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Trans-activating rev protein of the human immunodeficiency virus 1 interacts directly and specifically with its target RNA

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ABSTRACT **The 20-kDa phosphorylated rev protein from human immunodeficiency virus 1 has been shown to trans-activate posttranscriptionally the expression of viral structural proteins by selective stabilization and nuclear export of unspliced and incompletely spliced viral mRNA. We could demonstrate in gel-mobility and immunoprecipitation assays that the recombinant rev protein purified from a baculovirus expression system forms a distinct and specific complex with its target RNA (rev-responsive element), a 234-nucleotide sequence within the envelope coding region of human immunodeficiency virus 1. No complex formation could be observed using RNAs with similar secondary structure nor with other human immunodeficiency virus 1 recombinant proteins. Deletion analysis mapped this specific binding to the first 90 nucleotides of this rev-responsive element, which contains a U2 small nuclear RNA homologous region. We propose that the specific binding of rev to its target RNA sequence plays an essential part in releasing an incompletely spliced viral mRNA containing this target sequence to the cytoplasm.**

The human immunodeficiency virus 1 (HIV-1) encodes two nuclear trans-activating proteins, tat and rev, which are expressed from two differentially spliced overlapping reading frames and are indispensable for viral replication (1, 2). Whereas the tat protein may function at both the transcriptional and posttranscriptional levels in enhancing expression of sequences downstream of the HIV-1 long terminal repeat (3–5), rev clearly exerts its transactivation effect posttranscriptionally (6–8). As a 20-kDa phosphorylated protein (9), predominantly localized in the nucleoli (10, 11), rev promotes the synthesis of viral structural versus regulatory proteins by increasing the level of unspliced (gag-pol) and singly spliced (env, vif) mRNA in the cytoplasm. At the same time rev reduces the level of spliced RNA, thus repressing its own synthesis and that of other regulatory gene products including tat and nef (6, 8).

Based on recent results demonstrating a retention of unspliced mRNA in the nucleus in the absence of rev, a role for rev in facilitating the transport of unspliced viral mRNA is suggested (8, 11–13). A target RNA sequence, the rev-responsive element (RRE), within the HIV-1 envelope gene close to the 5′ splice site is indispensable for rev function and is predicted to form a complex secondary structure (8, 13). Since rev is indispensable for the formation of viral particles, an understanding of rev function should not only provide insight into the mechanisms of eukaryotic mRNA transport but also lead to possibilities for interfering with the function of this unique viral protein.

We addressed this question by investigating a possible direct interaction of rev and its target RNA sequence. A distinct and specific complex formation of target RNA and recombinant rev protein purified from a baculovirus expression system could be demonstrated in gel-mobility and immunoprecipitation assays. Deletion analysis showed that the first 90 nucleotides (nt) of the RRE (LT RRE) are sufficient to confer interaction with rev. We propose that rev interacts specifically with its target RNA sequence and that this represents a necessary step in activating transport of unspliced RNA to the cytoplasm.

MATERIALS AND METHODS

Recombinant rev Protein. rev protein was expressed in a baculovirus vector by contract to Repligen (Cambridge, MA). The cDNA coding sequence of the HIV-1 rev protein was obtained by isolating the 740-base-pair *Mst* II fragment from the pCV1 cDNA clone (3) containing the correct reading frame. It was blunt-ended by polymerization with the Klenow fragment of DNA polymerase I and inserted into the *Bam*HI site of the baculovirus transfer vector pVL941 (14). The natural polyhedrin ATG initiation codon was changed to ATT, so that initiation would occur within the inserted fragment. Insect cells (*Spodoptera frugiperda*, Sf9) were grown and infected with recombinant baculovirus (HT REV) at a multiplicity of infection of 3 essentially as described (15). The rev protein was purified by ion-exchange chromatography on DEAE-Sepharose and subsequent Sephacryl S-200 filtration. Western blot analysis with rev-specific antisera was performed according to standard procedures (16).

