Biochemical Characterization of Binding of Multiple HIV-1 Rev Monomeric Proteins to the Rev Responsive Element

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Received April 12, 1993; Revised Manuscript Received July 22, 1993®

ABSTRACT: Recombinant HIV-1 Rev protein was overexpressed in *Escherichia coli* using translational coupling to the β-glucuronidase gene and demonstrated to interact with high affinity and specificity with the Rev responsive element (RRE). A complex Rev-dependent binding pattern was observed using the gel shift assay which could be simplified to one or two primary bands in the presence of stoichiometric concentrations of RRE. Competition of these bands with a series of homopolymer RNA species demonstrated that Rev is essentially a poly-G binding protein, although poly-I was also shown to compete for specific RRE binding. The stoichiometry of the Rev-dependent gel shift complexes was determined using ^{125}I-labeled Rev. The stable, lowest mobility complex was determined to possess a ratio of between 7 and 8 Rev molecules per RRE containing RNA fragment while the two fastest migrating complexes contained ratios of one and two Rev molecules per RRE, respectively. Using the Hill equation as a model for cooperative interactions, a Hill coefficient of $n_{app} = 2$ was obtained from fitting of direct nitrocellulose filter binding assays, reflecting cooperatively bound Rev molecules on the RRE under equilibrium binding conditions. An increase in ionic strength from 0.0 to 0.3 M NaCl reduced cooperative Rev binding to the RRE, but specificity of Rev for the RRE relative to antisense RNA was increased 100 000-fold. At molar ratios of Rev to RRE above 2, Rev dissociated from the RRE with a $T_{1/2}$ of approximately 20–25 min. Below a molar ratio of 1, the off-rate increased to between 7 and 9 min, suggesting that binding of a second Rev monomer is sufficient to stabilize the initial bound Rev species on the RRE. The data all support the contention that although Rev possesses an intrinsic multimerization activity, it interacts with the RRE as a monomer and is stabilized on the RRE by binding of at least one additional Rev molecule.

Early stages of human immunodeficiency virus type 1 (HIV-1) replication require the production of two virally encoded regulatory proteins called Tat and Rev (Sodroski et al., 1986; Arya et al., 1985; Fisher et al., 1986; Dayton et al., 1986; Terwilliger et al., 1988). Rev down regulates the synthesis of completely spliced viral transcripts leading to increased expression of singly spliced and unspliced mRNA transcripts (Feinberg et al., 1986; Sodroski et al., 1986; Knight et al., 1987). In the absence of Rev, complete splicing of the *rev* and *tat* genes (each composed of two overlapping exons in alternative reading frames) enhances the expression of these proteins. Once synthesized, Rev localizes to the nucleus. Increased Rev concentrations result in increased cytoplasmic levels of full length mRNA transcripts coding for the *gag*, *pol*, and *env* gene products and subsequent production of active viral particles (Sodroski et al., 1986; Feinberg et al., 1986; Knight et al., 1987; Sadaie et al., 1988; Malim et al., 1988; Hammarskjöld et al., 1989; Emerman et al., 1989).

In vivo mutational analysis has shown that Rev contains several functional domains including a nucleolar localization sequence which is necessary for RNA binding, at least one phosphorylation site which is dispensable for protein activity, and a transactivation domain which may directly or indirectly play a role in the interaction with a host cellular factor, as well as a putative dimerization region (Hauber et al., 1988; Cullen et al., 1988; Malim et al., 1989a,b; Malim & Cullen, 1991; Cochrane et al., 1989).

Genetic studies have located a cis-acting RNA sequence within the envelope gene (*env*) which functions in a Rev-dependent manner (Rosen et al., 1988; Malim et al., 1989; Hadzopoulou-Cladaras et al., 1989). Deletions of part or all of this Rev responsive element (RRE)[1] resulted in a loss in the ability of Rev to inhibit the processing of reporter mRNA transcripts. The computer-generated prediction that the Rev responsive element contains substantial secondary structure, as well as *in vivo* experiments demonstrating that this region must be present on the messenger RNA transcript in the correct orientation, suggested that this region of RNA contained a potential binding site for the Rev protein (Malim et al., 1989a). The availability of small quantities of highly purified recombinant Rev, produced in *Escherichia coli*, have permitted the demonstration of high-affinity, specific binding of between 8 and 10 Rev molecules to the RRE using *in vitro* binding assays (Daly et al., 1989; Zapp & Green, 1989; Cochrane et al., 1990; Heaphy et al., 1990; Malim et al., 1990; Olsen et al., 1990a). Rev specificity for the RRE was determined to be at least 1000-fold greater than for a comparably sized antisense RNA fragment. Interestingly, Rev also displayed high-affinity binding to antisense RNA in the absence of added unlabeled competitor RNA, suggesting that the protein may either intrinsically bind all folded RNA molecules with high affinity

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® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

[1] Abbreviations: RRE, Rev responsive element; PMSF, phenylmethylanesulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MMTS, methyl methanethiosulfonate.

0006-2960/93/0432-10497$04.00/0

($K_D = 1 \times 10^{-9}$ M) or utilize protein–protein interactions to stabilize nonspecific RNA binding (Daly et al., 1989).

Several groups have demonstrated that Rev is capable of self-association in the absence and presence of the RRE containing RNA, suggesting that the Rev–RRE interaction may be cooperative in nature (Karn et al., 1991; Wingfield et al., 1991). Therefore, Rev affinity, specificity, and cooperativity for the RRE may all be important parameters which are fundamental for biological activity. In this paper we describe the expression, purification, and biochemical characterization of the RRE binding activity of recombinant Rev protein from *E. coli*.

MATERIALS AND METHODS

Construction of BG-rev Translational Couple Vector. PCR primers were used to add a *Mlu*1 restriction site, a Shine–Dalgarno sequence, an additional stop codon 5′ to the *rev* start codon, and a *Sal*1 site to the 3′ end of the gene. The amplified *rev* fragment was digested with *Mlu*1 and *Sal*1, purified in LTG agarose, and ligated to *Mlu*1–*Sal*1 digested pREV2.1 plasmid.

Expression of Rev in E. coli. The pBGrev expression plasmid was transformed into *E. coli* RGN714 (Repligen Corp.). Transformants were grown in L-broth containing 20 μg/mL chloramphenicol at 37 °C, and the level of expression was determined by SDS–PAGE. Large scale growth of RGN714 pBGrev was performed in a 10-L Chemap fermenter. The fermentation medium consisted of 20 g/L of peptone, 20 g/L casein hydrosylate, 20 g/L yeast extract, 2 g/L Na_2HPO_4, 2 g/L KH_2PO_4, 2 g/L K_2HPO_4, and 20 mg/L chloramphenicol. Growth was performed at 40 °C for 24 h with aeration at 10 L/min.

