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The Basic Domain of Rev from Human Immunodeficiency Virus Type 1 Specifically Blocks the Entry of U4/U6 · U5 Small Nuclear Ribonucleoprotein in Spliceosome Assembly

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Human immunodeficiency virus type 1 (HIV-1) encodes a regulatory protein, Rev, which is required for cytoplasmic expression of incompletely spliced viral mRNA. Rev binds to a *cis*-acting Rev-responsive element (RRE) located within the *env* region of HIV-1. It has previously been shown that a 17-amino-acid peptide, corresponding to the basic domain of Rev, specifically inhibited in vitro the splicing of mRNAs containing the RRE. In this reaction, the peptide acts after an ATP-dependent step in the spliceosome assembly resulting in an accumulation of a 45-50S splicing-deficient complex. Characterization of this complex revealed that the basic domain of Rev does not interfere with U1 small nuclear ribonucleoprotein binding but blocks the entry of U4, U5, and U6 small nuclear RNAs into the spliceosome. Binding of U2 small nuclear ribonucleoprotein was partially inhibited. The critical nature of the oligomeric structure of RRE has been investigated both in vitro and in vivo. Reporter genes that contained one, three, or six repeated-monomer high-affinity Rev binding sites (IIB) within an intron yielded a correlation among the oligomeric state of bound Rev; inhibition of splicing; ability to block the assembly of U4, U5, and U6 small nuclear RNAs in the spliceosome in vitro; and level of Rev response in vivo.

Human immunodeficiency virus type 1 (HIV-1) is the primary etiologic agent of the lethal disease AIDS (1, 15). It belongs to the group of complex retroviruses which encode a number of regulatory proteins in addition to the Gag, Pol, and Env proteins common to all retroviruses. The best-studied proteins are the Tat and Rev proteins from HIV-1. Tat mediates its function through direct binding to an RNA motif (TAR) in the 5′ end of the HIV-1 transcript, leading to transactivation of transcription from the viral long terminal repeat, whereas Rev acts posttranscriptionally by regulating the cytoplasmic appearance of unspliced and single-spliced HIV-1 mRNA in the cytoplasm (reviewed by Cullen [8]). There has been some controversy about what particular step of posttranscriptional RNA processing is regulated by Rev. Rev may modulate recognition of the precursor RNA by splicing components and thereby indirectly regulate the cytoplasmic transport of mRNA (4, 30), and/or Rev may activate an alternative transport mechanism, allowing unspliced introns to be exported to the cytoplasm (13, 14, 34).

Rev mediates its function through a *cis*-acting RNA target sequence termed the Rev-responsive element (RRE), which is located in the envelope gene of HIV-1 (11, 17, 34, 44) and has been shown to bind the RRE as a multimeric protein in vitro (5, 9, 10, 18, 35, 43, 50). RNA footprinting data suggest that RRE contains several discrete binding sites for Rev (25), and a minimal RNA substrate containing only a stem-loop structure of 35 nucleotides of the RRE has been demonstrated to bind monomeric Rev (2, 7, 19, 26). The internal loop and flanking nucleotides of this minimal Rev binding site have been identified as the main determinants for Rev recognition (2, 26, 48). Although Rev binds this minimal binding site with high affinity in vitro, a tandem repeat of 65 nucleotides of the RRE is necessary for Rev function in vivo (22).

The active signal for regulation by Rev is conferred by the viral protein in the RRE-Rev complex and not by RNA structure. It has recently been shown that Rev will regulate the appearance of cytoplasmic viral RNA when targeted to the nuclear precursor RNA by a heterologous RNA binding domain (37, 49).

The Rev protein has been divided into functional domains on the basis of mutational analysis. The tract of basic amino acids of Rev (residues 35 to 50) is important for RNA binding and nuclear and nucleolar localization (21, 35, 42). A synthetic peptide containing this domain of Rev (positions 34 to 50) (Rev 34-50) binds specifically to the RRE, interacting with essentially the same set of nucleotides as the total Rev protein, suggesting that this region of Rev contains the entire RNA binding site (26). Flanking the basic region are protein sequences important for oligomerization of Rev on the RRE (33, 42, 51). A leucine-rich sequence centered around residue 80 is believed to interact with a cellular factor on the basis of the observation that mutations in this region which do not affect RNA binding and Rev oligomerization can render Rev defective and an effective dominant inhibitor of wild-type Rev function (32).

We have previously investigated the function of Rev in a splicing system in vitro. The addition of the Rev protein specifically inhibited the splicing of a β-globin pre-mRNA containing an RRE by three- to fourfold. Furthermore, the addition of Rev 34-50 was highly functional and specifically inhibited splicing of the same RNA by 30-fold (27). In this assay, a pre-mRNA containing tandemly repeated monomer binding sites for Rev was as responsive to regulation as a pre-mRNA containing a complete RRE. Here, the mechanism of Rev-dependent inhibition of splicing has been investigated by analysis of the splicing complexes formed in the

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presence of Rev 34-50. We demonstrate that the basic domain of Rev does not interfere with U1 small nuclear ribonucleoprotein (snRNP) binding but inhibits splicing by effectively blocking the entry of the essential U4/U6 · U5 tri-snRNP particle in spliceosome assembly. We also show a clear correlation between the functionality of pre-mRNA with either one, three, or six tandemly repeated monomer Rev binding sites in vitro and in vivo, suggesting that inhibition of spliceosome assembly in vitro is a relevant process for Rev function in vivo.