Cell Culture and Transfection. HeLa and HeLa cells stably expressing HIV-1 tat (kindly provided by B. Felber, Frederick Cancer Research Center, Frederick, MD) were grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum and gentamicin (50 μg/ml). Transfections with the individual plasmids (1 μg per 10^6 cells) were done by employing standard Ca precipitation techniques (16). For the loading of the recombinant rev and gp160 proteins, a Ca precipitate was made with protein and plasmid and then layered on the cells. Cells were grown for 24 hr, then harvested, and processed for chloramphenicol acetyltransferase assay as described (17).

Plasmids. LT, LT-D4, LT-D5, and LT-D6 were constructed by polymerase chain reaction (PCR) amplification of the respective fragment from HIVHXB2 plasmid (18) using oligonucleotide primers that contained a *Bam*HI (antisense) and *Hin*dIII (sense) site, thus allowing forced cloning into pIBI31 downstream of the T7 promoter according to standard procedures (16). The cloned insert of LT was generated from nt 7755 to nt 8003, of LT-D4 was from nt 7845 to nt 7959, of LT-D5 was from nt 7845 to nt 8003, and of LT-D6 was from nt 7890 to nt 8003 (numbering according to ref. 19). For the FS plasmid a fragment spanning nt 679–859 was amplified by using the PCR technology from the HIVHXB2 plasmid (18) and, after treating the blunt-ends with kinase, ligated into the

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Abbreviations: snRNA, small nuclear RNA; HIV-1, human immunodeficiency virus 1; RRE, rev-responsive element; nt, nucleotide(s); PCR, polymerase chain reaction.

Sma I site of Bluescript (Stratagene). This fragment includes the major splice donor site of HIV-1. PET8C was kindly provided by William Studier (Brookhaven National Laboratory, Upton, NY) and contains the T7 major capsid leader sequence. pIIIAR was as described by Rosen *et al.* (20). pREV expresses the HIV-1 rev gene under the control of the HIV-1 long terminal repeat promoter and is as described (7).

RNA and DNA Binding Assay. [α-^{32}P]UTP-labeled RNA transcripts were obtained by *in vitro* transcription with T7 polymerase according to manufacturer's instructions (Stratagene) using purified inserts as templates: LT, LT-D4, LT-D5, and LT-D6 were digested with *Eco*RI and *Bam*HI to yield full-length inserts including the T7 promoter; templates for LT-D1 to LT-D3 were obtained by digesting the LT *Eco*RI–*Bam*HI fragment with *Hae* III (LT-D1), *Dde* I (LT-D2), or *Hin*fI (LT-D3). FS was prepared for *in vitro* transcription by digesting the FS plasmid with *Bam*HI and PET was prepared by digesting the PET8C plasmid with *Nco* I. The TAR (nt +1 to nt +80) template was obtained by truncating the T7-LTR-CAT plasmid with *Hin*dIII. Short RNA fragments (14–26 nt) sequentially covering LT-D1 were generated by using synthetic oligonucleotides (HPLC purified) containing the T7 polymerase recognition sequence as templates. Sequences were as follows: SD22, 5′-GCAAGCTCCTATTCCAACTC-CCTATAGTGAGTCGTATTAA-3′; SD23, 5′-CTGCTC-CCAAGCCCTATAGTGAGTCGTATTAA-3′; SD24, 5′-CCGTCAGCGTCATTGACGCTGCGCCCTATAG-TGAGTCGTATTAA-3′; SD53, 5′-TCGTCCTTCGTG-ATACCCTATAGTGAGTCGTATTAA-3′. For *in vitro* transcription the individual oligonucleotides were hybridized with an oligonucleotide complementary to the T7 recognition sequence (SD20, 5′-TTAATACGACTCACTATA-3′) and transcription was then performed as described above. Labeled RNA transcripts were purified on nondenaturing polyacrylamide gels (6% and 20% gels, respectively) and radioactivity was quantified by Cerenkov counting.