Purification of Rev. Rev was purified under denaturing conditions as previously described (Daly et al., 1990) with several modifications. Rev containing fractions from the preparative HPLC step were lyophilized and stored at −20 °C. Lyophilized Rev fractions were solubilized in buffer containing 0.01 M Tris-HCl, pH 7.6, and 8 M urea (buffer U) and loaded onto an S-Sepharose column equilibrated in buffer U. The column was washed in buffer containing 0.01 M Tris-HCl, pH 7.6, and protein was eluted with a 0–2 M NaCl gradient in 0.01 M Tris-HCl, pH 7.6. Protein typically eluted at approximately 1 M NaCl, and fractions were stored either at 4 or −20 °C. Rev purity was assessed by amino-terminal sequencing, by amino acid analysis, and by Coomassie and silver-stained SDS–PAGE. Protein was quantitated by Bradford assay (Bradford, 1976). Rev size and solubility were compared against Rev samples purified under nondenaturing conditions. Rev that was purified under nondenaturing conditions was a gift from Drs. M.-L. Hammarskjöld and D. Rekosh.

Amino Acid Composition of Rev. Samples containing purified Rev protein were hydrolyzed for 24 h in 12 M HCl at 90 °C. Following hydrolysis, samples were derivatized and analyzed according to the method of Bidlingmeyer et al. (1984).

Synthesis of Labeled Sense and Antisense RRE Containing RNA Fragments. pRRE280 and p280AS were created by cloning a 280 base pair PCR fragment corresponding to HXB3 nucleotides 7333–7612 into the *Eco*RV site of pBluescript SK(−) (Stratagene) in either orientation. These plasmids were linearized with *Spe*I and transcribed *in vitro* by T7 RNA polymerase, resulting in the synthesis of 367 base sense or antisense RNA. For synthesis of unlabeled RRE containing RNA, 100 μg of linearized pRRE280 was typically incubated in 1.5 mL of 40 mM Tris-HCl, pH 8.1, 20 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 50 μg/mL BSA, 1 mM each of ATP, GTP, CTP, and UTP, 20 units of RNase block II (Stratagene), and 2000 units of T7 RNA polymerase. The transcription reaction was performed at 37 °C for 1 h. Labeled RNA fragments were synthesized as suggested by the supplier using [α-^{32}P]UTP (50 μCi, 3000 Ci/mmol; NEN) and a final UTP concentration of 100 μM. Following the transcription reaction, the solution was loaded onto a 6% denaturing polyacrylamide gel and electrophoresed at 45 W for 3 h. The RNA was visualized and excised from the matrix followed by electroelution into TE buffer, ethanol precipitation, and resuspension in TE buffer. Following a heat renaturation step (70 to 25 °C), the integrity of the RNA was analyzed by electrophoresis on either denaturing or nondenaturing polyacrylamide gels.

Gel Shift Assay of Rev Activity. Gel shift assays were performed according to the method of Fried and Crothers (1981) with the following modifications. Samples containing protein and nucleic acid were typically electrophoresed on a 5% TBE (0.1 M Tris-borate, 0.001 M EDTA) nondenaturing polyacrylamide gel (4 cm × 6 cm × 1.5 mm). Samples contained 10 units of Rnasin (RNase inhibitor, Promega). Unless otherwise specified, binding of Rev to RNA species was performed in a 10-μL volume containing 0.01 M K_2HPO_4, 0.01 M NaH_2PO_4, 0.14 M KCl, and 10 μg of tRNA. Labeled RNA species were added to the reaction volume followed by addition of Rev protein. Samples were incubated for 5 min at room temperature. Prior to being loaded onto the gel, 0.5 μL of tracking dye containing 0.5% bromophenol blue, 0.5% xylene cyanol FF, and 50% glycerol was added to the sample. Samples were electrophoresed at 15 mA for approximately 3 h at room temperature after which the gels were dried and exposed for autoradiography.

Nitrocellulose Filter Binding. Rev binding to labeled RNA in the absence and presence of unlabeled nonspecific RNA was measured by the nitrocellulose filter binding assay of Riggs et al. (1968) with several modifications. To all samples, 10 units of Rnasin was added to inhibit nuclease activity. Binding reactions were carried out in binding buffer (PBS) containing 50 μg/mL BSA. Rev-containing samples were incubated for 15 min prior to filtering of the 0.5-mL volume through nitrocellulose filters (0.45-μm pore size; Millipore). No washing step was included after filtering of the samples. Filters were subsequently dried and radioactivity detected by scintillation counting. RRE concentrations were estimated to be below 1×10^{-10} M, based on the specific activity deduced from the labeling reaction. Unless otherwise noted, for direct binding experiments Rev concentrations were typically varied from 8×10^{-9} to 8×10^{-11} M. For competition filter binding experiments, the Rev concentration was held constant at 8×10^{-9} M in each sample. For dissociation rate experiments performed under pseudo-first-order conditions, Rev was incubated at the appropriate concentrations for 30 min with ^{32}P-labeled RRE supplemented with unlabeled RRE of known concentration. At time = 0, a 1000-fold molar base excess of either unlabeled RRE or poly-G RNA was added to the pre-formed complex, and the solution was inverted three times. Aliquots (0.5 mL) were removed and filtered at appropriate time intervals in duplicate. As Rev dissociates from the pre-formed complex, it is trapped by the excess competitor RNA resulting in a decrease in bound radioactivity over time. A final data point filtered at 3.5 h after addition of competitor RNA and representing the final equilibrium was subtracted from each time point. Data were plotted as log fractional saturation versus time in minutes.

Determination of Stoichiometry of Rev–RRE Interaction. Purified Rev was initially reacted with a 200-fold molar excess of methyl methanethiosulfonate to prevent oxidation of any available cysteine residues. The reaction was performed at 4 °C for 0.5 h. Following the reaction, unreacted reagent was removed by passing the protein over a G-25 Sephadex column equilibrated in 0.01 M Tris-HCl, pH 7.6, and 1 M NaCl. The eluted Rev was quantitated by absorption spectroscopy. The RRE binding activity of this treated Rev was judged by gel shift assay to be equivalent to that of untreated protein. A 5-μL aliquot of [^{125}I]NaI in 0.1 M NaOH (0.5 mCi; NEN) was added to 0.75 mL of buffer containing 0.01 M Tris-HCl, pH 7.6, and 1 M NaCl, followed by addition of a single bead containing immobilized chloramine T (Pierce) and allowed to incubate for 5 min. Following reaction, 0.5 mg of MMTS-reacted Rev was added to the mixture and reacted for 7 min at room temperature. The reaction was terminated by removal of the iodobead and unreacted radioisotope removed by passage of the sample through a G-25 Sephadex column. Fractions containing labeled Rev were quantitated by absorption spectroscopy and Bio-Rad protein assay. The specific activity of the protein, as determined by scintillation counting, was calculated to be 7×10^9 cpm/μmol.

