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Identification of a Domain in Human Immunodeficiency Virus Type 1 Rev That Is Required for Functional Activity and Modulates Association with Subnuclear Compartments Containing Splicing Factor SC35

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The activity of human immunodeficiency virus Rev as a regulator of viral mRNA expression is tightly linked to its ability to shuttle between the nucleus and cytoplasm; these properties are conferred by a leucine-rich nuclear export signal (NES) and by an arginine-rich nuclear localization signal/RNA binding domain (NLS/RBD) required for binding to the Rev-responsive element (RRE) located on viral unspliced and singly spliced mRNAs. Structure predictions and biophysical measurements indicate that Rev consists of an unstructured region followed by a helix-loop-helix motif containing the NLS/RBD and sequences directing multimerization and by a carboxy-terminal tail containing the NES. We present evidence that the loop portion of the helix-loop-helix region is an essential functional determinant that is required for binding to the RRE and for correct intracellular routing. Data obtained using a protein kinase CK2 phosphorylation assay indicated that the loop region is essential for juxtaposition of helices 1 and 2 and phosphorylation by protein kinase CK2. Deletion of the loop resulted in partial accumulation of Rev in SC35-positive nuclear bodies that resembled nuclear bodies that form in response to inhibition of transcription. Accumulation of the ΔLoop mutant in nuclear bodies depended on the presence of an intact NES, suggesting that both the loop and the NES play a role in controlling intranuclear compartmentalization of Rev and its association with splicing factors.

The Rev protein of human immunodeficiency virus type 1 (HIV-1) is a 116-amino-acid phosphoprotein that is essential for expression of the incompletely spliced mRNAs encoding the structural viral proteins. Direct interaction of Rev with a stem-loop structure termed the Rev-responsive element (RRE) located in this subset of mRNAs protects them from splicing and degradation, facilitates their nuclear-cytoplasmic transport, and promotes their utilization in the cytoplasm (reviewed in references 28 and 56).

Although Rev is detected primarily in the nuclei and nucleoli of human cells under steady-state conditions, it is known to shuttle between the nucleus and the cytoplasm (32, 49, 58), a property that is essential for its function as an RNA transporter. In addition to accumulating in nucleoli, Rev has been demonstrated to partially colocalize with the splicing factor SC35 in nuclear speckles (33) or in the vicinity of nuclear speckles (5, 20) and, when coexpressed with an HIV-1 RNA target, in the cytoplasm (41) as well as in subnuclear zones probably corresponding to active sites of transcription and processing (6, 41).

Results of numerous mutagenesis studies aimed at defining functional domains within the Rev protein have identified an amino-proximal arginine-rich region spanning amino acids 35 to 50, which serves as both a nuclear localization signal (NLS) and an RNA binding domain (RBD), and a leucine-rich sequence spanning amino acids 75 to 93, which functions as a nuclear export signal (NES). Additional residues important for multimerization and high-specificity binding to RRE flank the NLS/RBD (reviewed in references 28 and 56).

As shown in Fig. 1A, the PHDSEC secondary-structure prediction model (59, 60) indicates that Rev consists of an unstructured region spanning residues 1 through 8 (the amino-terminal tail), a helix-loop-helix region (amino acids 9 to 24, 25 to 34, and 35 to 61, respectively), and an unstructured carboxy-terminal tail spanning residues 62 to 116. Superimposing the secondary structure information with functional data places the NLS/RBD within helix 2, the NES in the carboxy-terminal tail, and sequences contributing to multimerization and RNA binding specificity in the helix-loop-helix. The PHDsec-generated secondary structure prediction generally agrees with earlier structural predictions and biophysical studies of Rev peptides performed by Auer et al. (1), which provided evidence for the presence of a helix-loop-helix motif within the amino-terminal half of the protein, although these authors proposed that the carboxy-terminal portion of the protein (i.e., beyond residue 65) is also helical.

Although the tertiary structure of Rev remains to be determined, mutagenesis studies provided evidence that the two helices might associate with each other, with Leu^{22} (located in helix 1), Ile^{59}, and Leu^{60} (located in helix 2) forming a contiguous hydrophobic patch that determines formation of multimeric Rev-RRE complexes (79, 80). Nuclear magnetic resonance studies carried out using a peptide spanning residues 34 to 50 demonstrated a direct interaction between a minimal RRE sequence and amino acids 34 (in the loop), 35, 38, 39, 40 to 44, 46, and 48 (all in helix 2) (2). While the loop linking the two α-helices has not previously been identified as a distinct functional domain, we reasoned that this region might play a role in directing correct orientation of the two flanking helices

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A

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Rev
                1         10         20         30         40         50         60
AA      |MAGRSGDSDEELIRTVRLIKLLYQSNPPPNPEGTRQARRNRRRRWRERQRQIHSISERIL|
PHD     |         HHHHHHHHHHHHHHHH            HHHHHHHHHHHHHHHHHHHHHHHHHHHH|
Rel     |999999755999999989999841589899842228996658999999999999998762|

                61         70         80         90        100        110
AA      |GTYLGRSAEPVPLQLPPLERLTLDCNEDCGTSGTQGVGSPQILVESPTVLESGTKEN|
PHD     |H                   E                        EE             |
Rel     |048998999887578641001568999999999858899961233565354798689|

RevΔLoop
                1         10         20  Δ24-34  40         50         60
AA      |MAGRSGDSDEELIRTVRLIKLLYRLQARRNRRRRWRERQRQIHSISERIL|
PHD     |         HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH|
Rel     |999999815999999999999999898876689999999999999988977|

                61         70         80         90        100        110
AA      |GTYLGRSAEPVPLQLPPLERLTLDCNEDCGTSGTQGVGSPQILVESPTVLESGTKEN|
PHD     |HHH                                 E    EEEE            |
Rel     |652589979999899975521367867879767613079943564532221698789|
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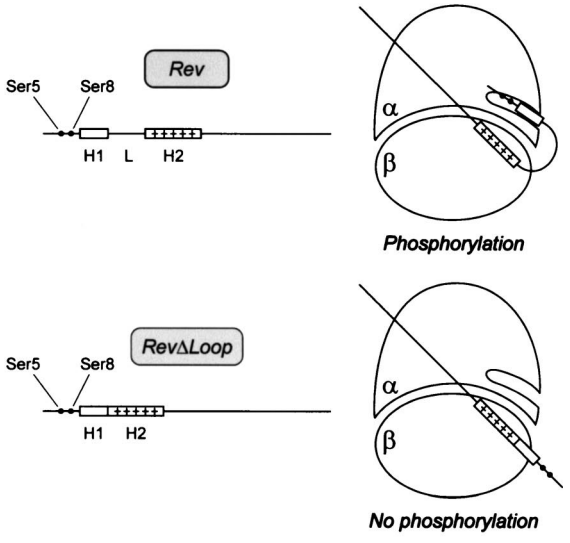
B



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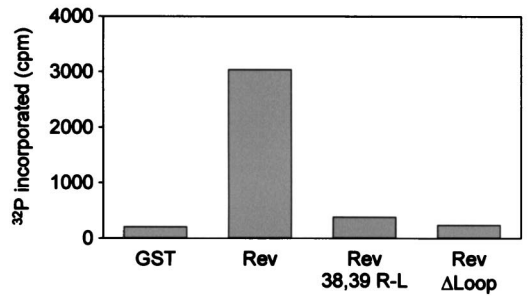