RNA–protein binding reactions were carried out at room temperature in a total volume of 30 μl containing 60 mM NaCl, 12 mM Hepes (pH 7.9), 12 mM dithiothreitol, 4 mM Tris·HCl (pH 8.0), 4 mM $MgCl_2$, 1 μg of poly(dI-dC)·poly(dI-dC), and 50 units of placental RNase inhibitor (Boehringer Mannheim). Typically 4000 cpm of labeled RNA was used. The binding reaction was allowed to proceed for 10 min at room temperature and then the mixture was electrophoresed on a nondenaturing 6% polyacrylamide gel. Gels were dried and subjected to autoradiography. DNA binding was done in a buffer containing 60 mM NaCl, 12 mM Hepes (pH 7.9), 4 mM Tris·HCl (pH 8.0), 4 mM $MgCl_2$, and 1 μg of poly(dI-dC)·poly(dI-dC). For immunoprecipitation analysis, the binding reaction was incubated with 10 μl of polyclonal rev-specific (Repligen), envelope-specific (Biotech Research Laboratories), or preimmune rabbit antiserum for 30 min at room temperature and then precipitated with protein A-agarose (Boehringer Mannheim) and washed five times in binding buffer. Labeled RNA was released in 0.3% SDS and analyzed by liquid scintillation counting. rev antisera were prepared at Repligen by immunization of rabbits with purified rev protein.

For isolation of the RNA–protein complex the binding reaction was performed as described using ^{32}P-labeled RNA. The complex was resolved on a nondenaturing 5% polyacrylamide gel and identified by autoradiography; the band was excised and directly loaded on a SDS/15% polyacrylamide gel in loading buffer containing RNase A. Western blot analysis using rev-specific antiserum at a dilution of 1:1000 was performed according to standard procedures (16). Visualization of the antigen–antibody complexes was achieved by incubation with ^{125}I-labeled protein A and subsequent autoradiography.

RESULTS

Recombinant rev Protein. The nonfusion rev protein expressed in a baculovirus system was purified to more than 80%, as judged by a Coomassie-stained SDS gel. Identity of the protein was confirmed by Western blot analysis (data not shown). To confirm the biological activity of the recombinant rev protein, HeLa cells stably expressing tat were cotransfected with pIIIAR, a rev-dependent chloramphenicol acetyltransferase-expressing plasmid containing the HIV-1 envelope sequence (16), and the recombinant rev or gp160 protein at 5 μg per 10^6 cells. With the rev protein, a 13.7-fold increase in chloramphenicol acetyltransferase activity could be seen, which was absent when a recombinant baculovirus gp160 preparation was used. This induction is comparable to what can be seen with a rev-expressing plasmid, pREV (data not shown).

rev Forms a Stable and Specific Complex With Its Target RNA. To approach the question of RNA–protein interaction, the sequence within the HIV-1 gene required for rev function was amplified by the PCR from the HIVHXB2 plasmid and cloned into pIBI31 downstream of the T7 promoter (Fig. 1*A*). By using an *in vitro* T7 polymerase transcription system, a 249-nt [^{32}P]UTP-labeled RNA transcript, LT, was obtained. Incubation of this RNA with the recombinant rev protein under appropriate binding conditions and subsequent analysis in a gel-retardation assay clearly demonstrated the formation of a stable RNA–protein complex. This complex formation could be observed with as little as 50 ng of rev protein and reached full saturation at 225 ng of protein (Fig. 1*B*). Since the rev protein displays a high net positive charge due to arginine-rich stretches, we sought to exclude nonspecific ionic interaction with the target RNA by varying the salt concentration. The complex remained stable over a broad range of salt concentrations (20–500 mM NaCl; data not shown), indicating that the interaction was nonionic in nature. Nonspecific competitor RNAs (tRNA) and other polynucleotides [poly(rA-rU)·poly(rA-rU) or poly(dI)·poly(dC)]

