are such that they would only be present in unspliced genomic RNA and not in spliced subgenomic mRNAs. Each of these regions is about 200 nucleotides long, but it may be that specific short sequences within these regions are essential for binding. There is no obviously significant sequence similarity between the two regions we have identified, and we suspect that it is RNA secondary structure that is recognized by Pr55^{gag}.

How faithfully the binding assay reflects true packaging remains to be determined. Very little is known about the sequence requirements for RNA packaging in vivo in the HIV-1 system. Deletion of sequences between the splice donor and the start of the *gag* gene of HIV-1 reduces the RNA content of virions to 1 to 25% of wild-type levels (2, 10, 39), while deletion of these sequences from our HIV-1 RNA probes decreases binding to Pr55^{gag} by only 30% under our standard assay conditions (Figs. 6 and 7). The relative specificity of binding that we observe, however, is dependent on the exact assay conditions that we select, and by making the conditions more stringent with additional NaCl we can reduce binding of this deletion mutant to 10% of the parental level (Table 1). Recently, a short region near the 5' end of the genome was shown to be sufficient to specify the packaging of RNAs into virions at relatively high efficiency (53). These experiments show that at least one of the binding sites in the *gag* gene detected here is not absolutely required for packaging in vivo. As has been shown in the M-MuLV system, the *gag* sequences may only stimulate binding initiated by the major packaging region (1, 3). Ultimately, the importance in vivo of these binding sites must be validated by testing mutant RNAs for packaging in animal cells.

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