Gel shift assays were performed in the presence of either radiolabeled Rev and unlabeled RRE, unlabeled Rev and radiolabeled RRE, or both radiolabeled Rev and RRE. Protein–RNA complexes were electrophoresed under nondenaturing conditions, followed by autoradiography. Gel slices containing protein–RNA complexes detected in the autoradiogram were excised and dissolved overnight in 150 μL of 30% H_2O_2 at 60 °C. Samples were quantitated by scintillation counting. Corrections were made for overlap of the ^{125}I into the ^{32}P channel and backgrounds were subtracted.

RESULTS

Expression and Purification of Recombinant HIV-1 Rev. The initial cloning of the *rev* gene into the expression plasmid pKK223 to create plasmid pKKrev did not result in a significant level of Rev expression (data not shown). To increase production of Rev, expression was coupled to the highly expressed, cloned *E. coli* β-glucuronidase (βG) gene in a translational couple design. The pREV2.1 expression plasmid carries the cloned β-glucuronidase promoter and ribosome binding site as well as the N-terminal domain of the β-glucuronidase structural gene. The translational coupling was accomplished by creating a stop codon in the middle of this BG sequence of pREV2.1 which was immediately followed by the *rev* coding sequence. A Shine–Dalgarno ribosome binding site was placed upstream of the newly synthesized BG stop codon with correct spacing relative to the *rev* start codon Figure 1A. To make this construction, PCR primers were synthesized to allow amplification of a fragment which added the Shine–Dalgarno and BG peptide stop codon 5′ to the *rev* gene and an additional stop codon to the 3′ end. The primers also created an *Mlu*1 site at the 5′ end of this PCR fragment and a *Sal*1 site at the 3′ end to allow in-frame cloning into the *Mlu*1 site in the β-glucuronidase sequence of pREV2.1. The amplified *rev* fragment was cloned into pREV2.1 to create pBGrev. The expression of Rev from pBGrev in *E. coli* RGN714 constituted approximately 5% of the total cellular protein.

To remove any associated trifluoroacetate ion associated with the protein and to refold the protein at high concentration, an additional step was added to the Rev purification protocol. This refolding step, performed using S-Sepharose ion-exchange

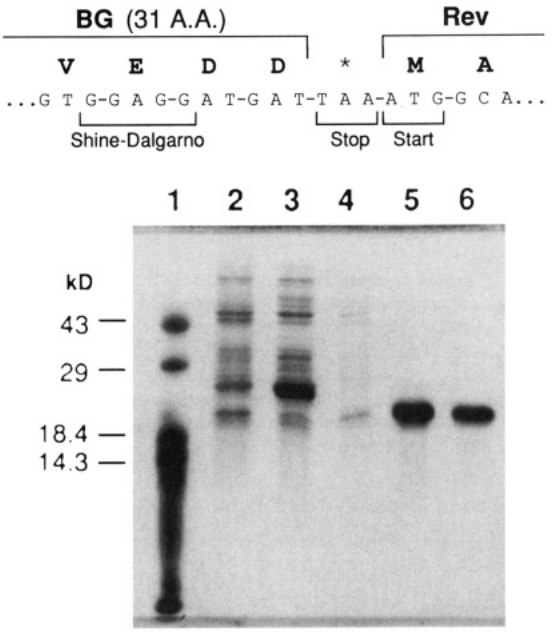

FIGURE 1: (A, top) Schematic illustration of the translational couple used for the overexpression of recombinant Rev using the *E. coli* β-glucuronidase gene product. (B, bottom) Composite 15% SDS–PAGE exhibiting the extent of Rev purification following each purification step. Lane 1, molecular weight markers; lane 2, lysed cells; lane 3, lysis supernatant; lane 4, ethanol precipitation; lane 5, carboxymethyl-Sepharose column; lane 6, C_{18} reverse-phase chromatography.

chromatography, was observed to increase the solubility of the protein to levels comparable to that of Rev purified under nondenaturing conditions. Rev was isolated in high ionic strength buffer at concentrations as high as 15 mg/mL. The ability of this protein to bind with high affinity and specificity to the RRE was unaffected by this procedure. The *in vivo* biological activity of the refolded Rev was observed to be equivalent to Rev purified under nondenaturing conditions (data not shown). Figure 1B is a composite SDS–polyacrylamide gel demonstrating the purification scheme for Rev at various stages. The purified protein was found to be greater than 95% pure as determined by C_4 reverse-phase chromatography, amino acid analysis, and amino-terminal sequencing analysis. In addition, N-terminal sequencing revealed that the initial methionine residue had been removed in *E. coli*. Typically, several hundred milligrams of highly purified Rev were obtained from 0.5 kg of cells. Rev protein, purified under nondenaturing conditions, was assessed for RRE binding, solubility, size on gel filtration chromatography, purity, and absorption spectra for comparison with the refolded species. In each of the studies, Rev purified under nondenaturing conditions was identical to the refolded Rev species at identical concentrations (data not shown).

Characterization of the Rev–RRE Interaction. It was initially observed that, in the absence of tRNA in the gel shift assay, Rev aggregated with the RRE at the top of the gel suggesting a strong tendency of Rev to self-associate at higher concentrations. Although it was found that tRNA disrupted Rev aggregation, it was also noticed that the order of addition of reagent affected the resulting gel shift pattern. Prebinding of Rev to either a labeled RRE containing fragment at low concentrations or an unlabeled nonspecific RNA at high concentrations resulted in distinct gel shift patterns. The samples of Rev prebound to the RRE were detected as aggregates at the top of the polyacrylamide gel, whereas Rev prebound to the unlabeled competitor produced only high

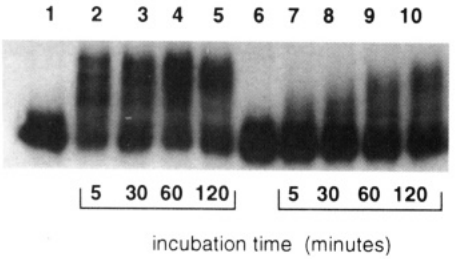

FIGURE 2: Time course of Rev binding to the RRE. Rev at 3×10^{-6} M was added to samples either in the presence of unlabeled tRNA and labeled RRE (lanes 2–5) or in the presence of unlabeled tRNA only (preincubation; lanes 7–10). Samples in lanes 2–5 were incubated for the indicated times prior to loading onto the gel. Samples in lanes 7–10 were incubated for 30 min prior to addition of labeled RRE at time = 0. Samples were subsequently loaded onto the polyacrylamide gel at the indicated times.