MATERIALS AND METHODS

Plasmids. The following plasmids have previously been described: pPIP7.A (41); pRRE (25); PIP/B1/RRE, PIP/B1/GLO, PIP/B1/1xIIB, PIP/B1/6xIIB, p1xIIB, and p6xIIB (27); and pgTat-CMV, pcRev-CMV, pcRevmut-CMV, and pβG-CMV (4). pgTat1xIIB, pgTat3xIIB, and pgTat6xIIB were prepared by replacing the *Kpn*I-*Hin*dIII fragment of pgTat-CMV containing the RRE in the sense orientation with the blunted *Kpn*I-*Bam*HI fragments of p1xIIB, p3xIIB, and p6xIIB, respectively. p3xIIB was prepared by the strategy described previously for p6xIIB (27). pgTatΔRRE was prepared by deleting the *Kpn*I-*Hin*dIII fragment of pgTat-CMV. pgTatRRE was prepared by deleting the *Ssp*I-*Bgl*II fragment of pgTat-CMV (see Fig. 1).

Preparation of Rev, Rev peptides, and RNA transcripts. Rev was prepared as described previously by Kjems et al. (25). Peptides, which were a generous gift from Alan D. Frankel, were prepared as described by Calnan et al. (3). Transcription and renaturation of RNA for the mobility shift assay were done by the methods of Kjems et al. (26). Biotinylated RNA for streptavidin affinity purification was synthesized in 200-μl reaction mixtures containing 5 μg of linear plasmid DNA, 40 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 4 mM spermidine, 10 mM dithiothreitol, 50 U of RNasin, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM GTP, 0.5 mM CTP, 0.05 mM biotin-11-UTP (10% of total UTP; Bethesda Research Laboratories), 50 μCi of α-^{35}S-UTP (3,000 Ci/mmol; New England Nuclear), and 200 U of T7 RNA polymerase (Stratagene). The RNA was separated from unincorporated nucleotides on a G-50 Sephadex spin column and purified on a 4% polyacrylamide–8 M urea gel. The final concentration of the RNA was calculated from the specific activity of incorporated ^{35}S label.

Gel mobility shift and in vitro splicing assays. Gel mobility shift assays and in vitro splicing assays were performed essentially as described previously (25, 26), except that the splicing reaction mixtures were preincubated for 20 min in the absence of ATP or Rev 34-50, as described in the legend to Fig. 2, followed by 90 min of incubation in the presence of ATP and Rev 34-50. The splicing products were fractionated on 6% polyacrylamide–8 M urea gels containing 75 mM Tris-borate (pH 8.3). Gels were quantitated with a Molecular Dynamics PhosphorImager and Image Quant software version 3.0.

Purification of splicing complexes and Northern (RNA) blotting. Streptavidin affinity purification of biotinylated RNA was performed essentially as described by Grabowski and Sharp (16), using the following modifications. RNA splicing was done in 200-μl reaction mixtures containing 1 μg of pre-mRNA (approximately 10^6 cpm), 30% (vol/vol) nuclear extract in buffer D (12), 1 mM ATP, 5 mM creatine phosphate, 2.5 mM $MgCl_2$, 25 U of RNasin, 0.3 mM dithiothreitol, 100 μg of *Escherichia coli* tRNA, and 10 μl of Rev dilution buffer (27) containing either no peptide or 0.4 μg of Rev 34-50 per μl. The samples were incubated for 20 min at 30°C and loaded onto a 5-ml 10 to 30% sucrose gradient in buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.6], 60 mM KCl, 3 mM $MgCl_2$). The gradients were centrifuged for 2 h and 40 min at 48,000 rpm in an SW 50.1 rotor, and 23 to 24 fractions of 205 μl each were collected from the top of the gradient. Forty microliters of the fractions was counted in a scintillation counter to determine the amount of ^{35}S-labelled mRNA in each fraction. Fractions 11 to 20 (containing complexes of approximately 35S to 65S) were pooled in pairs (12 and 13, 14 and 15, etc.) into five fractions, and the salt was adjusted to 360 mM KCl by adding 40 μl of 3 M KCl. Thirty microliters of a 1:1 suspension of streptavidin beads in NET2 buffer (50 mM Tris-HCl [pH 7.9], 0.05% [vol/vol] Nonidet P-40, 0.5 mM dithiothreitol), which had been preblocked by incubation with 0.5 mg of tRNA per ml and 0.2 mg of bovine serum albumin per ml in NET2 buffer at 4°C for 1 h, was added to each fraction and rotated at 4°C for 1 h. The beads were washed four times in washing buffer (0.05% [vol/vol] Nonidet P-40, 500 mM KCl, 20 mM HEPES-KOH [pH 7.6], 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA) over a total of 3 h, and specific bound complexes were released by incubating collected beads in denaturation buffer (20 mM Tris-HCl [pH 7.5], 4 M urea, 0.5% sodium dodecyl sulfate, 10 mM EDTA, 0.3 M NaCl) at 50°C for 5 min. The samples were extracted with phenol-chloroform, precipitated, and loaded onto an 8% denaturing acrylamide gel for Northern blot analysis. The RNA was transferred to GeneScreen filters (New England Nuclear) and probed with antisense transcripts of U1, U2, U4, U5, and U6 small nuclear RNAs (snRNAs) by the protocol of New England Nuclear. The anti-snRNA transcripts were prepared by the method of Konarska and Sharp (29). Quantification of U4 snRNP was generally performed after rehybridizing the filter with anti-U4 snRNA alone because of the comigration of a degradation product of U1 snRNA with U4 snRNA.

