Rev Binds Specifically to a Purine Loop in the SL1 Region of the HIV-1 Leader RNA*§

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The leader RNA sequence of human immunodeficiency virus type 1 (HIV-1) consists of a complex series of stem loop structures that are critical for viral replication. Three-dimensional structural analysis by NMR of one of these structures, the SL1 stem loop of the packaging signal region, revealed a highly conserved purine rich loop with a structure nearly identical to the Rev-binding loop of the Rev response element. Using band-shift assays, surface plasmon resonance, and further NMR analysis, we demonstrate that this loop binds Rev. HIV-1 appears to have a second Rev-binding site close to the major splice donor site that may have an additional role in the viral life cycle.

Human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, is classified as a complex retrovirus that uses a group of regulatory and accessory proteins to control its life cycle and to influence cellular function. The unspliced genomic RNA of the virus has been shown to contain a large number of cis-acting sequences that influence transcription, splicing, intracellular transport, genome dimerization, and packaging. The leader region in particular is highly structured and has been extensively studied by a number of different groups to attempt to analyze both the structures and their functional significance. One function that has been consistently ascribed to the leader is the presence of a sequence that is used to identify the RNA genome for encapsidation into the viral particle. This is termed the packaging signal, abbreviated as the Greek letter ψ. The encapsidation signal must distinguish the genomic RNA from viral as well as cellular messages. In HIV-1, using deletion mutagenesis, we identified a region involved in packaging (1) that was confirmed by other groups (2, 3). Since that time, a number of other regions inside and outside of the leader region have been implicated in packaging although largely in an enhancing role (4–8). Secondary structure analysis of the ψ region identified a series of conserved stem loops, and disruptive and compensatory mutation confirmed the importance of these structures in retaining packaging function (Fig. 1) (9). NMR-derived three-dimensional structures of the SL3 region have been published with and without complexed viral nucleocapsid protein (10, 11). In addition, NMR analysis of the major splice donor loop (SL2) and a fourth loop of uncertain functional significance (SL4) have been published (12–14). The first stem loop in the packaging region (SL1) has been the subject of intense analysis because of its involvement in the dimerization process of the genomic RNA facilitated through the “kissing” interaction of the palindromic sequences at the tip (15). Proximal to the terminal loop of SL1, there is a second loop structure consisting of an AGG triplet opposite a single bulged G residue. This region is 100% conserved in all HIV-1 sequences so far identified and is thought to be important in the viral life cycle and, in particular, is thought to be critical for the occurrence of dimer linkage (15). Proximal to this loop, the predicted structures differ between different groups. We demonstrated previously that a further loop (loop A) could be identified by biochemical analysis, phylogenetic comparison, and free energy minimization, and that sequential truncation of SL1 showed a progressive decline in virus viability, culminating in complete loss when loop A was removed (16). We recently published NMR evidence supporting this secondary structure (17). Loop A consists of an AGG facing a GG and is, again, 99% conserved in all known HIV-1 sequences and 100% conserved in known viable isolates. Intriguingly, the three-dimensional structure of this loop was found to be superimposable on another loop, that of the high affinity nucleation site of Rev on the Rev response element (RRE) found within the envelope gene sequence (18, 19). The Rev-RRE interaction is a vital part of the virus life cycle involved in the switch from early to late gene expression. Rev-RRE binding permits nuclear export of the unspliced and singly spliced viral messages (20). Rev also has been suggested to have additional effects such as enhancement of translation and packaging (21). It was, therefore, of great interest to determine whether the novel RNA structure we had identified in the leader region had the capability of binding Rev and whether that binding could be shown to be specific and to involve analogous nucleotide-amino acid interactions to those identified previously in the Rev-RRE complex.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—Recombinant Rev protein was obtained either from Bachem AG or through the European Union Program Cen-
turalized Facility for AIDS Reagents, National Institute for Biological Standards and Control, UK (Grants QLKZ-CT-1999-00689 and CP8228102). Both comprised His-tagged bacterially produced proteins, which on gel analysis were monocomponent. It was used at a final concentration of 100 ng/µl. Recombinant Gag protein was generated in vitro in Escherichia coli as described previously (10). It was used at a final concentration of 200 ng/µl.