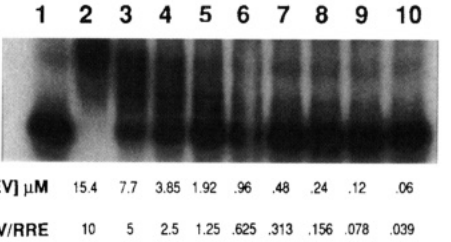

FIGURE 3: Gel shift analysis of the interaction of Rev with RRE in the presence of excess unlabeled specific RRE RNA. Varying concentrations of Rev were incubated with labeled RRE, supplemented with 1.5×10^{-6} M unlabeled RRE. Molar ratios of Rev:RRE were varied as follows: lane 2, 10; lane 3, 5; lane 4, 2.5; lane 5, 1.25; lane 6, 0.63; lane 7, 0.31; lane 8, 0.15; lane 9, 0.08; lane 10, 0.04.

Table I: Competition of Nucleic Acid Homopolymers for Rev Binding to the RRE[a,b]

competitor	concentration of competitor at 50% fractional saturation[c]	relative competitive strength[d]
RRE	7×10^{-7} M	1
poly-G	3×10^{-6} M	4.3-fold
poly(dG)	3×10^{-6} M	4.3-fold
poly-I	3×10^{-5} M	43-fold
poly-G_{12-18}	3×10^{-5} M	43-fold
poly-G_4	$>2 \times 10^{-4}$ M	>286-fold
poly-AU	$>2 \times 10^{-3}$ M	>2860-fold
poly-A	$>2 \times 10^{-3}$ M	>2860-fold

[a] Rev concentration was held constant at 9×10^{-9} M. [b] No unlabeled MS2 or tRNA was added to this assay. [c] Concentration of nucleic acid base required to inhibit 50% of the binding of Rev to ^{32}P-labeled RRE. [d] Amount of competitor nucleic acid base concentration required for 50% fractional saturation relative to the amount required by RRE.

mobility complexes. A third binding pattern was observed for samples in which the protein was added to samples containing both the labeled and unlabeled RNA species. This sample resulted in the formation of lower mobility complexes in the gel and is representative of a true equilibrium condition. Since previous filter binding experiments had shown that binding to the RRE was complete within 5 min (data not shown), the differences in the gel shift binding patterns under the three different conditions are likely to reflect a relatively slow dissociation rate for the protein, possibly due to protein–protein interactions. This is reflected in Figure 2, which shows that equilibrium is reached quickly when Rev is added in the presence of tRNA and RRE. However, when Rev was prebound to tRNA, equilibrium was reached only after several hours, and the observed gel shift pattern was observed to change over time. The gel shift pattern generated by adding Rev to tRNA and RRE was unchanged over time and was identical after 5 min to the pattern generated after 2 h following prebinding of Rev to tRNA (compare lanes 2 and 10). Due to the presence of protein–protein interactions in the gel shift assay, the dissociation of prebound Rev proteins to RNA is a slow process which may affect the interpretation of results. Therefore, in order to remove this artifact from the system, Rev was always added in the presence of both specific RRE and competitor RNA and was incubated for a sufficiently long time to achieve equilibrium.

The presence of multiple Rev proteins bound to the RRE was further examined by gel shift assay. As previously shown, at low Rev concentrations, multiple low-mobility species, relative to unbound RRE were detected. As the concentration of Rev was increased, the presence of these intermediate species was diminished, concomitant with an increase in the presence of a single low mobility complex, presumably a saturated Rev–RRE complex. Under equivalent conditions, specific complexes were unaffected by an increase in the amount of competitor tRNA up to a base concentration of 150 mM (not shown). However, if the binding reaction was performed in the presence of an increasing excess of unlabeled RRE, the gel shift binding pattern was significantly altered. As the ratio of protein to RRE was decreased from 10 to 0.625, the single predominant species which migrated at an R_f value of 0.67 relative to unbound RRE was replaced by two faster migrating species at R_f values of 0.89 and 0.85, respectively (Figure 3). Below a protein to RRE ratio of 1, the presence of even these two species was reduced. The lower mobility complexes, which cannot be challenged by unlabeled nonspecific competitor yet can be abolished by increasing the RRE concentration, may represent stably bound Rev molecules to nonspecific sites on the RRE resulting from an initial specific binding event followed by formation of Rev–Rev interactions.

Characterization of the Nucleotide Preference of Rev. A series of homopolymer RNA species were tested in the gel shift and filter binding assay to study whether Rev displayed any nucleotide preference. Titration of poly-A has little effect on Rev interaction with the RRE (Table I). Poly-A displayed a particularly poor ability to compete with the RRE for Rev binding. Poly-G, however, exhibited the highest competitive strength of any of the homopolymer RNA species, indicating that Rev possesses a preference for binding poly-G RNA. The relative competitive strength of each of the homopolymers was quantitated by competition filter binding assay. Poly-G as well as poly(dG) exhibit competitive strength comparable to RRE calculated by molar base concentration. Poly-I displayed an approximately 10-fold weaker competitive strength relative to poly-G. Relative competitive strength was also demonstrated to be affected by the length of the poly-G homopolymer. As the size of the competitor RNA was reduced to G_{12-18}, the ability to compete with RRE for Rev binding was reduced 10-fold compared to poly-G of length greater than 100 nucleotides (not shown). As the size of the poly-G was reduced to G_4, the RNA was no longer observed to compete with RRE for Rev binding, suggesting that length, perhaps affecting conformation of the poly-G, affects relative competitive strength.