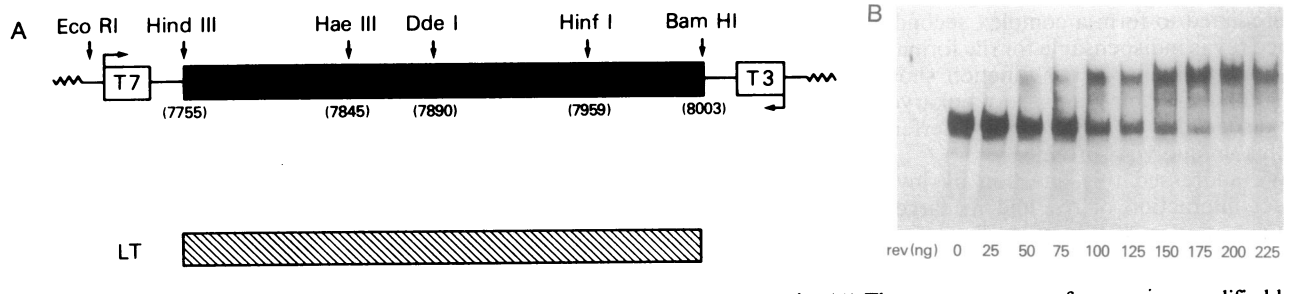

FIG. 1. Complex formation of rev-responsive target LT RNA and HIV-1 rev protein. (*A*) The target sequence for rev was amplified by a PCR employing primers with *Hin*dIII and *Bam*HI sites and cloned into pIBI31. Numbering indicates HIV-1 nucleotide position (according to ref. 19). LT denotes the full-length transcript of the RRE. (*B*) *In vitro*-transcribed [^{32}P]UTP-labeled LT RNA was incubated with 25–225 ng of purified recombinant rev protein (as indicated) and electrophoresed on a nondenaturing 6% polyacrylamide gel.

did not inhibit complex formation when employed up to 50,000-fold excess (data not shown). In contrast, unlabeled LT RNA could effectively compete with complex formation of labeled RNA and rev in a 5- to 100-fold excess (Fig. 2*A*).

To analyze the specificity of the binding further, various recombinant HIV-1 proteins and various RNA templates exhibiting a similar secondary structure were investigated. Recombinant HIV-1 gp160 and HIV-1 tat, both purified from a baculovirus expression system, as well as HIV-1 nef, purified from an *Escherichia coli* expression system, failed to bind to the rev-responsive LT RNA (Fig. 2*B*). Also no complex formation of the recombinant rev protein with the HIV-1 TAR region and with the T7 major capsid leader sequence RNA, which both form stem–loop structures, nor with a HIV-1 RNA stretch including the major splice donor site could be demonstrated (Fig. 2*C*). No binding of rev to labeled LT DNA could be observed in gel-shift assays using reaction conditions similar to those for RNA binding (data not shown).

Deletion Analysis of the Target RNA. For further delineation of the RNA binding region, 5′ deletions (LT-D4 to LT-D6) and 3′ deletions (LT-D1 to LT-D3) of the LT RNA were performed (Fig. 3*B*). By using gel-mobility assays, [^{32}P]UTP-labeled LT-D1, LT-D2, and LT-D3 RNA transcribed *in vitro* formed a complex with rev, as judged from the retarded migration, whereas LT-D4, LT-D5, and LT-D6 RNA did not bind (Fig. 3*A*). The same RNA transcripts were used for a control binding reaction with an equivalent amount of recombinant gp160, where no shifted bands were observed (Fig. 3*C*). To ensure that the complex formation was actually due to the rev protein rather than to a contaminating protein, immunoprecipitation assays of the protein–RNA complex were performed. Polyclonal anti-rev rabbit serum could precipitate a LT RNA–rev complex and a LT-D1 RNA–rev complex, as judged by liquid scintillation counting of the [^{32}P]UTP-labeled RNA. This could not be achieved with HIV-1 envelope-specific antiserum or preimmune serum. No complex could be precipitated from the reaction mixture of LT-D5 and rev (data not shown). To further prove that the protein binding to LT and LT-D1 was rev, the RNA–protein complex was resolved on a nondenaturing polyacrylamide gel, as described above, and identified by autoradiography. The complex was excised and directly loaded on a 15% polyacrylamide gel containing SDS.