The HIV-1 Rev peptide TRQARNrRWRERQR, corresponding to the arginine-rich 34–50 amino acid region of the Rev protein, was purchased from Bachem AG and microdialyzed against an aqueous solution containing 20 mM NaCl, 10 mM sodium phosphate (pH 6), and 0.1 mM EDTA. Short peptides corresponding to the arginine-rich RNA-binding domain of Rev have been shown previously to have good solubility properties and to bind specifically to stem-loop IIIB of the RRE with similar affinity (18, 22, 23).

Monoclonal Antibodies—Monoclonal antibodies directed against the Centralized Facility for AIDS Reagents at National Institute for Biological Standards and Control as described above.

DNA Preparation—DNA for in vitro transcription was prepared by polymerase chain reaction (PCR) amplification from the leader sequence of wild-type HXBc2 DNA and from a series of deletion mutants A1, A2, and A3 that sequentially truncate the SL1 sequence and that have been published previously (Fig. 5) (16). The primers used for amplification were: forward, TAATACGACTCACTATAGGAAACCAA-

Monoclonal Antibodies—Monoclonal antibodies directed against the HIV-1 Rev or HIV-1 Gag protein (p24 region) were obtained from the Centralized Facility for AIDS Reagents at National Institute for Biological Standards and Control, UK (Grants QLKZ-CT-1999-00689 and CP8228102). Both comprised His-tagged bacterially produced proteins, which on gel analysis were monocomponent. It was used at a final concentration of 100 ng/µl. Recombinant Gag protein was generated in vitro in Escherichia coli as described previously (10). It was used at a final concentration of 200 ng/µl.

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RESULTS

Rev Binds to the SL1 Stem Loop—In Fig. 2, Rev can be seen to produce a distinct retarded RNA band that is not observed when the A1 deletion mutant, which completely truncates SL1, is used. Cold unlabeled SL1 RNA in \( \times 5 \)-fold excess is able to compete away this binding, whereas unlabeled A1 RNA cannot. Cold RRE RNA can also compete the binding successfully again when in \( \times 5 \)-fold excess (Fig. 3). Rev thus binds to the intact SL1 sequence only when the SL1 region is present and can be competed off by another native RNA ligand.

Bound Rev-RNA Can Be Supershifted—Incubation of the Rev-RNA complex with monoclonal antibody to Rev demonstrates that the Rev loop A complex can be supershifted when the Rev loop is competed away by anti-Rev monoclonal antibody but not by anti-Gag antibody (Fig. 4).
strates a supershift of the RNA-protein complex (Fig. 4). To exclude nonspecific protein binding effects, a control antibody that binds to HIV-1 Gag was used, and this did not cause a supershift of the complex. The RNA species that is retarded in the presence of Rev is therefore a Rev-containing complex and not an effect of Rev on altering RNA structure in a nonspecific fashion.

**Sequential Truncation of SL1 Leads to Loss of Rev Binding**—A series of truncated RNAs was produced corresponding to those used in previously published packaging studies (16). A3 removes the terminal palindrome-containing loop. A2 further deletes SL1 to remove the first internal loop (loop B). A1 removes loops A and B. All truncations introduce a heterologous sequence at the tip of the truncation. Fig. 5 compares the effects of the truncations on binding of Rev and Gag protein. Gag will bind most efficiently to the SL3 region, which is retained in all the constructs. The gel demonstrates that A3 and A2 truncations still bind Rev, although the latter is slightly less efficient than the wild-type binding. The A1 truncation, as shown previously, abrogates Rev binding although retaining competence to bind Gag. The mapping identifies the loop A region as having the most influence on binding Rev.