FIG. 1. Predicted secondary structures of Rev and RevΔLoop and phosphorylation of wild-type Rev and RevΔLoop by protein kinase CK2. (A) Secondary structures predicted for wild-type Rev protein and RevΔLoop, obtained using the PHDsec method (59, 60), which is available over the Internet (http://dodo.cpmc.columbia.edu/predictprotein/). Unstructured (loop) regions, β sheets, and helices are indicated by blank spaces, Es, and Hs, respectively, on the PHD line; the Rel line reports the reliability of the structure predicted for each residue on a scale of 0 to 9 (lowest to highest reliability, respectively). (B) A model of the interactions between Rev and RevΔLoop and the catalytic (α) and regulatory (β) subunits of CK2. In the diagram of Rev, the two helices (H1 and H2) are indicated by boxes, separated by the intervening loop (L); the plus signs in the second helix indicate the polycation-like arginine-rich domain. Although the active CK2 tetramer is composed of two α and two β subunits, only one pair of subunits is depicted in this simplified model. (C) Results of in vitro phosphorylation assays carried out using CK2 and equivalent amounts of recombinant GST, GST-Rev, GST-Rev38,39R-L, and GST-RevΔLoop. Phosphorylation assays were performed as described previously (47) and then combined with SDS-PAGE sample buffer and subjected to SDS-PAGE followed by Coomassie brilliant blue staining to identify the GST and GSTRev bands. Quantification of ^{32}P in each band by using a Packard Instant Imager revealed the following levels of ^{32}P incorporation: GST, 202 cpm; GSTRev, 3,034 cpm; GSTRev38,39R-L, 379 cpm; and GSTRevΔLoop, 236 cpm.

by folding the amino terminus into a "closed" conformation, thereby favoring formation of the hydrophobic patch proposed by Thomas et al. (79, 80). To test this hypothesis, we analyzed the effect of ablation of the amino acids spanning the loop and consequent fusion of the first and second helices into a single helical structure, as predicted by the PHDsec model (Fig. 1A). Detailed characterization of this mutant, named RevΔLoop, confirmed that the loop sequence is an important structural and functional determinant. In addition to contributing to the ability of Rev to bind to its RNA target, the loop appears to modulate the protein's intracellular trafficking and its association with subcellular compartments, as its deletion led to prom-

inent accumulation of Rev in subnuclear domains containing the SC35 splicing factor. Taken together with previous reports indicating partial colocalization of Rev with splicing components, our results suggest that correct juxtaposition of the two helices by the loop region is required for progression of Rev from one subcompartment to the next within the intranuclear phase of its RNA transport pathway.

MATERIALS AND METHODS

Cells and transfections. The HeLa-derived cell line HLtat, which constitutively expresses the HIV-1 Tat protein (66), was transfected by calcium phosphate coprecipitation. Plasmids used in transfections were purified by chromatography (Jetstar; Genomed).

Plasmids. The vector portion of pLsRevΔLoop and other plasmids with the pLs prefix were derived from LdKL3pA (48), which is driven by the Tat-dependent HIV-1 promoter and thus is constitutively expressed in HLtat cells. The RevΔLoop insert was generated by joining a pair of PCR fragments coding for sequences amino terminal and carboxy terminal to the deletion. This cloning approach resulted in deletion of the last glutamine in the first α-helix and addition of a leucine (Fig. 1). The Rev-green fluorescent protein (GFP) fusions were generated by joining a PCR fragment coding for Rev, with a *Sac*II site added in place of its stop codon, to a PCR fragment coding for enhanced GFP (kindly provided by R. Tsien) containing a *Sac*II site in place of the initiation codon; the same strategy was used to clone Rev-Tat hybrids. PCR amplifications were carried out using Vent DNA polymerase (New England Biolabs) in a Perkin-Elmer GeneAmp 9600 thermal cycler. Restriction enzymes were purchased from New England Biolabs and Roche, and synthetic oligonucleotides were purchased from European Primer.

Cell treatments and indirect immunofluorescence. Cells were treated and harvested for indirect immunofluorescence 22 to 24 h after transfection. Heat shock was carried out by floating the tissue culture plates in a 42°C water bath for different time points; control cells were incubated at 37°C in a nonhumidified incubator for the same time periods. For immunofluorescence assays, cells were fixed for 20 min with 3.7% formaldehyde in phosphate-buffered saline (PBS), permeabilized for 10 min with 0.1% Nonidet P-40 in PBS, and then incubated with combinations of the following antibodies: rabbit anti-sRev serum (17), mouse anti-SC35 antibody (Sigma), fluorescein isothiocyanate (FITC)-anti-rabbit and FITC-anti-mouse antibodies (Sigma), Texas Red-conjugated anti-rabbit antibody (Jackson Immunoresearch), and Alexa 488-conjugated anti-mouse antibody (Molecular Probes). Cells were examined under epifluorescence using an Olympus IX70 or Zeiss Axioplan microscope. Confocal images were obtained using a Zeiss LSM510 microscope.

Production and purification of GST fusion proteins. Glutathione-*S*-transferase (GST) fusion proteins were produced in bacteria and isolated by affinity chromatography using glutathione-Sepharose and then eluted using glutathione according to standard protocols (68).

RNA gel mobility shift assays. Aliquots (200 ng) of GST or GST-Rev fusion proteins were incubated with a constant amount of ^{35}S-labeled RRE330 in 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10 mM dithiothreitol, 10 μg of yeast tRNA, 1.5 μg of heparin, and 10 U of RNase inhibitor (Roche) for 20 min at 30°C, and then separated by nondenaturing polyacrylamide gel electrophoresis (PAGE) as described previously (75).

In vitro binding and immunofluorescence assay. HLtat nuclei were isolated by lysis using a hypotonic buffer (5 mM Tris-HCl [pH 7.4], 1.5 mM KCl, 2.5 mM $MgCl_2$, 1% sodium deoxycholate, 1% Triton X-100) (78) as described previously (17). Following removal of the cytoplasmic extract, nuclei were rinsed with ice-cold hypotonic buffer, fixed for 20 min with 3.7% formaldehyde in PBS, and then rinsed with PBS. Binding assays were carried out by incubating the isolated nuclei for 30 min at 37°C with GST-Rev proteins (approximately 1 μg of each, diluted in 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 3 mM glutathione, and 200 μM phenylmethylsulfonyl fluoride), followed by indirect immunofluorescence to detect Rev.

RESULTS

Deletion of the loop region abrogates phosphorylation of Rev by CK2: insights into conformation. Secondary structure predictions, circular dichroism measurements, and mutagenesis studies all point toward the presence of a helix-loop-helix structure in the amino-terminal half of Rev. To examine the conformation of this portion of the protein, we devised an assay based on the mechanistic features of protein kinase CK2-catalyzed phosphorylation. We have previously shown that Rev is efficiently phosphorylated by CK2 at Ser^5 and Ser^8 (47). CK2 is a tetramer composed of two catalytic (α) and two regulatory (β) subunits. Its activity is typically enhanced in the presence of polycations, which are believed to act by neutralizing an acidic cluster located in the amino-terminal domain of the β subunit, which inhibits the catalytic site in the α subunit through a direct pseudosubstrate effect (8, 65). It was recently demonstrated that Rev is able to stimulate the phosphorylation of several CK2 substrates through its positively charged NLS/RBD in the second α-helix, probably by mimicking the effects of polycations (52). Interestingly, CK2-mediated phosphorylation of Rev, although strictly dependent on the presence of the β subunit, is not stimulated by addition of polycations, suggesting that the basic residues present on the Rev molecule itself provide an intramolecular polycation-like motif that directs efficient phosphorylation of Ser^5 and Ser^8 (44a). In light of the fact that the acidic cluster of the CK2 β subunit lies in close proximity to the enzyme's catalytic domain located on the α subunit (9), one could predict that efficient CK2 phosphorylation of Ser^5 and Ser^8 requires that the amino-terminal portion assume a particular closed conformation that allows correct placement of Ser^5 and Ser^8 within the α subunit's catalytic pocket and simultaneous binding of its polycation-like segment to the β subunit's acidic domain (Fig. 1B, Rev model).