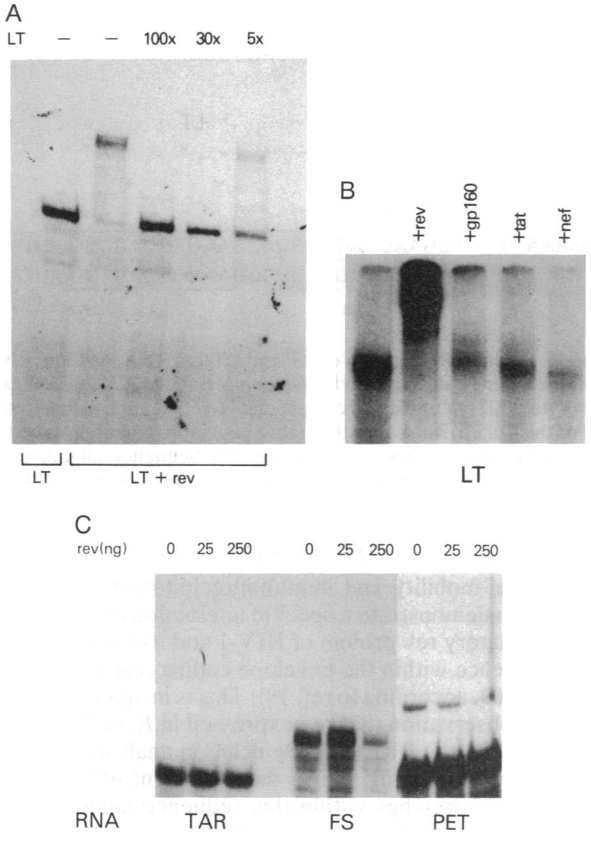

FIG. 2. Specificity of rev binding to its target sequence. (*A*) Complex formation of [^{32}P]UTP-labeled LT RNA with 250 ng of recombinant rev was incubated with 5- to 100-fold excess of unlabeled *in vitro*-transcribed RNA (as indicated) and electrophoresed on a nondenaturing 6% polyacrylamide gel. (*B*) Other recombinant HIV-1 proteins, gp160, tat, and nef, were compared to rev in gel-mobilty assays as described above using 250 ng of protein per assay. (*C*) *In vitro*-transcribed [^{32}P]UTP-labeled RNA with strong secondary structure such as the HIV-1 tat-responsive region (TAR), the T7 bacteriophage promoter sequence (PET), and a HIV-1 RNA containing the major splice-donor site (FS) were assayed as described above using recombinant rev protein at 0, 25, or 250 ng per assay (as indicated).