**A Mutation of Loop A Reduces Rev Binding**—A more subtle mutation of SL1, which would be predicted to have little effect on the global structure of the region, was introduced. This substitutes two A purines for two G purines in loop A such that the structure becomes GGGG instead of AGGA opposing GG (Fig. 6).

Using competition with unlabeled ψ RNA to compare the affinity of the mutant sequence with that of wild-type ψ, band-shift analysis shows that this mutant has a reduced but not abolished affinity for Rev, comparing lanes 2 and 6 or 3 and 7 (Fig. 6).

**NMR Spectroscopy Confirms Rev Binding to Loop A**—Upon addition of the 34–50 Rev peptide, significant changes in the imino proton spectra of an RNA construct containing loop A (AGGA-GG) are observed. New resonances appear for several imino protons (e.g. U697, G730, G733, G728) connected by exchange cross-peaks with the corresponding resonances in the isolated RNA characterized previously by NMR spectroscopy (17) (Fig. 7). Exchange broadening is also observed for almost all RNA protons in the one-dimensional and two-dimensional spectra. These changes clearly indicate Rev peptide binding to AGGA-GG. Indeed, similar alterations in the RNA spectra

![Fig. 5. Truncation of SL1 beyond loop A abrogates Rev binding. The point of truncation of SL1 is indicated by the vertical bar. All constructs are shown to bind Gag.](image1)

![Fig. 6. Comparison of GGGG mutant with wild type. * indicates radiolabeled RNA. Triangles represent decreasing concentrations of unlabeled competitor wild-type ψ RNA demonstrating higher affinity of Rev for the wild-type ψ than the GGGG mutant.](image2)
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were also observed for the RRE-Rev interaction (26, 27).

The AGGA-GG-peptide sample precipitates before a 1:1 stoichiometry can be attained, and this, together with spectral broadening, complicates the spectral assignments and NOE analyses of this complex. A number of exchangeable and non-exchangeable assignments could still be obtained for several RNA nucleotides (A695; stem and UUCG tetraloop nucleotides) and peptide residues (Thr1, Ala4, and Trp12). The RNA hairpin seems to retain the same general conformation when bound to Rev; similar chemical shift and NOE patterns for the UUCG tetraloop and stem nucleotides are observed as well as a strong NOE between A695 H2 and C696 H1, within internal loop A detected previously in the isolated oligonucleotide (17). Additionally, broad NOEs between aromatic RNA resonances and peptide resonances (at 1.6 and 3.1 ppm) are observed in the nonexchangeable NOESY spectra. These interactions confirm Rev binding to the AGGA-GG oligonucleotide.

To assess the specificity of the observed interaction, we also studied the interaction of Rev peptide with another SL1 RNA construct containing mutant loop B (GGA-G) instead of loop A. As with internal loop A, loop B is purine-rich, and the GGA-G oligonucleotide system was also characterized by NMR spectroscopy in our previous study (17). Although some changes are present at the later stages of the titration, these are clearly less extensive than for loop A, indicating significantly less affinity of the Rev peptide for this purine rich bulge (supplementary material). Furthermore, addition of excess peptide to the loop B-Rev complex did not produce further spectral changes or sample precipitation. These data confirm the specificity of Rev binding to loop A rather than loop B in the SL1 region.

Affinity of Rev for Loop A—Based on the exchangeable NMR spectra of AGGA-GG free and in complex with Rev peptide, the isolated AGGA-GG RNA and the AGGA-GG-peptide complex are in slow exchange on the chemical shift time scale; upon addition of Rev, new imino resonances appear (Fig. 7) that are connected by exchange cross-peaks to the imino resonances of free RNA. Because some of the exchanging resonances are separated by 0.1 ppm (50 Hz at 500 MHz), the loop A peptide-dissociation rate is below 100 s⁻¹. Assuming a diffusion-limited association rate, this indicates that the dissociation constant should be smaller than ~10 μM. The significant broadening observed for many exchangeable and nonexchangeable RNA resonances upon addition of Rev also indicates a peptide-RNA dissociation constant in the μM range.