Reasoning that deletion of the loop region might interfere with docking the substrate to the enzyme and correct placement of Ser^5 and Ser^8 at the catalytic site on the enzyme's α subunit (Fig. 1B, RevΔLoop model), we compared the ability of CK2 to phosphorylate wild-type Rev and RevΔLoop in in vitro phosphorylation assays carried out using the CK2 holoenzyme, [γ-^{32}P]ATP, and GST-Rev fusion proteins as substrates. Unfused GST was included in the assays as a control to quantify the extent of phosphorylation of the GST moiety, and GSTRev38,39R-L, in which two arginines in the polycation-like domain were replaced with leucines, was tested to verify the necessity of the polycation-like domain for efficient phosphorylation. As shown in Fig. 1C and as reported previously (47), GST-Rev was efficiently phosphorylated by CK2. In contrast, both GSTRev38,39R-L and GSTRevΔLoop served as very poor substrates for the kinase, with both mutants incorporating an amount of ^{32}P comparable to that observed for unfused GST. Thus, disruption of either the polycation-like stretch or the loop region had similar effects in abrogating CK2-mediated phosphorylation. Given that the deleted loop region does not contain any positively charged residues that might confer a polycation-like stimulatory effect, we concluded that the reduced phosphorylation of GSTRevΔLoop by CK2 provided physical evidence that the loop deletion substantially altered the structure encompassing the helix-loop-helix region, with consequent interference with proper alignment of the phosphoacceptor sites and polycation-like stretch with the holoenzyme's catalytic and regulatory sites, respectively.

The loop region controls the intracellular trafficking of Rev. Figure 2A shows the results of indirect immunofluorescence assays with the Hela-derived cell line HLtat transfected with plasmids expressing either wild-type Rev or RevΔLoop. Wild-type Rev exhibited the primarily nucleolar distribution that has been detected in many previous studies. In contrast, RevΔLoop exhibited a mixed cytoplasmic and/or nuclear distribution that was accompanied by accumulation in brilliantly stained spheroid structures in the nucleus. Nuclear bodies containing the mutant Rev protein were observed predominantly in cells showing either a diffuse nuclear-cytoplasmic pattern or a strong nuclear signal accompanied by a weaker cytoplasmic signal. The number of RevΔLoop-containing bodies in each nucleus varied from 9 to 20, with an average of 13 per nucleus; they were localized mainly in the periphery of the nucleus.

The previous observation that wild-type Rev partially colo-

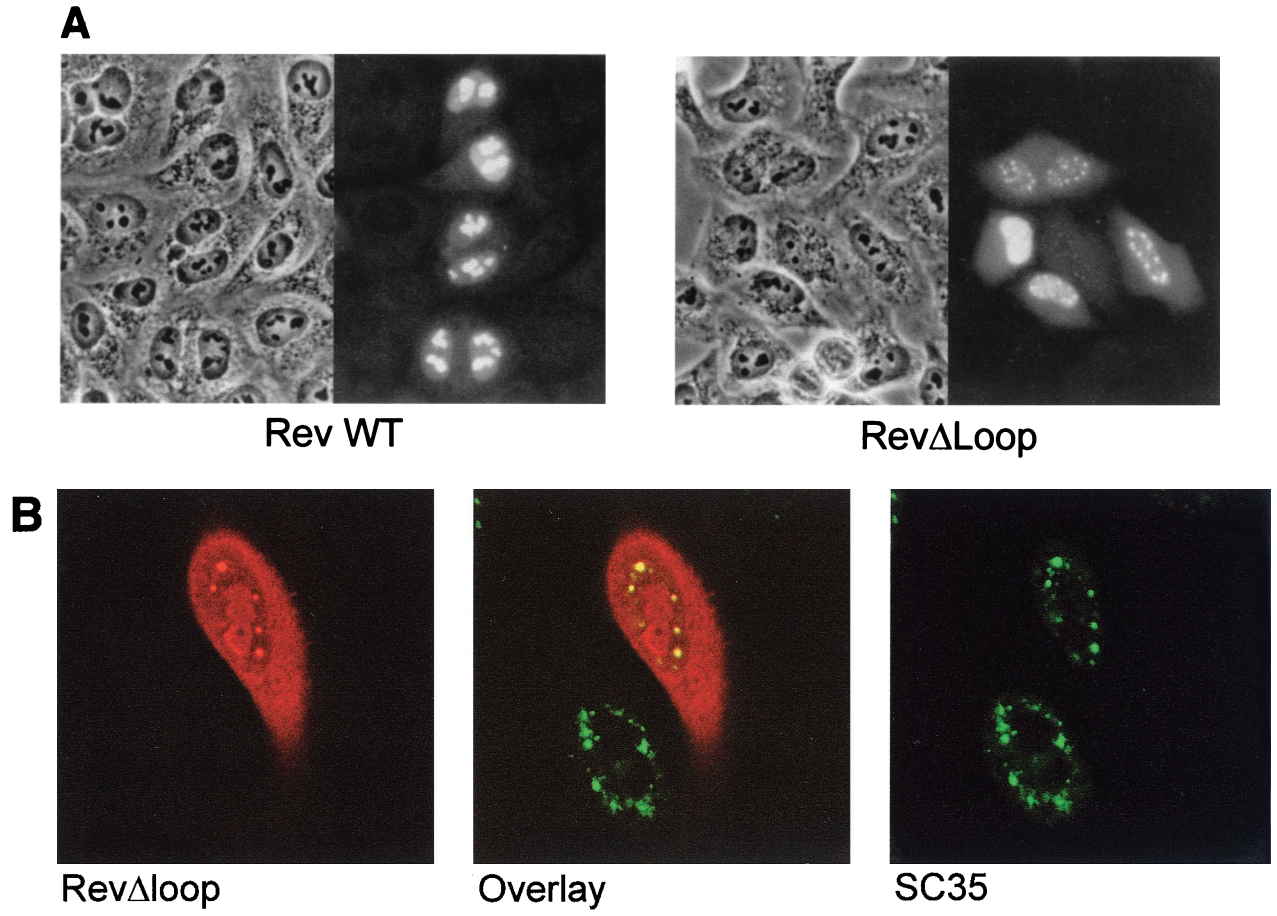

FIG. 2. Altered intracellular targeting pattern exhibited by RevΔLoop. (A) HLtat cells transfected with a plasmid expressing wild-type (WT) Rev (pBsRev) (17) or RevΔLoop (pLsRevΔLoop) and analyzed by indirect immunofluorescence after staining with rabbit anti-sRev serum (17) followed by FITC-conjugated anti-rabbit antibody. (B) Confocal images of HLtat cells transfected with pLsRevΔLoop; immunofluorescence was carried out using a combination of rabbit anti-sRev and Texas Red-conjugated anti-rabbit antibodies and mouse anti-SC35 and Alexa 488-conjugated anti-mouse antibodies.

calizes with SC35 (6, 33) prompted us to examine the distribution of this splicing factor in the context of RevΔLoop expression. Results of indirect immunofluorescence assays showed that, in most cells, SC35 accumulated in irregularly shaped speckle-like clusters in the nucleoplasm that superimposed over a pattern of minuscule grains (Fig. 2B). This pattern resembled the interchromatin granules and perichromatin fibrils (reviewed in references 39 and 67) that have been documented in previous studies (11, 25, 71). Interestingly, in a substantial proportion of the RevΔLoop-transfected cells, the SC35 signal was distributed in a small number of regularly shaped nuclear bodies instead of in interchromatin granules and perichromatin fibrils. Visualization of the pattern for RevΔLoop showed that these brilliantly staining nuclear structures corresponded exactly to the bodies containing RevΔLoop (Fig. 2B). The SC35-positive nuclear bodies were specific to RevΔLoop-expressing cells and were not detected in nontransfected cells or in cells transfected with wild-type Rev (data not shown). In addition, three-dimensional reconstructions of images generated by laser-scanning confocal microscopy showed that while "normal" SC35 speckles showed an irregular flake-like morphology, RevΔLoop SC35-positive nuclear bodies showed a very regular spheroid shape (data not shown). RevΔLoop was not detected in bodies resembling normal SC35-containing nuclear speckles. These observations indicated that the mutant Rev protein disrupted the normal intracellular distribution of SC35. To our knowledge, this is the first description of a Rev mutant that presents this peculiar subnuclear distribution and induces a redistribution of SC35.