Transfections and nuclease S1 analysis. COS A6, which is a clonal line derived from COS7, was transfected by the protocol of Chang and Sharp (4). Cells were harvested 48 h after transfection, and cytoplasmic mRNA was prepared by the Nonidet P-40 lysis method described by Maniatis et al. (36). The RNA was treated with DNase to remove contaminating chromosomal DNA. The nuclease S1 assay was essentially done as described by Chang and Sharp (4), using 5 μg of cytoplasmic RNA and 100,000 cpm of each 3′-end-labelled *Bam*HI-*Stu*I DNA fragment of pgTat-CMV and *Bam*HI-*Nco*I DNA fragment of pβG-CMV for each reaction. The protected fragments were separated on 6% polyacrylamide–8 M urea gels.

RESULTS

Rev 34-50 acts after an ATP-dependent step in spliceosome assembly. In a previous study, it was shown that Rev 34-50 specifically inhibits the splicing of an RRE containing pre-mRNAs in vitro if the peptide is present during spliceosome assembly (27). To investigate the particular step of spliceosome assembly at which the addition of Rev 34-50 interferes, a splicing reaction mixture containing two mRNAs, PIP/B1/RRE with an RRE and PIP7.A without an RRE (Fig. 1A), was preincubated for 20 min in the absence of either ATP, Rev 34-50, or both (Fig. 2); the preincubation was followed by 90 min of incubation in the presence of all components. The yield of lariat product RNA was used as an appropriate measure for RRE-specific inhibition of splicing. Preincuba-

FIG. 1. Constructs. (A) Structure of pre-mRNAs derived from PIP.7A, which contains a modified version of the first intron in the major late transcript of adenovirus behind a T7 promoter. (B) Structure of pre-mRNAs derived from pgTat-CMV, which contains the Tat gene of HIV-1 behind a cytomegalovirus promoter. Exons and introns are denoted with thick and thin lines, respectively, and the test fragments, which were inserted in PIP7.A or which replaced an internal fragment in pgTat-CMV, are denoted by shaded bars (not drawn to scale). Names and sizes (in base pairs) of various inserts and names of respective plasmids are indicated. Abbreviations for the inserted elements: RRE, wild-type RRE; βGlo, a control fragment from the second intron of the rabbit β-globin gene; 1xIIB, helix IIB of the RRE (26); 3xIIB, three tandem repeats of IIB RNA; 6xIIB, six tandem repeats of IIB RNA. Restriction sites within PIP7.A and pgTat-CMV which were used for constructions are indicated. The dashed line indicates the position of the DNA fragment used for nuclease S1 analysis, and the asterisk denotes the location of a radioactive end label. (C) Schematic drawing of the RRE and the structure of the IIB domain which constitutes a single high-affinity binding site for Rev and Rev 34-50. The 3xIIB and 6xIIB elements contain three and six direct repeats of the IIB element, respectively. Putative base pairs are connected by dashes or dots. The 5'-terminal GG sequence of IIB does not derive from the RRE sequence but was added to stabilize the duplex structure.

tion of the substrate RNAs with nuclear extract and ATP before the addition of Rev 34-50 yielded only a marginal Rev 34-50 effect (Fig. 2, lanes 5 to 8). However, if either ATP or both ATP and Rev 34-50 were absent during the preincubation of substrate RNAs with nuclear extract, the splicing of the pre-mRNA containing the RRE was specifically inhibited upon addition of the peptide (Fig. 2, lanes 1 to 4 and 9 to 12). This implies that Rev 34-50 competes kinetically with an ATP-dependent step of spliceosome assembly.