Spectra of Rev loop B mixtures showed no shifts indicating any interaction between these two molecules confirming that the loop A interaction was specific and not merely a nonspecific purine interaction with an arginine-rich peptide (data available as supplementary material).

The affinity of Rev for loop A has also been determined using surface plasmon resonance. Visual inspection of the traces obtained shows that the interaction is characterized by moderately rapid on and off rates. Scatchard analysis of equilibrium binding leads to estimates for the Kd of 1 and 2 μM from data sets recorded on channels with differing levels of coupled HIV-Rev (Fig. 8).

The affinity of recombinant Rev peptide (amino acids 34–50) against purified loop A RNA was also calculated using band-shift assays comparing bound and unbound RNA concentrations with known molarity of peptide. This was calculated using imaging intensifier methodology and gave a Kd of 0.11 μM (data not shown).

DISCUSSION

Our data confirm the hypothesis we proposed previously that loop A of the HIV-1 leader RNA can adopt a Rev-binding conformation (17). We have now shown by direct band-shift assays that Rev binds specifically to this region. Additionally, the chemical shift variations, spectral broadening, and NOE interactions observed by NMR spectroscopy are all consistent with binding of the Rev peptide to an SL1 construct containing internal loop A. A comparison of the Rev peptide interaction with a similar RNA oligonucleotide containing a different purine-rich bulge suggests that the Rev-loop A interaction is specific, confirming the band-shift results. Although spectral broadening and sample precipitation obviates an ideal NMR study at 1:1 stoichiometry, there is good agreement between our findings and those of Battiste et al. (26) and Peterson et al. (27), who also detected spectral broadening and changes in the imino resonances of nucleotides adjacent to the Rev-binding bulge. Refinement of the conditions for a NMR spectroscopy study of the interaction between internal loop A and the 34–50 Rev peptide or suitable analogues is currently in progress.

The SL1 loop already is predominantly in the “bound” conformation with the novel purine pairs, which the RRE only adopts after interaction with Rev (17). It was expected, therefore, that the affinity for binding of Rev to loop A would be different from that of the RRE because the SL1-Rev interaction would be expected to have a different biological role. Interestingly, unlike the Rev-RRE system, we see evidence for only a single binding event of Rev, although again the experimental conditions make it difficult to increase Rev concentration to seek evidence of multiple or cooperative binding patterns. The favorable conformation of SL1 may obviate further Rev assembly along the RNA. The lower affinity for loop A compared with the RRE does not imply lack of specificity, because both the band-shift data and the NMR comparison of loop A to loop B binding of Rev demonstrate clear single-site binding to this region in the context of both oligonucleotide and a longer in vitro transcript. Experimental data comparing the binding of Rev to loop A and loop B are available as supplementary material.
The Rev-RRE system is a widely studied and highly efficient system for transporting lentiviral RNA from nucleus to cytoplasm. Rev, however, has had a number of other properties ascribed to it. Initially it was believed to subserve two functions, that of inhibiting RNA splicing as well as directing nuclear export; it may be, however, that the export function acts to reduce splicing simply by efficient removal of the RNA from the splicing environment. Rev has also been claimed to enhance RNA loading on ribosomes for translation (28). In the past, we have shown that the RRE region itself enhances RNA packaging and can render a previously unpackageable RNA competent for encapsidation (21). More recently, a role for Rev in packaging has again been implicated in vector studies in which packaging has been implicated in vector studies in which the leader of HIV-1 and HIV-2 may undergo conformational changes during passage through the cell-forming structures, which do or do not favor dimerization and presumably affect packaging (31–33). We have demonstrated that the leader have continued to generate different structural in-