Inhibition of transcription by DRB reproduces the pattern of SC35-containing nuclear bodies. According to the current knowledge of nuclear architecture, nuclear interchromatin granules correspond to storage-assembly sites of splicing factors such as SC35, while perichromatin fibrils represent the actual sites of transcription and splicing (reviewed in references 39 and 67). Previous studies of SC35-containing interchromatin granules and perichromatin fibrils demonstrated that SC35 changes its distribution upon inhibition of RNA polymerase II-driven transcription by treatment with α-amanitin or 5,6-dichlorobenzimidazole riboside (DRB), accumulating in regularly shaped nuclear bodies lacking fibril-like connections (12, 38, 51, 70, 72). The disruption of the intranuclear distribution of SC35 upon inhibition of transcription is in agreement with the known close temporal and spatial relationship between transcription and splicing (reviewed in references 4 and 50). Figure 3 shows the effects of DRB treatment on the distribution of RevΔLoop and SC35 in transfected HLtat cells. The results showed that the overall distribution of RevΔLoop did not appreciably change upon treatment with DRB for 3 h, with many cells containing the protein in nuclear bodies (Fig. 3, compare A and B). Examination of the SC35 pattern showed that treatment with DRB for 3 h resulted in a redistribution of

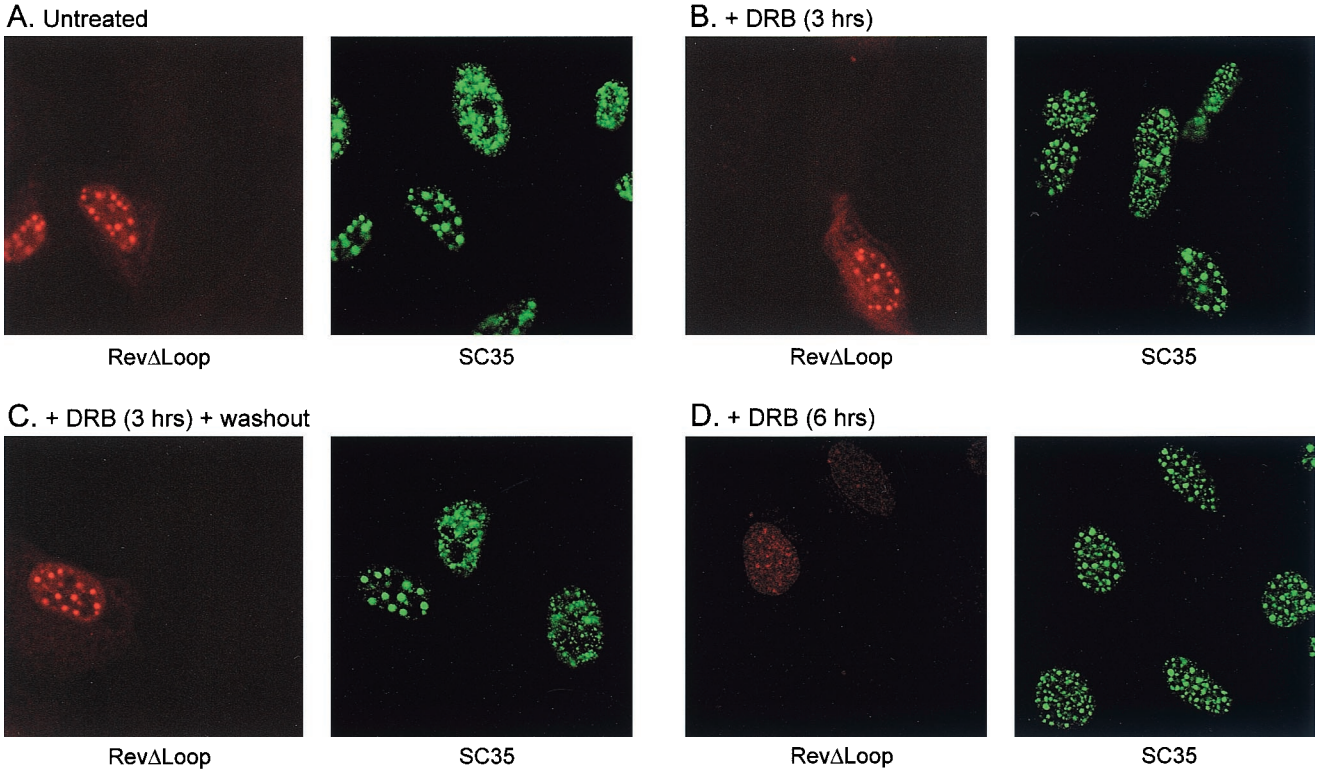

FIG. 3. Effect of DRB on the intracellular accumulation of RevΔLoop and SC35. Approximately 18 h after transfection with pLsRevΔLoop, HLtat cells were incubated in the absence (A) or presence of 100 μM DRB for either 3 h (B) or 6 h (D), or with 100 μM DRB for 3 h followed by a 1-h incubation in the absence of the drug (C). The cells were then subjected to indirect immunofluorescence using a combination of rabbit anti-sRev and Texas Red-conjugated anti-rabbit antibodies and mouse anti-SC35 and Alexa 488-conjugated anti-mouse antibodies and analyzed by confocal microscopy.

SC35 from interconnected clumps and granules (Fig. 3A) into isolated, spheroid nuclear structures that were morphologically indistinguishable from those containing RevΔLoop (Fig. 3B). This pattern of intranuclear bodies lacking fibril-like connections appeared to be identical to that previously observed in DRB-treated HeLa cells (70).

The effects of DRB on transcription and nuclear speckle morphology are known to be reversible upon removal of the drug. Cells treated with DRB for 3 h followed by incubation in the absence of the drug for 1 h showed a normal SC35 staining pattern, with the exception that the cells containing RevΔLoop in nuclear bodies also contained SC35 in the same structures (Fig. 3C). Upon treatment with DRB for 6 h, very few cells expressing RevΔLoop were detected, and accumulation of the protein in nuclear bodies was no longer evident; the SC35-containing nuclear structures became more numerous and smaller and resembled the pattern previously observed in MDCK cells treated with DRB (38). The increased number and smaller dimensions of the SC35-containing nuclear spots observed after 6 h of treatment of HLtat cells with DRB (Fig. 3D) might partly reflect the documented activity of this drug as an inhibitor of protein kinases, whose targets include RNA polymerase II (14).

Effects of heat shock on the distribution of RevΔLoop and SC35. Heat shock is known to exert different effects on the distribution of various nuclear speckle components: for example, while SC35 remains associated with speckles in heat-shocked cells, the pattern of snRNP antigens becomes more diffuse and shifts to the nucleoplasm (71). This prompted us to compare how RevΔLoop and SC35 would respond to heat

TABLE 1. Effect of heat shock on the distribution of RevΔLoop

Intracellular distribution of RevΔLoop	Control culture		Heat-shocked culture	
	No. of cells without nuclear bodies	No. of cells with nuclear bodies	No. of cells without nuclear bodies	No. of cells with nuclear bodies
Diffuse (nuclear plus cytoplasmic)	11	22	29	3
Cytoplasmic, nuclear excluded	9	2	1	0
Nuclear	2[a]	0	0	0
Nuclear > cytoplasmic	10	6	23[b]	0
Nuclear plus nucleolar > cytoplasmic	4	0	5	0
Total	36	30	58	3

[a] One of these cells exhibited a mottled pattern in the nucleus.
[b] Six of these cells exhibited a mottled pattern in the nucleus.