Determination of the Stoichiometry of the Rev–RRE Interaction. In order to determine the stoichiometry of the Rev–RRE interaction, double-labeling experiments were performed using the gel shift technique. Cook et al. (1991), using subfragments of the RRE, demonstrated stoichiometries of three Rev proteins bound to the stem II RNA and one Rev protein bound to the F8 RNA fragment. Radiolabeled Rev

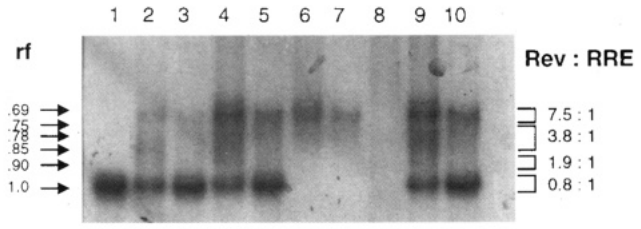

FIGURE 4: Determination of the stoichiometry of the Rev–RRE interaction using ^{125}I-labeled Rev and ^{32}P-labeled RRE in the gel shift assay. Samples containing 10 μg of unlabeled tRNA and 1.08 $\times 10^{-6}$ M RRE were incubated with either ^{125}I-labeled or unlabeled Rev in PBS binding buffer followed by electrophoresis on a 5% TBE nondenaturing polyacrylamide gel. Gel slices corresponding to positions of Rev–RRE complexes were excised, dissolved in H_2O_2, and quantitated by scintillation counting. Rev concentrations were as follows: lane 1, none; lanes 2, 4, 6, 8, and 9, 5.8×10^{-6} M; lanes 3, 5, 7, and 10, 2.9×10^{-6} M. Lanes 2 and 3 contain unlabeled Rev; lanes 4–10 contain ^{125}I-labeled Rev protein. Lanes 1–5, 9, and 10 contain ^{32}P-labeled RRE; lanes 6 and 7 contain unlabeled RRE; lane 8, no RRE. The specific activity of RRE was 4.37×10^9 cpm/μmol. The specific activity of Rev was 7.00×10^9 cpm/μmol. Corrections were made for 18% overlap of ^{125}I into the ^{32}P channel during scintillation counting.

was incubated with either unlabeled or ^{32}P-labeled RRE and electrophoresed under nondenaturing conditions. Slices of polyacrylamide corresponding to the positions of protein-RNA complexes were quantitated by scintillation counting. The results shown in Figure 4 indicate that the predominant, stable Rev–RRE complex, which migrates at an R_f value of 0.69 relative to unbound RRE, corresponds to a stoichiometry of 7.45 ± 0.5 Rev molecules per RRE molecule. The complex observed at an R_f value of 0.9 relative to unbound RRE is consistent with a single Rev molecule bound to the RRE. The second fastest migrating complex ($R_f = 0.85$) was determined to contain a stoichiometry of two Rev molecules bound to the RRE. Gel slices, located at positions between the octamer and dimer species, yielded stoichiometries consistent with complexes containing between three and six Rev molecules per RRE. Individual bands within this range could not be reproducibly isolated, and the results therefore represent the average of several Rev:RRE ratios. Equivalent stoichiometry results were indirectly obtained by comparing samples containing unlabeled Rev and labeled RRE with samples containing labeled Rev and unlabeled RRE at identical concentrations. The results demonstrate that Rev is able to bind to the RRE as a monomer but is capable of forming a stable oligomeric complex, a result consistent with the previously determined stoichiometry using the nitrocellulose filter binding assay (Daly et al., 1989).

Effect of Ionic Strength on Rev–RRE Binding Parameters. The ability of purified Rev to interact specifically with the RRE was previously tested using the nitrocellulose filter binding assay (Daly et al., 1989). Typically, the efficiency of retention of total input counts on the filters at saturating Rev concentrations was approximately 95%. As previously noted, care was maintained to assay that binding reactions had reached equilibrium conditions. In the absence or presence of unlabeled nonspecific competitor RNA, Rev demonstrated high affinity for the RRE. The equilibrium dissociation constant determined from the concentration of Rev at 50% fractional saturation was determined to be approximately 2×10^{-10} M for binding to the RRE. In addition, the shape of the binding curves obtained was not hyperbolic but rather sigmoidal, a result suggesting the presence of positive cooperativity even in the presence of an excess of nonspecific unlabeled competitor (1.45×10^{-5} M base). Under identical conditions using an antisense RNA species of comparable length, no Rev binding in the presence of unlabeled competitor was observed. In the absence of excess unlabeled competitor RNA, Rev bound to the antisense RNA with an equilibrium binding constant of approximately 1×10^{-9} M. Similar Rev–RRE binding affinities were obtained using smaller, 0.1-μm pore size filters, suggesting that a primary higher affinity ($<10^{-10}$ M) binding event was not being masked by a low efficiency of retention on the filter (data not shown).

In order to analyze the cooperativity in the Rev–RRE filter binding curve in greater detail, the data were fit to a sequential interaction model (Segel, 1975). In the Hill equation, the sequential binding of n substrate molecules to equivalent binding sites changes the intrinsic dissociation constant by a factor of $f_1, f_2, ..., f_n$. In the case of binding of n Rev molecules to the RRE and the assumption of very marked cooperativity (factors $f_1, f_2, ..., f_n$ being very small numbers), the concentration of all Rev–RRE complexes with less than n Rev molecules will be negligible at any Rev concentration which is appreciable compared to the K_D ([Rev][RRE]/[Rev–RRE]) of the single binding event.

Therefore, Y, the ratio of Rev-complexed RRE to total RRE (RRE_0) will be dominated by the $[Rev]^n$ term. In the equation

$$Y = [Rev]^{n_{app}}/(K_D' + [Rev]^{n_{app}})$$

K_D' comprises the factors $(f_1^{n-1}, f_2^{n-2}, ..., f_n)$ and the intrinsic dissociation factor K_D. As shown in Figure 5A, this equation accurately fits the data with an average apparent $K_D' = 1.88 \times 10^{-8}$ M and $n_{app} = 2.087$ at 0.15 M NaCl, indicating that, in the case of perfect cooperativity, the minimum number of Rev molecules bound per RRE molecule would be two. This result is consistent with the results obtained in the stoichiometry gel shift experiment and makes a mechanism of sequential interaction of Rev with the RRE most probable. Gel shift and double-labeling experiments show that the first Rev protein can bind to the RRE as a monomer followed by cooperative binding of a second Rev species.

Direct binding isotherms at varying ionic strength were analyzed for positive cooperatively bound Rev molecules on the RRE. In buffer containing 0.3 M NaCl, the Hill coefficient was 1.44, somewhat less than the Hill coefficient of 2.09 (Figure 5B) obtained in the presence of 0.15 M NaCl. In the presence of 0.4 M NaCl, the binding curve was no longer sigmoidal, suggesting that under these conditions positive cooperativity was abolished. At salt concentrations less than 0.15 M, successful competition of nonspecific RNA for binding to Rev precluded fitting of the binding isotherms. However, the observation that nonspecific RNA could successfully compete with RRE for Rev binding at low salt concentration implied that ionic strength could affect the ability of Rev to distinguish the RRE from nonspecific RNA. On the other hand, an increase in salt concentration from between 0.3 and 0.4 M NaCl up to 0.8 M NaCl lead to an exponential reduction in binding affinity (Figure 5C). An extrapolation of the semilogarithmic plot (Figure 5D) of salt concentration versus K_D up to 1 M NaCl resulted in a dissociation constant of 1.4×10^{-4} M in 1 M NaCl containing buffer. As electrostatic interactions between Rev and RRE should be eliminated at high salt concentration, it can be concluded that the remaining binding affinity, which corresponds to –5.2 kcal/mol, comprises approximately 50% of the total free energy for the Rev–RRE interaction in 0.15 M NaCl containing buffer (–10.5 kcal/mol).