Rev 34-50 blocks the entry of U4, U5, and U6 snRNAs into the spliceosome. Splicing proceeds through a number of intermediate steps which involve formation of a spliceosome containing snRNP complexes (U1, U2, U4/U6, and U5 snRNPs). The content of splicing complexes formed in the presence of Rev 34-50 was investigated by an affinity selection protocol described by Grabowski and Sharp (16). Biotin and radioactive ^{35}S were incorporated into PIP/B1/RRE RNA (Fig. 1), and the substrate RNA was incubated with nuclear extract for 20 min in the absence and presence of Rev 34-50. Spliceosome complexes containing the ^{35}S-labelled substrate RNA were resolved by sedimentation through density gradients. In the absence of Rev 34-50, the pre-mRNA was assembled into 40S and 60S prespliceosome and spliceosome complexes, respectively. The amount of pre-mRNA in the 60S peak was much smaller than that in the 40S peak. This was due to the relatively large amount of substrate RNA (approximately 1 μg) added to the reaction mixture. In the presence of Rev 34-50, only one broad peak sedimenting at approximately 45S was formed, in accordance with earlier observations (Fig. 3A) (27). Complexes containing biotinylated mRNA were affinity selected from fractions across the sucrose gradients by using streptavidin beads. The samples were analyzed by a Northern blot which was probed with a mixture of ^{32}P-labelled RNA complementary to snRNAs (Fig. 3B). The level of nonspecific binding of snRNA was determined with control experiments using nonbiotinylated pre-mRNA performed in parallel (Fig. 3C). The 40S and 60S complexes formed in the absence of Rev 34-50 contained mainly U1 and U2 snRNAs and all five snRNAs, respectively. In contrast, the 45S peak formed in the presence of Rev 34-50 contained predominantly U1 snRNA and no fully assembled spliceosomes were detected. The 45S complex which formed in the presence of Rev 34-50 exhibited a tail of faster-sedimenting complexes containing not only U1 snRNA but also a significant amount of U2 snRNA. This suggests that the pre-mRNA-U2 snRNA complex partially forms in the presence of Rev 34-50 and that the assembly pathway is arrested at this point. A similar series of experiments were performed in which spliceosome assembly on pre-mRNAs of similar length lacking the RRE (PIP/B1/GLO) was compared with assembly on pre-mRNA containing the RRE, both in the presence of Rev 34-50 (Fig. 3D). Only when the RRE was absent from the pre-mRNAs were U4, U6, and U5 snRNAs observed sedimenting as part of a mature spliceosome in the 60S region. Thus, blocking of the entry of these snRNAs by Rev 34-50 was strictly RRE dependent.

Intact Rev also inhibits the binding of U4/U6 and U5 snRNPs, but the effect is less specific compared with that observed with the Rev 34-50 peptide (data not shown). This result correlates with the previously published observation which showed that intact Rev is less specific as an inhibitor of mRNA splicing in vitro, probably because of the limited solubility of the protein at the required concentrations (27). In a control experiment, Rev 34-50 was replaced with a basic peptide containing 15 arginine residues (R15). This peptide binds the RRE with high affinity but with low specificity (26), and its addition inhibits splicing at high concentrations and only in an RRE-independent manner (27). Analysis of splicing complexes showed that the addition of inhibitory levels of R15 reduced the binding of all five snRNPs in an RRE-

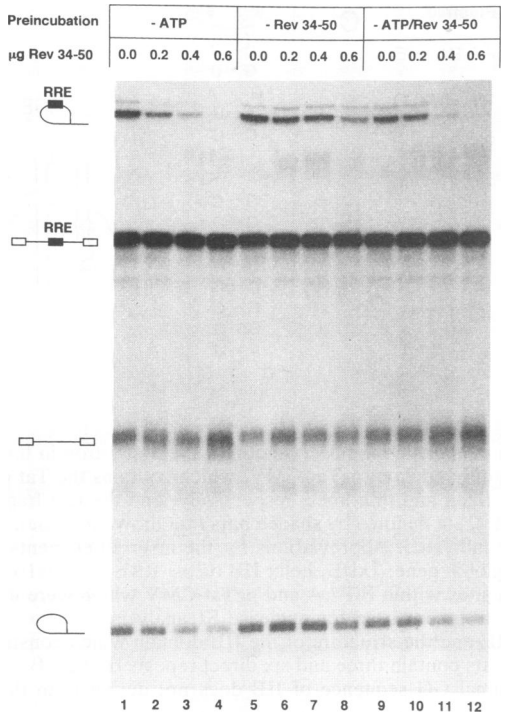

FIG. 2. ATP dependence of Rev 34-50 inhibition of splicing. PIP/B1/RRE pre-mRNA and PIP7.A pre-mRNA (internal control lacking the RRE) were incubated together under splicing reaction conditions for 90 min at 30°C in the presence of the indicated amount of Rev 34-50. Otherwise, complete splicing reaction mixtures were preincubated for 20 min in the absence of ATP (lanes 1 to 4), in the absence of Rev 34-50 (lanes 5 to 8), or in the absence of both ATP and Rev 34-50 (lanes 9 to 12) and at zero time the remaining components were added. The identities of the products are indicated schematically. Exons and introns are denoted by open boxes and thin lines, respectively, and the RRE is indicated by a solid box. The efficiency of splicing can be measured by the level of lariat formation. Specific Rev 34-50 inhibition of splicing is determined from the ratio of the amounts of lariat RNAs from pre-mRNA containing the RRE and pre-mRNA lacking the RRE.

independent manner, suggesting a general interference with snRNP assembly (data not shown).

Multiple copies of IIB can function as the RRE both in vitro and in vivo. The specificity of Rev function is mediated by the RRE. A previous analysis of Rev binding to the RRE by RNA footprinting techniques suggested the presence of multiple binding sites (25). The highest-affinity binding site was determined to be the hairpin IIB subregion, which is 35 nucleotides in length (2, 7, 19, 26). To test the importance of multiple binding sites for biological activity, a number of artificial RREs (1xIIB, 3xIIB, and 6xIIB) containing one, three, and six copies of the high-affinity binding site for Rev (26) were constructed. Incubation of increasing amounts of Rev protein with the 6xIIB RNA generated multiple complexes as resolved by a gel mobility assay. These complexes were formed over the same protein concentrations and were similar in mobility to the complexes formed with the wild-type RRE (Fig. 4). More explicitly, both RRE and 6xIIB generated approximately six complexes over the same titration range of Rev, although formation of the first four complexes with the wild-type RRE occurred at a lower Rev concentration than formation of a similar set of complexes with 6xIIB RNA.