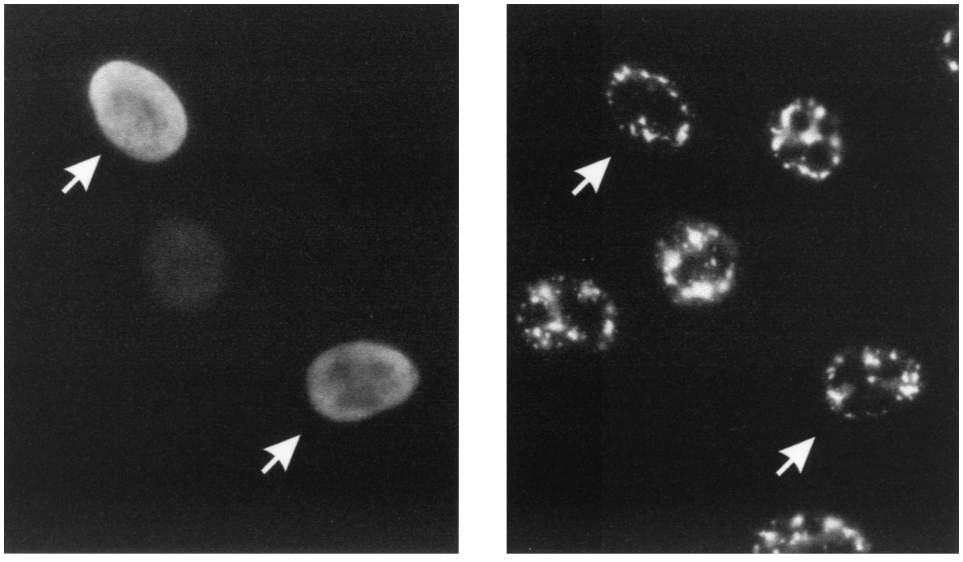

FIG. 4. Effect of an NES mutation on intracellular targeting of RevΔLoop. Shown is an indirect immunofluorescence assay of HLtat cells transfected with pLsRevΔLoopBL, carried out using a combination of rabbit anti-sRev and Texas Red-conjugated anti-rabbit antibodies and mouse anti-SC35 and Alexa 488-conjugated anti-mouse antibodies.

shock. Cells transfected with RevΔLoop were subjected to heat shock at 42°C for 10, 20, 30, or 50 min and then analyzed by indirect immunofluorescence using anti-sRev and anti-SC35 antibodies. Consistent with previous findings (71), the pattern of SC35 staining did not change substantially upon heat shock for up to 50 min, with the exception of a diminution in the pattern of small grains (data not shown). In contrast, nuclear bodies containing RevΔLoop became less evident after heat shock for 20 min. This effect became more obvious after heat shock for 30 min, as illustrated in Table 1, which reports the distribution of RevΔLoop observed in 10 randomly selected microscopic fields of control and heat-shocked cultures. In the nontreated culture, RevΔLoop-containing nuclear spots were detected in about 45% of the cells expressing the protein, compared to only 5% in the heat-shocked culture. Upon heat shock, RevΔLoop was detected mainly in a diffuse pattern throughout the cell or with a more pronounced nuclear accumulation, in some cases accompanied by a mottled pattern in the nucleus. Thus, RevΔLoop responded to heat shock in a manner more similar to that reported for snRNP antigens than that of SC35.

In cells incubated at 42°C for 50 min, the overall intensity of the RevΔLoop signal appeared to be decreased, and no nuclear spots were observed in 47 RevΔLoop-expressing cells examined in 10 microscopic fields (data not shown). The primarily nucleolar distribution of wild-type Rev was not appreciably affected by heat shock for up to 50 min (data not shown).

Accumulation of RevΔLoop in nuclear bodies requires an intact NES. Export of Rev from the nucleus to the cytoplasm is directed by its NES (22). The NES mediates association of Rev with the general nuclear export factor CRM1/exportin1 (23, 26, 54, 73), the nucleoporin-like protein Rab1/hRIP (7, 24), and the eukaryotic initiation factor 5A (eIF5A) (61), and it is responsible for directing Rev-RNA complexes through an export pathway used by 5S rRNA and U snRNAs (22). Mutants of the NES show wild-type nuclear/nucleolar localization and are able to interact with the RRE but act as transdominant inhibitors of Rev function (30, 43, 48, 53, 82) and are trapped in the nucleus (74, 77).

The data presented above demonstrated the importance of the loop region for proper intranuclear trafficking and nucleolar accumulation. To test the possible role of the NES in directing the trafficking of Rev through different subnuclear domains, we examined the subcellular localization of mutant RevΔLoopBL, which was derived from RevΔLoop by introduction of a previously described dominant negative mutation in the NES (48) that is known to disrupt the export leg of Rev (74). As shown in Fig. 4, RevΔLoopBL accumulated primarily in the nucleus but was not detected in nuclear bodies; SC35 exhibited a normal intranuclear distribution in speckles and grains. This observation indicated that, in addition to directing nuclear export, the NES contributes to modulating the association of RevΔLoop with nuclear bodies, thereby implying that this domain might also play a role in controlling association of wild-type Rev with components of the transcription-splicing machinery.

Deletion of the loop region results in loss of Rev function. We next examined whether the substantially altered intracellular targeting of RevΔLoop resulted in changes in Rev function. This question was addressed by comparing the abilities of wild-type Rev and RevΔLoop to augment expression of the Rev-dependent reporter plasmid pcGagRREpA (3), which codes for an mRNA consisting of the HIV *gag* gene linked to the RRE (Fig. 5). The transfections were carried out using increasing quantities of the Rev plasmids, with the highest quantity corresponding to saturation conditions for Rev-mediated activation of Gag-RRE expression, as established in control assays (data not shown); the transfections also included a plasmid coding for β-galactosidase, which served as a standard for transfection efficiency. As shown in Fig. 5 (lanes 5 to 7), Gag protein was not detected in transfections carried out using RevΔLoop, thus demonstrating that Rev function strictly requires the presence of the loop region.

The observation that RevΔLoop was functionally inactive prompted us to test its ability to competitively inhibit the activity of Rev in a *trans*-dominant manner. The results showed that cotransfection of a 10-fold excess of the RevΔLoop plasmid had no effect on the ability of Rev to induce expression

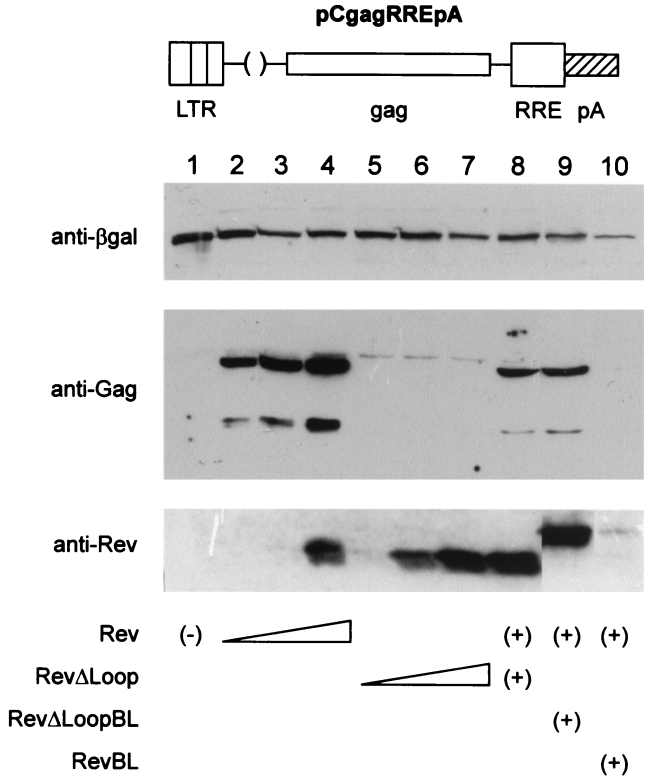

FIG. 5. Deletion of the loop results in functional inactivation of Rev. HLtat cells were transfected with the HIV-1 RRE reporter plasmid pCgagRREpA, which produces Gag in a Rev-dependent manner (3), in the absence (lane 1) or presence of 10 ng, 100 ng, or 1 μg of pBsRev (lanes 2 to 4) or pLsRevΔLoop (lanes 5 to 7); the assays in lanes 8 to 10 were carried out using 100 ng of pBsRev and 1 μg of either pLsRevΔLoop (lane 8), pLsRevΔLoopBL (lane 9), or pLBL (lane 10). The transfection standard pCMV-βgal was included in all transfections. One day after transfection, the cells were lysed in SDS-PAGE sample buffer and subjected to SDS-PAGE and Western blot analysis using mouse anti-β-galactosidase antibody (Roche) (upper panel), mouse anti-p24 antibody (Cellular Products) (middle panel), and rabbit anti-sRev serum (lower panel), followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech) and a chemiluminescence-based detection system (Pierce). Activation of pCgagRREpA by Rev is reflected by the detection of the two prominent Gag bands of 55 and ~37 kDa; the band migrating just above p55gag is a cellular protein. Transfection of 100 ng of pBsRev or 1 μg of pLBL produced amounts of protein at the limit of detection using this method. Assays were repeated three times with similar results.

of Gag-RRE (Fig. 5, lane 8), which contrasts with the strong inhibitory effect exerted by a 10-fold excess of a plasmid expressing the previously described *trans*-dominant NES mutant RevBL (48) (Fig. 5, lane 10). A double mutant containing both the loop deletion and the NES mutation (RevΔLoopBL) also failed to inhibit Rev function (Fig. 5, lane 9). Expression of each Rev plasmid was verified by Western blotting using rabbit anti-sRev serum (Fig. 5, lower panel). The results indicated that the failure of RevΔLoop to activate Gag expression and the inability of either RevΔLoop or RevΔLoopBL to inhibit Rev function did not result from inefficient expression of these mutants, as they were present in readily detectable quantities. In contrast, efficient production of Gag was obtained in the presence of Rev levels below the detection limit of the Western blot assay, and very low amounts of RevBL were able to completely inhibit Rev function.