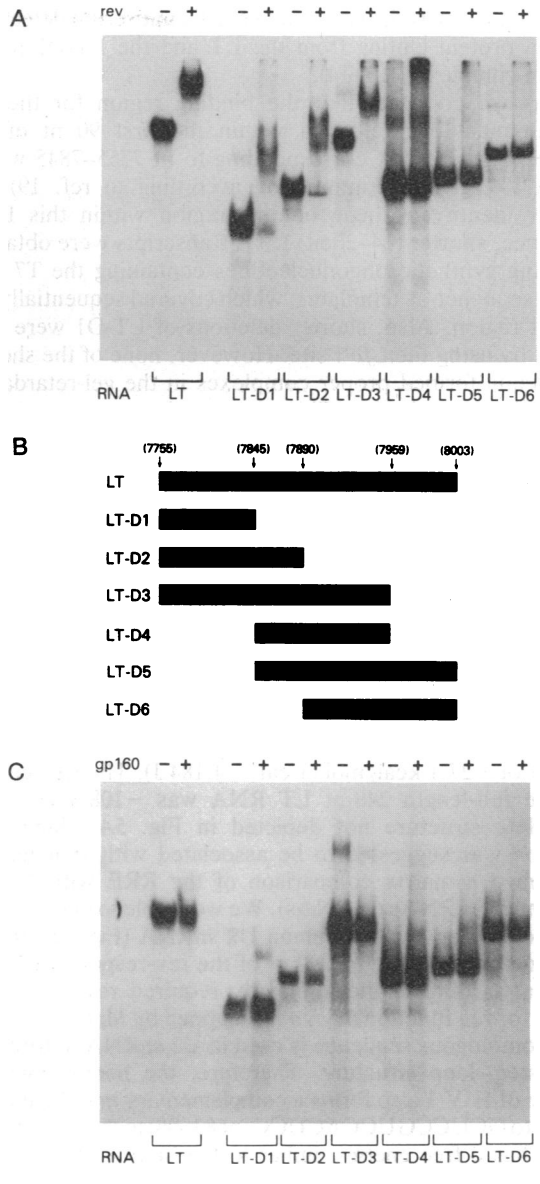

FIG. 3. Deletion mapping of the rev-responsive LT RNA. The 5′ and 3′ deletions are as depicted in *B*. Numbering indicates position in HIV-1 (according to ref. 19). (*A*) [^{32}P]UTP-labeled RNA transcripts of LT and LT-D1 to LT-D6 were incubated with 250 ng of recombinant rev protein and analyzed in a gel-retardation assay on a nondenaturing 6% polyacrylamide gel. (*C*) As control, the same RNA transcripts as in *A* were incubated with 250 ng of recombinant gp160 protein and analyzed in the same way.

Immunoblot analysis with a rev-specific antiserum detected a 20-kDa protein eluting from the LT and the LT-D1 RNA–protein complexes (Fig. 4).

Thus we conclude that the binding region for the rev protein must be contained within the first 90 nt of the rev-responsive RNA corresponding to nt 7755–7845 within the HIV-1 genome (numbering according to ref. 19). To further identify a single binding region within this RNA sequence, shorter (14–26 nt) RNA transcripts were obtained by using synthetic oligonucleotides containing the T7 promoter sequence as templates, which covered sequentially the LT-D1 region. Also, shorter deletions of LT-D1 were generated by using the *Cfo* I site. However, none of the shorter transcripts formed proper complexes in the gel-retardation assay (data not shown). This suggests that a more complex structure than what can be conferred by short RNA stretches may be necessary for RNA binding.

Sequence Analysis. Secondary structure of an RNA is supposed to play an important role for providing suitable contact points for appropriate portions of the interacting protein. By applying the algorithm by Zuker and Stiegler (21) and energy rules by Salser (22), a computer analysis of the theoretical secondary structure of the full-length LT RNA and the 90-base LT-D1 RNA, which seemed sufficient to confer binding to rev, was done. They both display a stable stem structure sharing structural similarity, as is demonstrated in Fig. 5*A*, with a calculated free energy (ΔG) for LT-D1 of −27.1 kcal/mol (1 cal = 4.184 J). The free energy for the full-length 249-nt LT RNA was −108.3 kcal/mol (complete structure not depicted in Fig. 5*A*). Since rev function was suggested to be associated with splicing, we performed a matrix comparison of the RRE with U1–U6 small nuclear RNAs (snRNAs). We were able to detect a 90% homology over 20 nt to human U2 snRNA (Fig. 5*B*) that is contained within the first 90 nt of the rev-responsive RNA, but that is outside the minimally required rev-responsive region for rev function *in vivo*, as mapped by Malim *et al.* (8). This homologous sequence is used in U2 snRNA to form the third stem–loop structure. Therefore, the homologous sequence of HIV-1 also forms a complementary antisense RNA strand to GCUCCGUCCACUCC (nt 83–96) in the U2 snRNA, which has a free energy similar to the original base pairing within the snRNA (numbering for U2 is as found in Microgenie Genebank, Beckman; nt 63 corresponds to nt 112 in ref. 23).

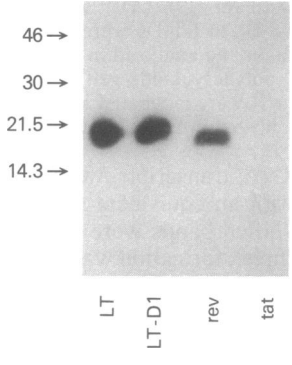

FIG. 4. Identification of rev in the RNA–protein complex. After resolution of the LT RNA–protein complex and LT-D1 RNA–protein complex (reaction performed as above) on a nondenaturing polyacrylamide gel, the band was cut out and directly loaded on a 15% polyacrylamide gel containing SDS. Western blot analysis was performed with rev-specific antiserum (as above) and visualization of the antigen–antibody complex was achieved with ^{125}I-labeled protein A. The control lanes contain 100 ng of the rev preparation (rev) and 100 ng of the tat preparation (tat). Molecular masses in kDa are shown.