Upon loss of positive cooperative Rev binding to the RRE at elevated ionic strength, it was anticipated that multimer-

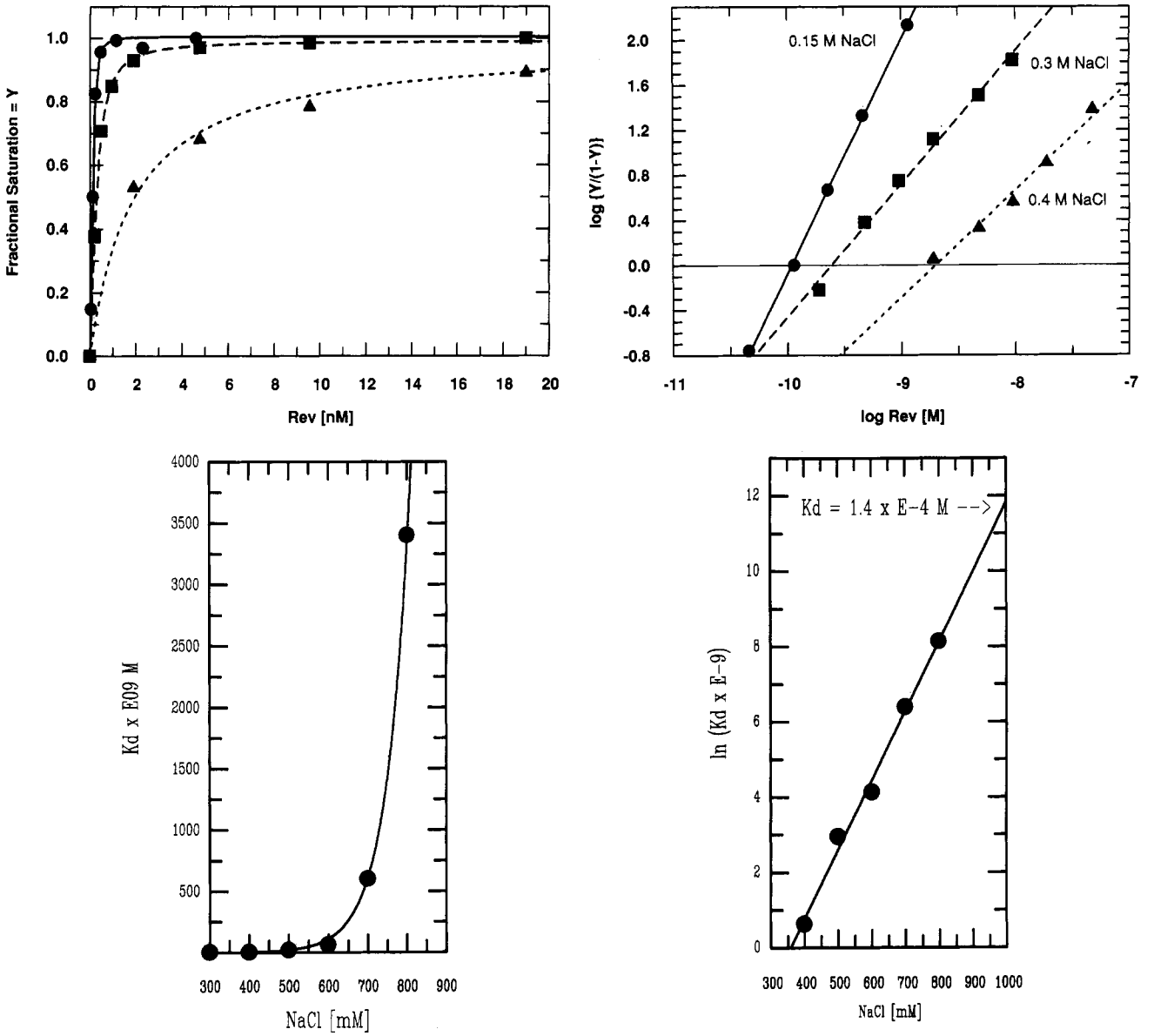

FIGURE 5: (A, top left) Measurement of direct binding of Rev to the RRE in the presence of unlabeled nonspecific competitor RNA at varying ionic strength using nitrocellulose filter binding techniques. Varying concentrations of Rev protein were diluted into PBS buffer supplemented with 50 μg/mL BSA and 2 mM DTT in the presence of ^{32}P-labeled RRE and unlabeled MS2 RNA (1.45×10^{-5} M base) and incubated for 0.5 h at room temperature. Samples in triplicate were filtered through 0.45-μm filter disks, and the radioactivity retained on the filters was quantitated by scintillation counting. Data were theoretically fit to a sequential interaction model. (●), 0.15 M NaCl; (■), 0.3 M NaCl; (▲), 0.4 M NaCl. (B, top right) Hill plot analysis of theoretical fitting of binding data under varying ionic strength conditions from panel A. (C, bottom left) Plot of measured equilibrium dissociation constants for Rev binding to the RRE versus salt concentration. In all experiments, the MS2 RNA concentration was 1.45×10^{-5} M base. (D, bottom right) Semilogarithmic plot of equilibrium dissociation constant for Rev binding to the RRE versus salt concentration with extrapolation to 1 M NaCl. MS2 RNA concentrations in each sample were 1.45×10^{-5} M base.

ization would also be affected. In this system, positive cooperativity reflects the ability of a previously bound protein to facilitate the binding of the next protein. On the other hand, multimerization appears to be an intrinsic property of Rev which is apparent in the gel shift assay as multiple bound proteins on the RRE following an initial specific binding event. Multimerization in the absence of RRE probably still exists (as observed in formation of filamentous structures; Wingfield et al., 1991; Karn et al., 1991); however, the availability of a template and a defined orientation point (RRE) facilitates visualization of discreet bands in the gel shift assay. Using gel shift analysis, it was observed that Rev incubated with the RRE in the presence of elevated salt concentrations (where positive cooperativity was abolished) maintained the ability of the protein to multimerize on the RRE (data not shown). The observation suggests that the presence of cooperativity may affect the mechanism of binding of Rev to the RRE but most likely has little effect on the number and location of monomers on the RRE. The ability of the protein to multimerize on the RRE is most likely an intrinsic property of the protein.