Effect of the loop deletion on the ability of Rev to bind to the RRE and nucleolar components. To investigate the molecular basis for the functional inactivity of RevΔLoop, the mutant was next tested for its ability to bind to RRE RNA in an RNA gel-shift assay carried out using purified recombinant GST-RevΔLoop fusion protein and radiolabeled full-length RRE (RRE330) (3). GST-Rev served as a positive control for Rev-RRE complex formation, and GST-Rev38,39R-L, which lacked two helix-2 arginine residues that are essential for RRE binding (42), served as a negative control. The assays also included untagged GST, to control for possible binding of the tag moiety to the probe, and GST-Rev14-16EED, a helix-1 mutant previously shown to bind to RRE with reduced specificity (75). As shown in Fig. 6A, both GST-Rev and GST-Rev14-16EED retarded the migration of the RRE probe, indicating the formation of Rev-RRE complexes. However, like Rev38,39R-L, RevΔLoop failed to shift the migration pattern of the RRE probe, indicating that this mutant is unable to bind to RRE.

The inability of RevΔLoop to bind to RRE RNA was also tested using an in vivo RNA binding assay developed by Madore et al. (42). This approach is based on a chloramphenicol acetyltransferase (CAT) reporter construct named pSLIIB/CAT, whose expression is driven by a modified HIV-1 promoter containing a minimal RRE element in place of the Tat-binding portion of the Tat-responsive element, the RNA element required for Tat-driven transcriptional activation. Efficient transcription of the CAT mRNA depends on coexpression of a Tat-Rev fusion protein whose Rev portion binds to RRE and correctly positions the Tat moiety within the context of the promoter-transcription factor complex, thus rendering it functionally active. To facilitate cloning steps, we modified this assay slightly by using Rev-Tat hybrids instead of Tat-Rev hybrids described in previous studies. All transfections included a plasmid coding for β-galactosidase, against which CAT activity was normalized. As shown in Fig. 6B, RevΔLoopTat behaved similarly to Rev38,39R-LTat (included as a negative control for RRE binding) and was unable to efficiently activate CAT expression from pSLIIB/CAT, thus confirming a defect at the level of RRE binding.

In a previous study carried out using an in vitro nuclear binding assay, we demonstrated the ability of both Rev and the NES mutant RevBL to interact with nucleolar RNA in the absence of active cellular sorting processes; in contrast, the RRE-binding-domain mutant Rev38,39R-L was unable to bind to nucleoli in this assay (17). This observation, taken together with the fact that Rev mutants unable to bind to RRE are generally also defective for nucleolar accumulation, indicates a correlation between the abilities of the protein to bind to RRE and to accumulate in the nucleolus. To determine whether the greatly reduced nucleolar accumulation of RevΔLoop observed in vivo was mirrored by a failure to bind to nucleolar components in vitro, similar nuclear binding assays were performed using RevΔLoop expressed as a GST-Rev fusion protein; GST-RevBL and GST-Rev38,39R-L served as positive and negative controls for nucleolar binding, respectively. The results showed that GST-RevΔLoop bound to the isolated nuclei in discrete patches corresponding to nucleoli (Fig. 6C). As expected, GST-RevBL also yielded an intense nucleolar binding pattern, while GST-Rev38,39R-L failed to bind to nucleoli. The ability of GST-RevΔLoop to interact with nucleoli in vitro suggested that, in contrast to Rev38,39R-L, the defect in nucleolar accumulation of RevΔLoop observed in vivo reflected an impairment in active sorting processes. Analysis of aliquots of the GST fusion proteins by SDS-PAGE and Coomassie brilliant blue staining confirmed that a similar amount of each protein was applied to the nuclei (Fig. 6C).

In this in vitro assay, RevΔLoop did not bind to nuclear bodies resembling those detected in cells transfected with RevΔLoop (Fig. 2). To determine whether RevΔLoop and