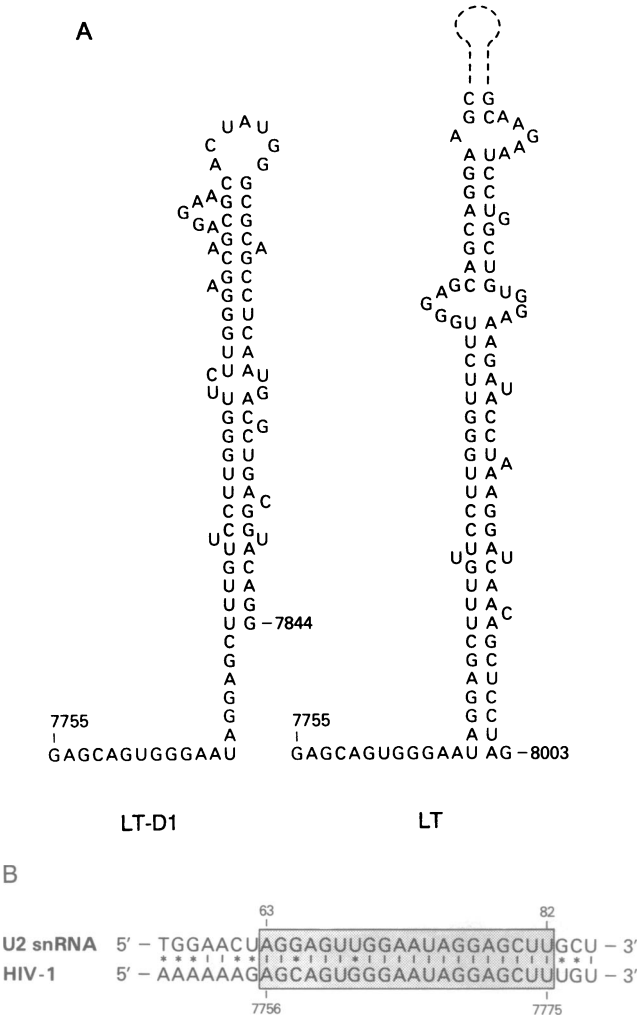

FIG. 5. Sequence analysis of LT and LT-D1 RNA. (*A*) The RNA was folded using the algorithm by Zuker and Stiegler (21) and Salser's energy rules (22). Numbering corresponds to HIV-1 according to ref. 19. For the full LT RNA only the stem of the more complex structure (indicated by dashed lines) is depicted. (*B*) Sequence alignment of HIV-1 RNA (numbering according to ref. 19) and human U2 snRNA (numbering as in Microgenie Genebank, Beckman).

DISCUSSION

By using gel-mobility and immunoprecipitation assays, we were able to demonstrate a specific interaction of the recombinant regulatory rev protein of HIV-1 and its target RNA, a 249-nt sequence within the envelope coding region of HIV-1 (nt 7755–8003, according to ref. 19). This is in agreement with the recent observation that rev expressed in *E. coli* can form a complex with the RRE (24). By deletion analysis, we have mapped the rev binding site to the first 90 nt of this RRE. Shorter RNA stretches within this sequence failed to form complexes when analyzed in gel-mobility assays. The first 90 nt (LT-D1) can form a stable stem structure that is comparable to structures found within full-length RRE (LT). It should be stressed, however, that a theoretical as well as experimental elucidation of the secondary structure of an isolated RNA strand always has to be artificial to some degree, since the effect of flanking sequences, folding of the nascent RNA as it comes off its template, and its potential binding to nuclear proteins are difficult, if not impossible, to take into consideration. Our results suggest that some kind of secondary structure of the RNA (loops, pseudoknots, bulges, or mismatches) in connection with sequence specificity may be required to provide a three-dimensional array of contacts

for the interaction with appropriate structures of the rev protein. This interaction is likely to be an essential step for the subsequent activation of the transport of unspliced or singly spliced viral mRNA to the cytoplasm, thus effecting the transition to the synthesis of viral structural proteins.