Competition nitrocellulose filter binding assays were used to measure Rev specificity for the RRE versus antisense RNA at varying ionic strength. When MS2 nonspecific RNA was used as the competing species, a 5000-fold difference in the specificity of Rev for the sense RRE relative to antisense RRE was observed under standard binding conditions (0.15 M NaCl). In the absence of NaCl, no difference in specificity could be detected between RRE and antisense RNA. As the ionic strength was increased, specificity of Rev for the RRE also increased Figure 6. At 0.3 M NaCl, the specificity was observed to increase to approximately 100 000-fold relative

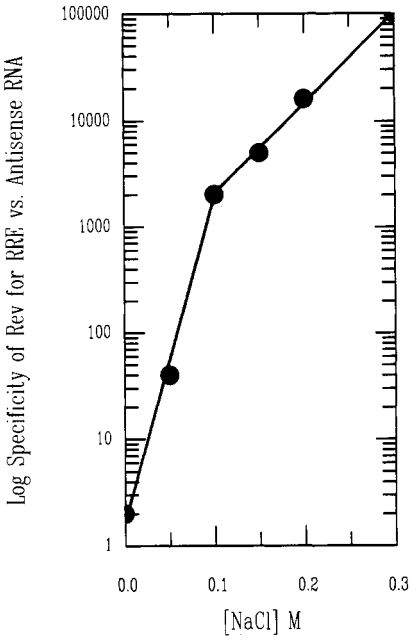

FIGURE 6: Plot of the log of the difference in specificity between Rev binding to sense and antisense RNA using MS2 RNA as competitor versus ionic strength.

to antisense RNA. Limitations within the assay precluded examination of specificity at higher ionic strength. However, since the ability to discriminate sense from antisense RNA directly correlates with ionic strength and inversely correlates with cooperative Rev binding to the RRE, it is possible that salt effects may play a role determining protein conformation.

Oligomerization of Rev. The oligomeric state of recombinant Rev was examined by gel filtration chromatography. Because Rev is insoluble at neutral pH at high protein concentration and low ionic strength, the protein was diluted and chromatographed in buffer containing 1 M NaCl. Typical Rev chromatograms exhibited a highly asymmetrical peak with a slight leading shoulder followed by a gradual decrease in absorbance over elution time (data not shown). This trailing phenomenon, consistent with a protein species comprised of many dissociating and associating oligomeric states is also consistent with protein interaction with the column matrix. Although nonspecific interaction with the column matrix at high ionic strength is considered unlikely, it cannot be ruled out. Actual determination of Rev sizes at varying concentrations of protein therefore was not attempted. However, it is apparent that retention time on the column is concentration dependent, suggesting that Rev oligomeric state is also concentration dependent. These data are consistent with sedimentation equilibrium data which showed a concentration dependent change in sedimentation rates of Rev and that the smallest kinetic species is the Rev monomer (Lee et al., 1993).

Measurement of Rev–RRE Dissociation Rates at Varying Stoichiometry. The nitrocellulose filter binding assay was adapted for the measurement of the dissociation rate of the protein from the RRE. Rev was initially incubated with labeled RRE supplemented with a quantitated amount of unlabeled RRE for 30 min to establish an equilibrium. Upon addition of an excess of unlabeled RRE or excess of poly-G RNA which initiated the dissociation experiment, samples were filtered at varying time intervals. Presumably, as Rev molecules dissociate from the labeled RRE molecules, they are trapped by the excess of unlabeled RRE or poly-G. The result was a decrease in the amount of radioactivity detected on the filter as a function of time (Figure 7). A 3.5-h time

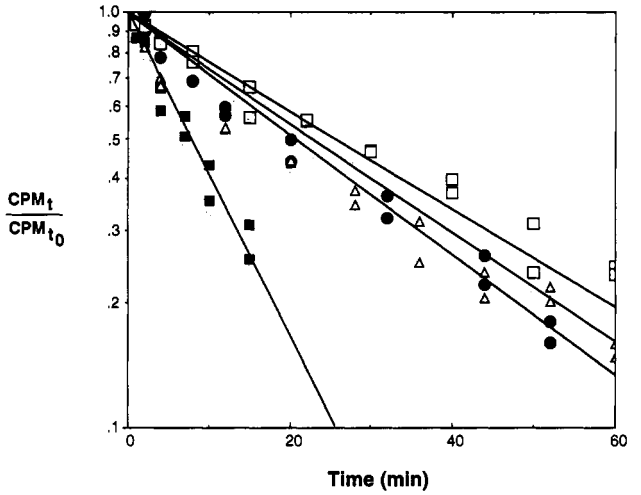

FIGURE 7: Effect of varying stoichiometry on the rate of dissociation of Rev from the RRE. Rev, at varying protein to RRE ratios, was preincubated with labeled RRE supplemented with 1×10^{-9} M unlabeled RRE for 30 min prior to addition of unlabeled poly-G RNA. At time = 0, a 1000-fold excess of unlabeled poly-G RNA was added to the complex, and 0.5-mL aliquots were filtered in duplicate at varying times. Data are plotted as the log fractional saturation versus time. Ratios of Rev to RRE were as follows: (■), 1:1 ratio, 1×10^{-9} M Rev; (△), 2:1 ratio, 2×10^{-9} M Rev; (●), 4:1 ratio, 4×10^{-9} M; (□), 8:1 ratio, 8×10^{-9} M Rev.

point, reflecting the final resulting equilibrium, was subtracted from each of the data points, which were subsequently converted to fractional saturation relative to the starting equilibrium. Data were plotted as the log fractional saturation vs time. Under pseudo-first-order conditions, the dissociation rate will be linear with respect to time and the dissociation half-life determined at 50% fractional saturation. For the dissociation of Rev from the RRE at a starting molar ratio of eight Rev monomers per RRE fragment, a $T_{1/2}$ of approximately 20–25 min was obtained, suggesting a relatively slow dissociation rate. No difference in the off-rate was determined using either unlabeled RRE or poly-G RNA as the competitor RNA species. The $T_{1/2}$ of between 20–25 min was converted to a k_{off} of between 5.8×10^{-4} and 4.6×10^{-4} s^{-1}, which, assuming an equilibrium dissociation constant of 2×10^{-10} M, predicts a k_{on} of approximately 3×10^6 M^{-1} s^{-1} for the interaction of Rev with the RRE. Diffusion controlled binding typically ranges from 10^6 to 10^8 M^{-1} s^{-1}. The binding of Rev to the RRE appears to fall in the slow range of diffusion binding, and therefore probably includes no facilitating binding mechanism. Additional off-rate experiments were performed at varying starting molar ratios. No change in the dissociation rate was observed for either the 4:1 or 2:1 molar ratios relative to the fully saturated RRE fragment. However, below a molar ratio of one Rev protein per RRE fragment, the $T_{1/2}$ was decreased to between 7 and 9 min, suggesting that the second Rev protein bound to the RRE is sufficient to stabilize the first Rev by approximately 3–4-fold. Additional Rev monomers do not appear to further stabilize the Rev–RRE complex.