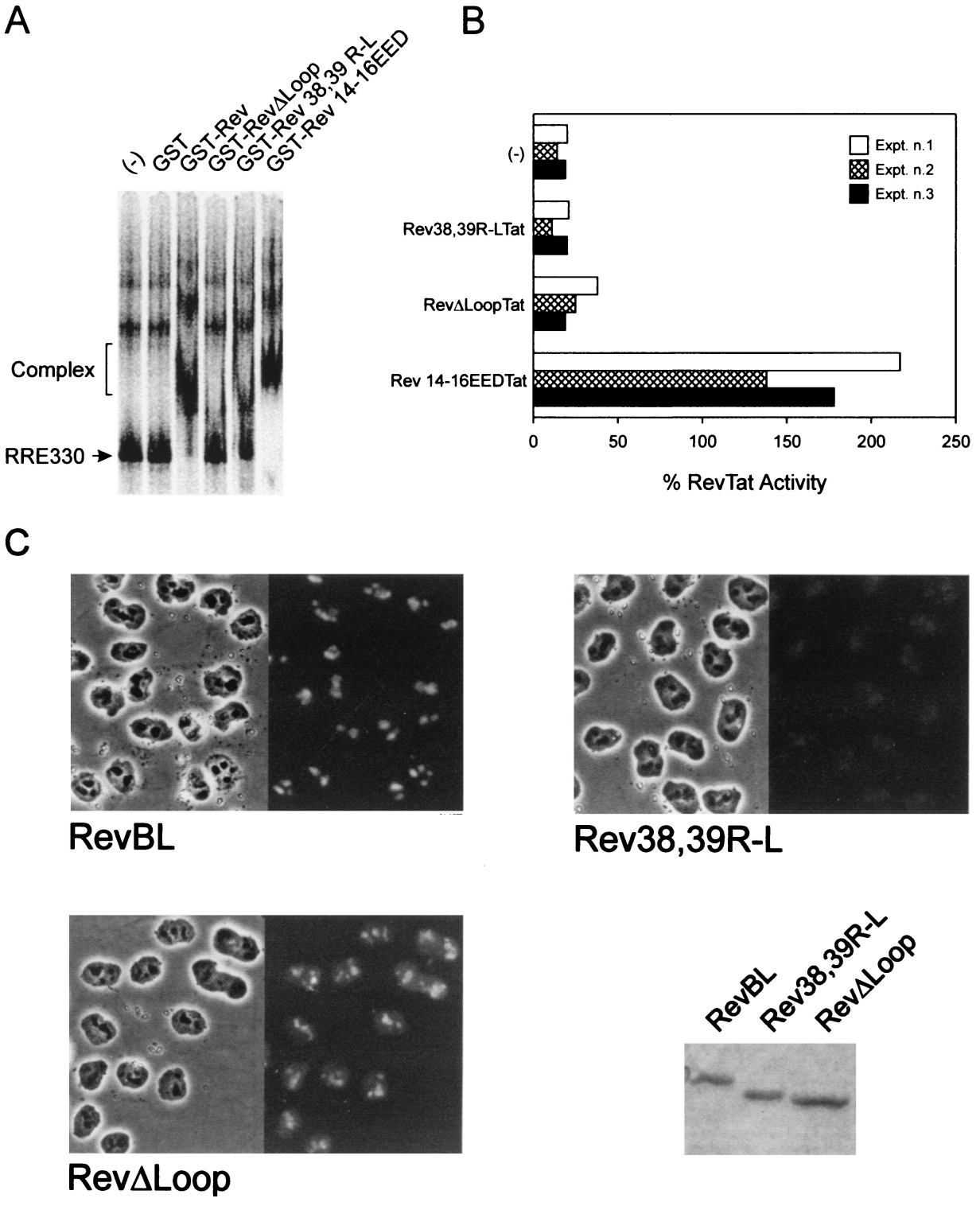

FIG. 6. Effect of the loop deletion on the ability of Rev to bind to the RRE and nucleolar components. (A) Radioanalytic image of a gel mobility shift assay carried out by incubating ^{35}S-labeled RRE330 (3) with unfused GST or the indicated GST-Rev fusion proteins. (B) Results of in vivo RRE binding experiments performed by transfecting HLtat cells with pSLIIB/CAT (4 μg) (42) and pCMVβgal (1 μg) either alone (−) or in the presence of a plasmid expressing Rev38,39R-LTat (2.5 μg), RevTat (1 μg), RevΔLoopTat (2.5 μg), or Rev14-16EEDTat (1 μg). CAT production was measured in CAT assays using [^{14}C]chloramphenicol as a substrate; acetylated and nonacetylated forms of chloramphenicol were separated by thin-layer chromatography and quantitated using a Packard Instant Imager. The resulting percent conversion values were normalized against β-galactosidase production, which was measured as described elsewhere (62), and then compared to the activation obtained in the presence of RevTat, which was set at 100%. Data shown are the results of three experiments. (C) Results of assays carried out to test the ability of the indicated GST-Rev proteins to interact with isolated HLtat nuclei and a Coomassie-stained SDS-12.5% polyacrylamide gel containing the same quantities of the fusion proteins used in the binding assay.

SC35 remained in the isolated nuclei after the extraction procedure, we carried out indirect immunofluorescence assays on both intact RevΔLoop-transfected cells and extracted nuclei prepared from duplicate transfections. Results showed that the number of extracted nuclei containing RevΔLoop was substantially lower than the number of intact cells containing RevΔLoop-positive nuclei; furthermore, nuclear bodies containing RevΔLoop were not observed in the extracted nuclei (data not shown). These observations suggested that the extraction procedure (i.e., treatment with a hypotonic buffer containing Triton X-100 and deoxycholic acid) removed most of the RevΔLoop protein from the nucleus and nuclear bodies. In contrast, SC35 was readily detected in the extracted nuclei, primarily in the pattern characteristic of nontransfected cells, as well as in nuclear bodies in a few of the RevΔLoop-positive nuclei, even though RevΔLoop was not detected in these structures (data not shown). The apparent differential release of SC35 and RevΔLoop from the nucleus and nuclear bodies is in line with the results of the heat-shock experiments that showed differential release of the two proteins.

Coexpression of wild-type and loop-deleted Rev proteins leads to reciprocal changes in their intracellular distribution. Stauber et al. (75) demonstrated the redistribution of the intracellular localization of mutant Rev proteins upon coexpression of wild-type Rev in an assay that utilized GFP and blue fluorescent protein hybrids to allow direct visualization of targeting in living cells. For example, coexpression of mutant Rev14-16EED (mainly cytoplasmic) with wild-type Rev (mainly nucleolar) results in increased accumulation of the mutant in nucleoli (75). Such a redistribution is indicative of the formation of either indirect associations between Rev molecules mediated by bridging cellular partners or direct Rev-Rev multimerization, a property known to be essential for Rev function (15, 16, 18, 42, 44, 81, 84).

We carried out similar assays to assess the ability of RevΔLoop to alter the intracellular distribution of wild-type Rev. HLtat cells were transfected with wild-type Rev-GFP either alone or in combination with untagged mutant RevΔLoop; 1 day later, the living cells were examined by direct fluorescence to visualize the GFP fusion protein and then lysed and subjected to immunoblotting analysis using anti-Rev antibodies to verify coexpression of the untagged RevΔLoop protein (data not shown). When expressed alone, RevGFP was detected primarily in the nucleoli (Fig. 7A), as described previously (74). Coexpression of RevΔLoop resulted in a partial redistribution of RevGFP from the nucleoli to nuclear bodies similar to those observed in cells transfected with untagged RevΔLoop (Fig. 7A). This alteration in targeting indicated that, although wild-type Rev dominated over RevΔLoop at the level of nuclear retention, the two proteins appeared to affect each other's distribution within the nucleus, yielding a mixed pattern of nucleolar accumulation typical of wild-type Rev and of nuclear bodies characteristic of RevΔLoop.

In addition to assessing the ability of a mutant Rev protein to change the distribution of wild-type Rev, this assay can be set up as a nuclear trapping system in which the test mutant protein is coexpressed with an identical Rev protein whose nuclear export is blocked by a second mutation in the NES; in this case, redistribution is reflected by increased nuclear retention of the test protein due to its association with the NES mutant, which is unable to exit from the nucleus (74, 75). Thus, HLtat cells were transfected with with RevΔLoopGFP either alone or in the presence of unfused RevΔLoopBL which carried both the loop deletion and NES mutation. RevΔLoopGFP was visualized in living cells (Fig. 7B), and coexpression of RevΔLoopBL was verified by immunoblotting

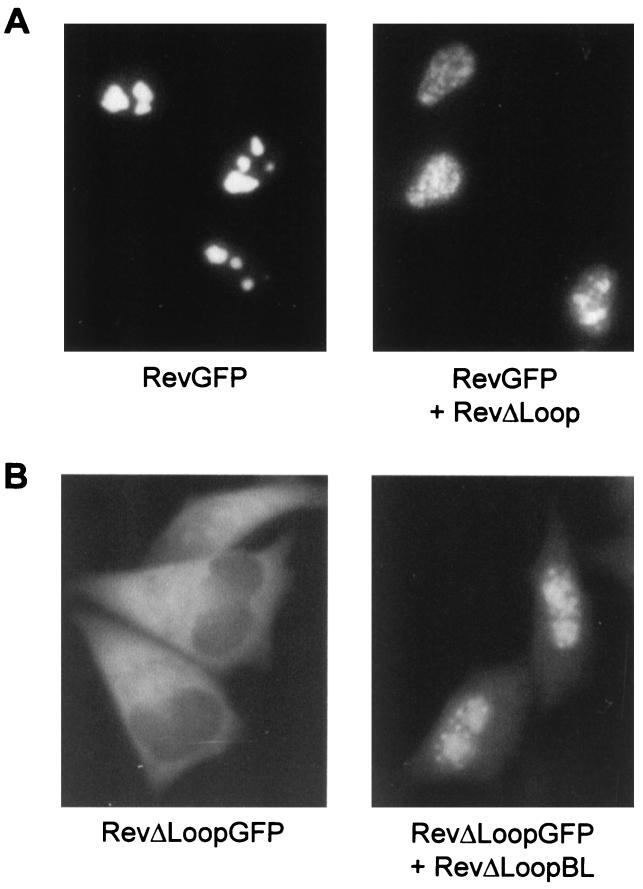

FIG. 7. Coexpression of wild-type and loop-deleted Rev proteins leads to reciprocal changes in their intracellular distribution. HLtat cells were transfected with plasmid pLsRevGFP with or without pLsRevΔLoop (A) or pLsRevΔLoopGFP with or without pLsRevΔLoopBL (B). One day later, the cells were examined as living cultures, photographed, and then harvested for immunoblotting analysis using rabbit anti-sRev serum to confirm expression of both proteins (data not shown).

as described above (data not shown). As shown in the left panel of Fig. 7B, the intracellular distribution of RevΔLoopGFP differed somewhat from that of unfused RevΔLoop, with the GFP hybrid accumulating primarily in a diffuse pattern throughout the cell and only a minority of the transfected cells showing nuclear bodies. Cotransfection of RevΔLoopGFP with RevΔLoopBL resulted in a prominent increase in the accumulation of RevΔLoopGFP in both the nucleus and in nuclear bodies, compared to cells transfected with RevΔLoopGFP alone (Fig. 7B, right panel). The reciprocal changes in the intracellular distributions of wild-type Rev and loop-deleted mutants suggest that RevΔLoop is capable of forming indirect or direct associations with other Rev molecules.