We have previously shown that the Rev 34-50 peptide will specifically suppress the splicing of an RNA substrate containing the 6xIIB element in an assay in vitro (27). An investigation of the splicing complexes formed on a pre-mRNA containing six copies of IIB RNA (PIP/B1/6xIIB) in the presence of Rev 34-50 yielded results very similar to those for the wild-type RRE containing pre-mRNA (PIP/B1/RRE) shown in Fig. 3 (data not shown). To test whether this correlation extends to Rev function in vivo, 1xIIB, 3xIIB, and 6xIIB elements were inserted into the intron of an HIV-1 expression vector. The constructs were transfected into COS cells together with a plasmid expressing either functional Rev or nonfunctional mutant Rev, and the level of cytoplasmic unspliced HIV-1 mRNA was determined by nuclease S1 protection analysis (Fig. 5A). The degree of Rev response, the increase of unspliced/spliced cytoplasmic RNA following cotransfection with functional Rev compared with mutant Rev, was measured for each mRNA construct (Fig. 5B). Constructs without an RRE or with one copy of IIB showed no Rev response. Three copies of IIB yielded an intermediate response (3.8), whereas six copies of IIB yielded an almost full Rev response (5.1) compared with the response obtained with the RRE (6.9). These results show that sequences within IIB are sufficient for Rev function and that a correlation between the number of Rev binding sites and the Rev response exists both in vitro and in vivo.

DISCUSSION

The Rev protein regulates the level of intron-containing RNA in the cytoplasm. This regulation occurs at the stage of pre-mRNA splicing and/or transport to the cytoplasm. RNA splicing and transport may, however, be closely interconnected in the nucleus, and the distinction between splicing factors and transport factors may be ambiguous. We have previously reported that Rev and a related peptide, Rev 34-50, will specifically inhibit in vitro splicing of a pre-mRNA containing the RRE. In this study, the function of the Rev 34-50 peptide in a splicing system in vitro has been investigated. The basic domain of Rev blocks the entry of U4/U6 and U5 snRNPs in the assembly of the spliceosome. This inhibition is specific for RNAs containing an RRE and for the Rev 34-50 peptide. The interference of spliceosome assembly by Rev 34-50 probably does not reflect simple blockage of accessibility. The binding of U1 snRNP and, partially, U2 snRNP, which interacts directly with the pre-mRNA, is not affected by Rev 34-50. It has previously been shown that the position of the RRE can be varied in different pre-mRNAs without a significant loss of Rev 34-50 inhibition of splicing (27). Furthermore, structures within introns do not necessarily interfere with splicing since extensive RNA secondary structures can be attached to or inserted into intron sequences without blocking RNA splicing (28, 46). These data in total suggest that a specific step in spliceosome assembly, before association of the particle U4/U6 · U5 tri-snRNP complex, is affected by the basic domain of Rev.

The assembly of spliceosomes occurs through a number of intermediate complexes (29, 40). Initially, an ATP-independent commitment complex which contains only U1 snRNP and a number of splicing factors is formed (23, 39). This complex interacts with U2 snRNP and other splicing factors in an ATP-dependent process to form the A complex. The U4/U6 · U5 multi-snRNP complex associates with complex A to form the spliceosome (complex B). It is likely that the addition of Rev 34-50 peptide interferes with a step after formation of the commitment complex, possibly with the