terpretations. Beerens and co-workers (34) have used their data to derive a structure in which the base of the stem sub-tending the primer-binding site is extended and to derive base pairs using bases that we predict to be involved in SL1 forma-
tion. Clever et al. (35) have also studied this region with de-
etailed mutagenesis and have concluded that there is no evi-
dence for this primer-binding site extension but that the bases we identify as occurring in loop A are critical for RNA packag-
ing. Other recent work has suggested that the loop A we de-
scribed and modeled using NMR is not substantiated on phy-
logenetic grounds (36). Because the helical regions on either side of loop A are conserved in HIV-1 and the AGGA-GG bulges are 100% conserved in 99% of all sequences, and because the variants are minor purine-for-purine substitutions, it is diffi-
cult to see the basis for this argument. We would argue that the structure derived from a combination of phylogenetic analysis, free energy minimization, and biochemical probing (by which we initially modeled this region) has been reinforced by an NMR-based structural solution and now by Rev binding. This makes a compelling case that the original SL1-1 model we proposed (9, 16) exists as a functional entity and, in particular, that loop A exists and that Rev binds specifically to it. It is also encouraging that a recent elegant NMR analysis of the loop B region provides additional support for our structure, published previously (37).

The function for Rev binding to SL1 is as yet unknown, although to our knowledge one other group has also found evidence for Rev binding in this region.\footnote{J. Kjems, personal communication.} A clear possibility is that Rev acts to enhance export of both spliced and unspliced viral mRNAs through its loop A interaction in the early phase of the viral life cycle but that the higher affinity interaction with the RRE becomes dominant when sufficient Rev has ac-
cumulated in the nucleus to bind in a multimeric form to the RRE. This would provide a switch from early to late gene expression based on differing affinity and numbers of bound

FIG. 8. Measuring the affinity of loop A RNA binding to HIV Rev by surface plasmon resonance. Loop A RNA at the concentrations indicated was injected (bar) for Time(s) over sensor surfaces with ~1000 response units (RU) or with no (control) HIV Rev bound. An overlay plot of the sensorgrams obtained after subtraction of their respective control sensorgrams is shown in the main panel. The spikes seen at the start and end of the injection are artifacts because of mistiming of the start/end of the injection between the HIV Rev labeled channel and the control channel. The inset shows a Scatchard plot derived from the response seen at equilibrium for the data shown in the main panel. A linear fit of these data gives a value for the $K_d$ of 2 μM with $R^2 = 0.9625$. 

The finding of a putative Rev-binding structure in the HIV-1 leader raises interesting questions. This area of the RNA has been modeled differently by a number of groups, and the nu-
cleotides can, on computer prediction, be folded into a number of different base-pairing conformations (9, 30). There is recent evidence that the leader of HIV-1 and HIV-2 may undergo conformational changes during passage through the cell-form-
ing structures, which do or do not favor dimerization and presumably affect packaging (31–33). We have demonstrated that the leader changes its conformation significantly when the Gag protein binds to the RNA, unwinding the helical regions and allowing Gag protein to bind along the RNA, which can then act as a scaffold element in viral assembly (10). Recent articles on the leader have continued to generate different structural
Rev proteins and is a plausible hypothesis that can be tested. This would also provide an interesting comparison with other RNA export mechanisms in other retroviruses in which all of the messages have an export signal on them, such as the Mason-Pfizer monkey virus constitutive transport element (38). The proximity of loop A to the major splice donor raises interesting possibilities regarding the effect of Rev on splicing documented previously; however a packaging function may also be possible. Whether Rev binding to SL1 has important implications for pathogenesis will depend on the specific role it may have in the life cycle of the virus. HIV-1-based vectors can also be possible. Whether Rev binding to SL1 has important implications for pathogenesis will depend on the specific role it may have in the life cycle of the virus. HIV-1-based vectors can be packaged in the absence of Rev indicating that it may not be critical for this process. However, in the context of wild-type virus replication, there may be an important role for Rev in the leader that makes it a further target for therapeutic interaction. Further work is currently under way to clarify this and to seek analogous structures in other lentiviruses.

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