As the RNA is transcribed from its template, multiple nuclear proteins bind to the transcript. Association with the nuclear matrix takes place and processing is initiated. Only fully processed mRNA (capped, methylated, polyadenylylated, and spliced) will be released from the nuclear matrix and transported to the cytoplasm through a nuclear pore complex. The critical step is the release from the nuclear matrix with translocation across the nuclear envelope being a facultative step (for review, see ref. 25). Thus rev has to shunt incompletely processed unspliced mRNA to the cytoplasm by affecting the retention by the nuclear matrix. By recognizing the specific interaction of rev with its target RNA sequence, as we describe in this paper, several possible mechanisms for rev function can be considered.

Binding of rev to the RNA may inhibit the adhesion to the nuclear matrix and thus accelerate the cytoplasmic transport of unspliced RNA. Association of the RNA with the nuclear matrix is not well understood; specific nuclear proteins, snRNA, or intron sequences have been suggested, but none of them could be convincingly validated. However, in each case, interference by rev binding to its target RNA sequence is a conceivable mechanism. On the other hand, if the RNA is already committed to the nuclear matrix, rev may act to effect the release of the transcript and thus bypass the splicing machinery. Double-stranded RNA regions have been implicated in attaching an RNA to the nuclear matrix with release of a mature mRNA associated with a topoisomerase II-like activity (25, 26). Thus one could speculate on an unwinding activity of rev, either directly or by activation of other cellular factors. This hypothesis is especially attractive, since the binding region of the RRE coincides with a complex secondary structure with long double-stranded regions (Fig. 5*A*). Thus this sequence represents a regulatory element serving two functions: one is to attach the pre-mRNA to the nuclear matrix in the absence of rev, allowing splicing to proceed until the sequence is removed as intron with subsequent release of the mRNA; in the presence of rev, unwinding of the double-stranded regions releases the unspliced mRNA from the matrix and allows the facultative step of translocation across the nuclear envelope.

Sequence homology to the U2 snRNA offers another possibility. Hybridization to the complementary sequence in the U2 stem may render the splicing process ineffective and allow the release of the unspliced RNA. This hypothesis is supported by the observation that an antisense oligonucleotide aimed at this specific U2 stem is able to increase the level of unspliced simian virus 40 mRNA in the cytoplasm in a *Xenopus* oocyte system (27). Rev may then function by rendering the otherwise cryptic sequence in RRE available for base pairing with U2 snRNA. Also it should be noted, that binding of U2-specific cellular proteins, A′ and B″, has been located to the last two (3′) hairpin–loop structures in U2 (28, 29), which would be destroyed by base pairing. Inhibition of spliceosome assembly in yeast was indeed shown to facilitate nuclear export of mRNA (30). Concerted action of rev and its target sequence might thus be a sequence-specific way to circumvent spliceosome-mediated nuclear retention.

The various mechanisms involving rev are not necessarily mutually exclusive to circumvent nuclear retention, which may also be quantitatively modulated by cis-acting negative sequences (13, 20). The observation that rev responsiveness is retained even when splice donor and acceptor sites were deleted (13, 20) or when the RRE is shifted to an exon (8) suggests that rev can act independently of splicing but does not rule out a yet undefined interaction with the spliceosome.

Further investigations will be needed to elucidate the mechanisms underlying rev function, which should also shed light on the general cellular control of transporting specific RNAs to the cytoplasm. The evidence presented here for a direct and specific interaction of rev with its target RNA raises several attractive mechanisms that can be investigated. Furthermore, the mapping of the interacting sequence should allow the design of agents that can specifically interfere with rev binding. This might hold some promise in inhibiting viral replication by blocking the synthesis of viral structural proteins.

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