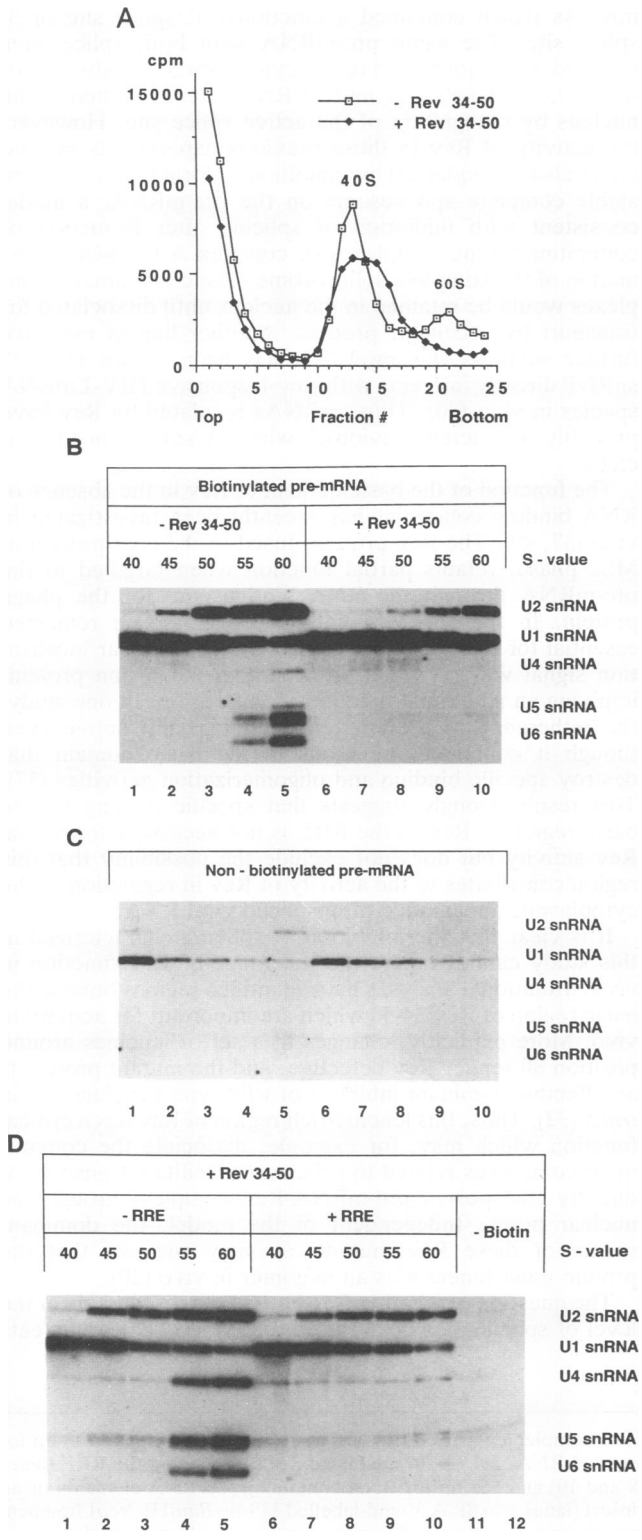

FIG. 3. Analysis of splicing complexes. (A) Two hundred-microliter splicing reaction mixtures which had been incubated for 20 min at 30°C and which contained approximately 1 μg of biotinylated and ^{35}S-labelled PIP/B1/RRE pre-mRNA were analyzed by sedimentation in sucrose gradients to compare the relative efficiencies of splicing complex formation in the absence of Rev 34-50 (−Rev 34-50) and in the presence of 4 μg of Rev 34-50 (+Rev 34-50). The positions of the 40S and 60S peaks are indicated. (B) Northern blot showing the snRNA composition of affinity-purified splicing complexes in the absence of Rev 34-50 (lanes 1 to 5) and in the presence of Rev 34-50 (lanes 6 to 10) as indicated. Splicing complexes formed on PIP/B1/RRE pre-mRNA were affinity purified from fractions 12 and 13, 14 and 15, 16 and 17, 18 and 19, and 20 and 21 of the sucrose gradient shown in panel A. The sedimentation range of these fractions is approximately 35S to 65S as indicated. Recovered complexes were denatured, and the eluted snRNAs were electrophoresed on an 8% polyacrylamide–8 M urea gel. The RNA was electroblotted onto a nitrocellulose membrane and probed with a mixture of antisense U1, U2, U4, U5, and U6 snRNAs that yielded approximately equal autoradiographic signals for the five snRNAs. (C) The same experiment as in panel B, using nonbiotinylated pre-mRNA as a control for nonspecific binding of snRNA to streptavidin beads. (D) Same experiment showing the RRE specificity of the effect of Rev 34-50. Splicing complexes formed on biotinylated PIP/B1/GLO pre-mRNA (−RRE, lanes 1 to 5) were compared with complexes formed on biotinylated PIP/B1/RRE pre-mRNA (+RRE, lanes 6 to 10), both in the presence of 4 μg of Rev 34-50. Lanes 11 and 12 are nonbiotinylated control samples which were treated in the same manner as the samples in lanes 5 and 10.

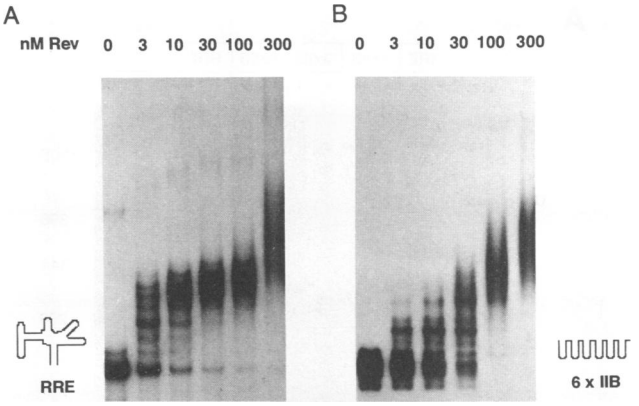

FIG. 4. Gel mobility shift assay showing the binding of Rev to RRE and 6xIIB RNA probes transcribed from pRRE and p6xIIB plasmids, respectively. Radioactively labelled RNA containing the RRE (A) or six repeated copies of IIB RNA (B) was incubated in the indicated concentration of Rev as described in Materials and Methods. RRE and 6xIIB indicate the positions of the free probe, and the putative structures of the RNA probes are drawn schematically.

formation of the A complex. The rationale for this is mainly that Rev 34-50 competes with an ATP-dependent step in spliceosome assembly prior to the binding of the U4/U6 · U5 snRNP complex. Furthermore, the level of complex A with tightly bound U2 snRNA is diminished in the presence of Rev 34-50. Kinetic considerations also support this conclusion. The Rev 34-50 peptide must be present in the splicing reaction as early as 1 min after initiation of the incubation with nuclear extract to function optimally; if the Rev peptide was added 15 min after the start of the incubation, only marginal inhibition of splicing was observed (27). This time scale is too long if the peptide had to inhibit the rapid formation of the ATP-independent commitment complex in 1 to 2 min (23, 39) (using PIP7.A) but is consistent with inhibition of formation of complexes A and B at 10 to 15 min.