DISCUSSION

High level expression of Rev was dependent on the translational coupling of the Rev to the β-glucuronidase gene in a two cistron construction. The translational coupling approach has been used to overexpress several heterologous proteins in *E. coli* (Spanjaard et al., 1989; Schoner et al., 1984; Makoff et al., 1990) and provides a convenient method to overcome poor translational efficiency of a cloned gene. HIV-1 recombinant Rev has been expressed in high quantity

as a soluble protein in *E. coli* and purified to greater than 95% purity. The high affinity and specificity of this protein species for the Rev responsive element demonstrates that the steps involved in the purification do not affect the integrity of the protein. The equilibrium dissociation constants for the Rev–RRE (0.2 nM) and Rev–antisense RNA (1 nM) interactions have been determined, as has the overall difference in specificity between RRE and antisense RNA. With similar binding constants, yet a large difference in specificity between RRE and antisense RNA, it was initially assumed that multimerization was directing specificity. The possibility that multimerization was occurring through misfolding of a large proportion of the protein was dismissed through circular dichroism studies which showed that Rev possesses 50% α-helix and 25% β-sheet structure (Daly et al., 1990). These results were subsequently confirmed by Wingfield et al. (1991). Through testing of the purified Rev in a cell based p24 Elisa assay, our refolded Rev displayed biological activities comparable to Rev which was synthesized within the cells from a plasmid containing the Rev gene (Daly et al., 1993). We therefore conclude that the refolded Rev is both correctly refolded and biologically active.

Rev dependent bands observed in the gel shift assay are most probably a manifestation of multiple Rev molecules stabilized on the RRE through protein–protein interactions following a primary, high affinity and specificity binding event by a monomeric Rev species (Cook et al., 1991; Kjems et al., 1991; Malim & Cullen, 1991). The binding of additional monomeric Rev proteins may be in part due to sequence- or structure-dependent recognition of the RRE as well as stabilization by Rev–Rev interactions. Huang et al. (1991) demonstrated that only the stem II region within the RRE was required for Rev-dependent biological function, suggesting that multimeric Rev binding to the stem I region of the RRE was not essential for activity. It is possible, however, that Rev–Rev interactions within the stem II RNA are responsible for biological activity. Rev has been observed to bind with high affinity to antisense RNA in the absence of excess unlabeled RNA. It is likely that common, nonspecific recognition motifs may exist on many folded RNA molecules including antisense RNA and tRNA. On the basis of the homopolymer competition experiment within this study, it is clear that Rev possesses a preference for guanine-rich regions of RNA. This observation is not novel. Several studies have previously measured high-affinity interactions of arginine-rich peptides with guanine rich RNA molecules (Porschke, 1978, 1979; Seeman et al., 1976; Helene, 1977; Helene & Maurizot, 1981, review). Indeed, Swanson and Dreyfuss (1988) have shown that different hnRNP proteins possess an intrinic ability to discriminate different homopolymer RNA sequences, presumably reflecting an ability to recognize specific sequences within target RNA molecules. Therefore it is not surprising for Rev to bind with high affinity to both RRE and antisense RNA fragments, both of which possess guanine-rich sequences.

The theoretical binding curve for the positive cooperative interaction (Hill equation), with $[\text{Rev–RRE}]_{max}$ (the maximum concentration of complex), K_d', and n_{app} as free variables for the calculation, fits very well to the experimental results from the filter binding experiments. The apparent Hill coefficient of approximately 2 indicates that the smallest stable complex under the experimental steady state conditions for filter binding assays is two Rev molecules bound to the RRE. However, the experimental binding curves can also be fit if a higher Rev stoichiometry within the complex along with reduced cooperativity is imposed. In this system, positive cooperativity may be abolished without apparent loss in multimerization. The precise stoichiometry of interaction can not be deduced using the fitting equation. Similarly, the Hill model for theoretical curve fitting can not clearly differentiate between a binding mechanism occurring via secondary binding sites on the RRE or stabilization of the RRE structure through protein oligomerization. However, the theoretical data are consistent with the observation of monomeric Rev–RRE complexes which could be detected under more rigorous mobility shift conditions. In the more realistic environment of the filter binding experiment, where samples were incubated until equilibrium was attained prior to measurement of complexed Rev, an equilibrium Rev-RRE complex consists of at least two and possibly up to eight Rev molecules per RRE fragment.

The dissociation rate experiments support the theoretical fitting analysis from the binding isotherms. Complexes of two or more Rev molecules per RRE fragment dissociate from the RNA with a rate which is significantly slower than that of a singly bound Rev. Since no difference in the off-rate was observed between ratios of Rev:RRE of either 2:1, 4:1, or 8:1, we must conclude that the second Rev bound to the RRE affects the binding properties of the first.

Alternatively, several groups have suggested that Rev exists as a defined oligomer (trimer or tetramer) in solution (Holland et al., 1990; Nalin et al., 1990; Olsen et al., 1990b; Zapp et al., 1991). Several oligomeric Rev proteins would then be predicted to assemble on the RRE with a final stoichiometry of eight Rev monomers/RRE fragment. Zapp et al. (1991) conclude that such oligomerization is required for both RNA binding and Rev function. Our results indicate that Rev possesses a propensity to self-associate (depending of Rev concentration and buffer conditions) in solution as well as bound to the RRE in agreement with the results of several groups (Karn et al., 1991; Wingfield et al., 1991; Lee et al., 1993). When Rev binding to the RRE was studied as a function of protein concentration, monomeric Rev binding to the RRE was observed (Malim & Cullen, 1991). Limitations within the assay system utilized by Malim and Cullen (1991), however, precluded the ability to observe more complex Rev–RRE species.

These studies, together with previous work (Daly et al., 1990; Holland et al., 1990; Cook et al., 1991; Malim & Cullen, 1991), indicate that Rev binds as a monomer to a primary binding site and is stabilized by binding of a second Rev molecule leading to additional multimerization of Rev molecules on the RRE containing RNA. Our working hypothesis is that functional Rev–RRE complexes must contain multiple Rev molecules. We are currently testing this hypothesis to determine the nature of the protein–RNA complex which is capable of inhibiting splicing or is functional in RNA transport.

ACKNOWLEDGMENT

We thank Drs. Kathleen Sue Cook and G. King Farrington for technical assistance and many helpful discussions, Betsy Bothwell and Steve Mousterakis for help with graphics, and Colleen Maloney for administrative assistance. We also thank Marie-Louise Hammarskjöld and Dave Rekosh for supplying Rev protein purified under nondenaturing conditions.

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