DISCUSSION

The present study demonstrates that deletion of the loop portion of Rev's helix-loop-helix region alters the protein's intracellular trafficking and destroys its ability to associate with the RRE but does not affect its ability to bind to nucleoli in vitro and does not disrupt its NES. RevΔLoop shows a partial distribution in distinct spheroid bodies in the nucleus; these bodies also contain SC35 and resemble the spheroid nuclear structures observed in cells treated with inhibitors of transcription.

The finding that RevΔLoop was poorly phosphorylated by

CK2 (Fig. 1) provided strong evidence that the introduced mutation disrupted the spatial relationship between Ser^5 and Ser^8 in the amino-terminal tail and the polycation-like stretch of positively charged amino acids in the second helix, thus indicating that the loop region is indeed required for correct alignment of the protein's amino-terminal domains.

Previous studies yielded mixed results concerning the role of individual amino acids within the loop region. A mutant containing substitutions of Tyr^{23} (in helix 1) and loop residues Ser^{25} and Asn^{26} by Asp-Asp-Leu was found to be functionally inactive (mutant M4; see reference 43) and defective for multimerization and/or binding to RRE (42, 44, 53, 83). Taken together, these results indicated a role for Tyr^{23}, Ser^{25}, and/or Asn^{26} in multimerization and possibly RRE binding. In a detailed mutational analysis that included a portion of the loop region, Thomas et al. (80) observed that paired substitutions of either Gln^{24} and Ser^{25} or Ser^{25} and Asn^{26} by leucines (mutants RevSLT13 and RevSLT14, respectively) did not impair Rev function, thus arguing against an essential role for loop residues 25 and 26; the signficance of Tyr^{23} to RRE binding and multimerization remains to be established. Olsen et al. (53) showed that the deletion of loop residues 32 to 34 resulted in a functionally inactive protein unable to bind to RRE but able to multimerize, while deletion of residues 28 to 31 destroyed functional activity, RRE binding, and multimerization.

The four prolines and one glycine residue found in the loop region are strong helix-breaking residues. While glycine allows high flexibility of the amino acid chain, prolines severely limit rotation, thus imparting specific conformational constraints which can be controlled by *cis-trans* isomerization. These properties suggest that the prolines in the loop might play a critical role in controlling orientation, which would in turn affect juxtaposition of the two helices. An early study of the amino-terminal region showed that substitution of Pro^{27}, Pro^{28}, and Pro^{29} by alanines resulted in a mutant that accumulates in nucleoli but is functionally inactive (29, 30). Among eight mutants containing paired substitutions spanning residues 25 to 32 that were tested by Thomas et al. (80), only those that affected the proline triplet (residues 27 to 29) were functionally impaired. We observed that deletion of Pro^{27}, Pro^{28}, and Pro^{29} or both Pro^{27}, Pro^{28}, and Pro^{29} and also Pro^{31} resulted in severe impairment of Rev's ability to activate expression of pCGagRREpA but did not have a substantial effect on the intracellular distribution of the protein (data not shown). Further studies of this region will be necessary in order to identify individual loop residues involved in mediating intranuclear trafficking of Rev and to understand the possible role of proline isomerization in controlling Rev's function and intracellular trafficking.

The spheroid SC35-positive nuclear bodies were observed exclusively in cells expressing RevΔLoop, with untransfected cells showing the typical accumulation of SC35 in speckles and grains (Fig. 2). Studies of the dynamics of speckle morphology using GFP-tagged SF2/ASF revealed that the majority of these structures remained stationary over an 8-h observation period, with the exception of changes in their peripheral extensions and relationships to other small nuclear particles, which likely reflect recruitment of splicing factors to new sites of transcription. Treatment of cells with α-amanitin to block RNA polymerase II activity causes the speckles to become round and static (51). Interestingly, HLtat cells treated for 3 h with the RNA polymerase II inhibitor DRB exhibited SC35-containing nuclear bodies that were morphologically indistinguishable from the RevΔLoop-containing nuclear bodies (Fig. 3) as well as the SC35-positive nuclear structures observed in previous studies of α-amanitin-treated cells. Alterations in the subnuclear distribution of SC35 in response to treatment with inhibitors of transcription are not unexpected, given the close temporal and spatial relationships between transcription and splicing (4, 50). It is tempting to speculate that the accumulation of SC35 in nuclear bodies in cells expressing RevΔLoop reflects inhibition of transcription and/or splicing by the mutant due to inappropriate sequestration of transcription-processing factors, a possibility that is currently being tested.

Herpes simplex virus type 1-infected cells exhibit a redistribution of splicing components into punctate nuclear structures resembling the RevΔLoop-containing bodies (45). This effect has been attributed to ICP27 (IE63) (55), an immediate-early herpes simplex virus type 1 regulatory protein that favors the cytoplasmic accumulation of intronless RNAs and inhibits splicing of cellular mRNAs (reviewed in reference 21). Like Rev, ICP27 is an RNA binding protein (10) that shuttles between the nucleus and the cytoplasm (69) and contains an NLS (46) as well as an NES (64). ICP27 has been demonstrated to associate with snRNPs (63), suggesting that it might inhibit accumulation of spliced mRNAs by interfering with spliceosome assembly. Evidence indicates that Rev also exerts direct effects on splicing of viral mRNAs. Chang and Sharp (13) proposed that Rev interacts directly with splicing processes, on the basis of cotransfection assays with mRNAs containing splice sites of differing efficiencies. These results were extended by studies demonstrating the ability of Rev or a peptide corresponding to amino acids 35 to 50 of the protein to inhibit splicing in an in vitro assay (34). The Rev 35–50 peptide was later shown to block entry of the U4/U6 · U5 snRNP into the spliceosome complex (35). Further cotransfection studies demonstrated that Rev-dependent expression of the env mRNA depends on the presence of splice sites defining an intron (27, 40). Splicing factor SF2/ASF has been shown to bind to the RRE in the presence of Rev, suggesting that this interaction might contribute to Rev-mediated inhibition of viral mRNA splicing (57); the finding that the functional activity of Rev depends on active transcription of its target RNA provides further evidence that the protein begins to exert its influence at an initial stage of RNA processing (31).

The observation that RevΔLoopBL accumulated primarily in the nucleus (Fig. 4), in contrast to the partial cytoplasmic localization observed for RevΔLoop (Table 1), strongly suggests that RevΔLoop is competent for both nuclear import and export, properties that are directly linked to the ability of Rev to engage and transport RRE-containing RNAs. Further evidence for efficient shuttling of RevΔLoop came from the observation that treatment of RevΔLoop-expressing cells for 8 h with 10 nM leptomycin B, a drug that by binding to CRM1/exportin 1 (37) interferes with Rev's export leg (23), resulted in a primarily nuclear distribution of the protein, with nuclear bodies evident in some of the RevΔLoop-expressing cells (data not shown). Upon incubation for 11 h with the drug, RevΔLoop was still detected mainly in the nuclei, but nuclear bodies were no longer evident; staining with anti-SC35 antibody confirmed the presence of nuclear speckles with a normal morphology in leptomycin B-treated cells (data not shown). This loss of nuclear bodies is in line with the observation that RevΔLoopBL failed to accumulate in these structures, and it indicates that a NES-dependent, leptomycin B-sensitive component of the Rev export pathway involving the protein's NES is required to direct the protein toward interactions with splicing complexes.

The association of wild-type Rev with splicing factors is not readily visible by conventional epifluorescence microscopy, suggesting either that this association might be very brief or that a small population of Rev molecules participates in these

interactions at a given time. In this regard, it remains to be determined whether the association of Rev with splicing factors might represent a transient storage phase secondary to its accumulation in nucleoli, which, according to Kubota et al. (36), serve as storage sites for the protein. Our analysis of RevΔLoop suggests that, in conjunction with the NES and NLS/RBD, the loop region might play a role in modulating the partitioning of Rev molecules between such storage deposits and sites of functional activity, possibly through conformational changes in the helix-loop-helix region that permit Rev to proceed rapidly from the nucleolus to transcription-splicing complexes and subsequent points along the route leading to nuclear export of Rev-RRE complexes.

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