We have previously suggested that Rev might dissociate spliceosome complexes from pre-mRNA to facilitate their transport to the cytoplasm (4). This proposal explained the findings that Rev regulated the cytoplasmic levels of pre-

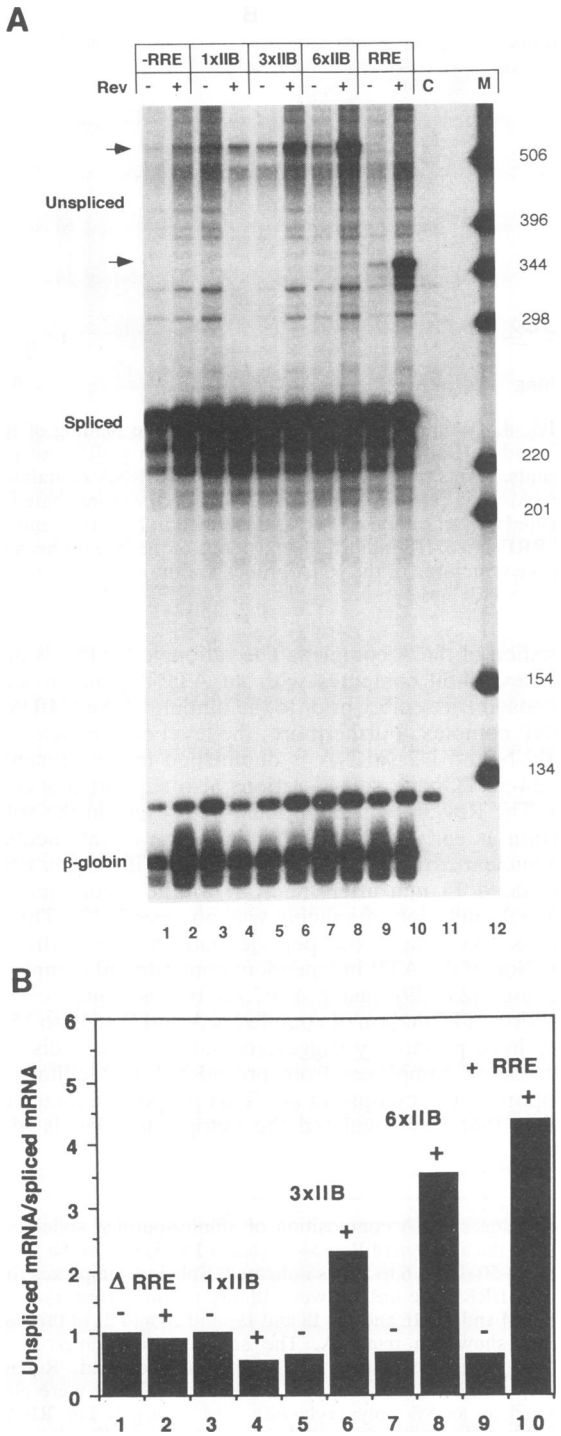

FIG. 5. In vivo Rev response with various RREs. (A) Cytoplasmic mRNA prepared from transiently transfected COS cells was analyzed by nuclease S1 protection. The cells were transfected with a combination of three different plasmids: (i) pgTatΔRRE (without RRE, lanes 1 and 2), pgTat1xIIB (lanes 3 and 4), pgTat3xIIB (lanes 5 and 6), pgTat6xIIB (lanes 7 and 8), or pgTatRRE (with RRE, lanes 9 and 10); (ii) pcRev-CMV (encoding functional Rev; indicated by a plus sign) or pcRevmut-CMV (encoding nonfunctional Rev truncated at the *Bam*HI site; indicated by a minus sign); (iii) pβG-CMV (encoding β-globin; added to all samples to control transfection efficiency). A 3′-end-labelled 880-bp *Bam*HI-*Stu*I probe was used to distinguish unspliced Tat mRNA from spliced Tat mRNA (Fig. 1B). The complementarity of this probe to the RNA extends 248 bp for spliced RNA, 361 bp for unspliced RNA containing the RRE (lanes 9 and 10) and 553 bp for RNA containing IIB RNA elements or no insert (lanes 1 to 8). A 3′-end-labelled 119-bp *Bam*HI-*Nco*I fragment which was specific for the second exon of wild-type β-globin was included in the hybridization mixture to detect the control RNA. C and M denote a control in which cytoplasmic RNA was replaced with *E. coli* tRNA and a size marker lane, respectively. (B) The ratio of unspliced mRNA/spliced mRNA was quantitated for each lane in panel A and normalized to 1 for lane 1. The level of Rev response is measured by the induction of this ratio in the presence of Rev (indicated by a plus sign) compared with that in the absence of functional Rev (indicated by a minus sign).

mRNAs which contained a functional 5′ splice site or 3′ splice site. The same pre-mRNA with both splice sites mutated was transported to the cytoplasm in the absence of Rev. Thus, these single mutant RNAs were retained in the nucleus by recognition of the active splice site. However, the activity of Rev in those previous experiments in vivo could also be explained by inhibition of formation of a more stable complete spliceosome on the pre-mRNA, a model consistent with inhibition of splicing after formation of commitment-type complexes or complex A but before formation of the complete spliceosome. The commitment complexes would be retained in the nucleus until dissociated for transport by a cellular process. Another line of evidence further supports this model. It has been shown that U1 snRNP directly interacts with Rev-responsive HIV-1 mRNA species in vivo (30). Thus, mRNAs regulated by Rev have probably interacted previously with U1 snRNP in the nucleus.

The function of the basic domain of Rev in the absence of RNA binding constraint has recently been investigated in vivo (37, 49). The Rev protein, fused to the coat protein of MS2 phase, retains partial function when targeted to the pre-mRNA through the RNA binding site for the phage protein. In these experiments, a basic domain remained essential for Rev function, even when the nuclear localization signal was provided elsewhere in the fusion protein, implying an additional function of this region. In one study, the tethered Rev protein remained partially active even though it contained mutations in the basic domain that destroy specific binding and oligomerization activities (37). This result strongly suggests that specific binding by the basic region of Rev to the RRE is not necessary for partial Rev activity but does not exclude the possibility that this region contributes to the activity of Rev in regulation of the cytoplasmic appearance of unspliced viral RNA.

It is clear that the inhibition of splicing characterized in this study cannot reflect the full range of Rev function in vivo. Mutational analyses have identified regions outside the basic region of Rev 34-50 which are important for activity in vivo. More explicitly, changes in a set of leucines around position 80 render Rev defective, and the mutant protein is an effective dominant inhibitor of wild-type Rev function in *trans* (32). Thus, this leucine-rich region of Rev has a critical function which may, for example, dissociate the commitment complexes related to splicing to facilitate transport or directly transport a pre-mRNA-Rev complex through the nuclear pores. Independent of the model, the dominant nature of these Rev mutants strongly suggests that the protein must function as an oligomer in vivo (20).

The question of whether Rev has any direct function at the level of splicing has been raised (6, 31, 45). The arguments

for Rev function in processes other than splicing are based mainly on observations that sequences within the *gag* region can function as inhibitory sequences (INS, CRS, or IR) for the cytoplasmic appearance of mRNA and that the inhibition can be overcome by the presence of the RRE in *cis* and the Rev protein in *trans*. It is argued that the lack of any known splice sites in these model viral pre-mRNAs indicates that Rev probably does not act at the level of splicing. However, it is impossible to exclude the presence of potential splice sites in such pre-mRNA and thus it is possible that commitment complexes or nonproductive spliceosome-like complexes do play a role in regulation by Rev. For example, as suggested here, part of Rev's activity may be to regulate the assembly of these complexes. Other types of retroviruses lacking known Rev-like proteins also contain sequences which inhibit splicing and thus balance the synthesis of unspliced and spliced viral RNAs (24, 38, 47). These viruses may overcome the retention of the partially spliced viral precursors in the nucleus by utilization of a cellular Rev-like factor or unknown viral protein activities.

The specificity of Rev function is mediated by the RRE, which has been intensively studied by mutagenesis. Originally, the RRE was defined as a highly structured 234-nucleotide target sequence (34, 44) which was required for full biological activity in vivo. More recently, it was demonstrated that two copies of a 65-nucleotide subfragment of the RRE were fully functional in vivo (22). The observation that six copies of a 39-nucleotide fragment, IIB, which contains a minimal binding site for a monomer of Rev, are also fully functional in vivo and in vitro suggests that Rev function is due to multiple tandem complexes of RNA and the Rev protein. The activity of Rev is highly dose dependent, because one copy of the IIB target RNA was inactive in vivo, three copies were only partially active, and six copies were almost fully active. Similar observations have been obtained with multiple MS2 phage coat protein binding sites for Rev-MS2 fusion proteins (37, 49). In those studies, four copies of the MS2 operator binding site protein yielded up to 50% of the Rev response obtained with an equivalent reporter constant containing the wild-type RRE (37).

Previous experiments have suggested that the RRE has multiple binding sites with variable binding affinities for Rev. The total RRE probably binds four or more Rev proteins. Many of the binding sites in RRE are of such low affinity that Rev will remain associated with them only when the Rev-RRE interactions are stabilized by Rev-Rev interactions. This suggests that Rev stably oligomerizes in the presence of the RRE. Furthermore, mutational changes in Rev which do not reduce the affinity of binding to a monomer site but do interfere with oligomerization would cause defects in activity in vivo (33, 42, 51). It is interesting that the strong dose dependence of the binding of oligomers of Rev would provide a basis for sharp regulation of function dependent on the concentration of cellular Rev (33).

A splicing system responsive to Rev in vitro is a valuable tool in the elucidation of Rev function and the potential isolation of Rev inhibitors. The reaction further analyzed by this study is dependent on a specific binding of a Rev peptide and the presence of an RRE-type element. Furthermore, the correlation that multiple sites for Rev binding are critical for specific activity strengthens the validity of the in vitro reaction. Finally, the observation that the Rev peptide blocks the splicing reaction at a specific point before formation of a complete spliceosome is another indication of the utility of the in vitro system.

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