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# Identification of Sequences Important in the Nucleolar Localization of Human Immunodeficiency Virus Rev: Relevance of Nucleolar Localization to Function

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**The human immunodeficiency virus *rev* gene product regulates the expression of viral structural genes. It was recently shown that Rev regulates the export of viral structural mRNAs from the nucleus to the cytoplasm. Analysis of Rev subcellular localization reveals marked accumulation in the nucleolus, suggesting a role for the nucleolus in this export process. We report here the identification of amino acid residues critical to the nucleolar localization of Rev. Consistent with this finding, a Rev/β-galactosidase fusion protein, harboring this region of Rev, localized entirely within the nucleolus. Of most significance, mutations that eliminated nucleolar localization markedly diminished Rev function, even though accumulation in the nucleoplasm was retained. These findings support a model whereby Rev-induced export of human immunodeficiency virus structural mRNAs from the nucleus to the cytoplasm is likely to involve nucleolar events.**

The human immunodeficiency virus (HIV) encodes several key regulatory proteins required for the regulation of gene expression. Expression of two of these proteins, Tat and Rev, has been shown to be essential for virus replication (1, 7, 25–28). Rev is a small, positively charged nuclear protein that is produced from a doubly spliced mRNA. Although the *cis*-acting target sequence for this protein has been determined (10, 16, 21), its precise mechanism of action remains unclear. Recent studies have shown that Rev functions posttranscriptionally, facilitating transport of the mRNAs encoding the viral structural proteins from the nucleus to the cytoplasm (8, 11, 16). The ability of Rev to induce the expression of specific mRNAs is a result of the interaction of both positive and negative regulatory elements contained within the structural gene mRNAs (21). Studies on the Rev protein have shown that it localizes to a great extent in the nucleolus (8, 18). Deletion analysis has also shown that, like other nuclear proteins, Rev contains a basic stretch of amino acids that functions as a nuclear targeting signal (18).

In this study, site-directed mutagenesis was used to localize potential nucleolar determinants of Rev and examine the role of nucleolar localization for Rev function. Our results indicate that Rev does contain regions essential for nucleolar targeting, distinct from the nuclear targeting domain and that accumulation in the nucleolus appears to be important for Rev function.

## MATERIALS AND METHODS

**Construction of Rev mutants and β-galactosidase fusion products.** Using the plasmid BL-SVRev (derived from pH1Art) (6), site-directed mutagenesis was carried out as described by Kunkel (15). Promoter sequences and polyadenylation signals are from simian virus 40 and were originally derived from plasmid pSV2CAT (9). The fusion protein Rev38-51/β-galactosidase was made by synthesizing an oligonucleotide with a 5′ *Hin*dIII and a 3′ *Bam*HI overhang encoding the amino acids shown (Fig. 1) and by ligating this to the *Hin*dIII-*Bam*HI site present between the simian virus 40 promoter and the *Escherichia coli* β-galactosidase gene. The Rev1-58/β-galactosidase fusion plasmid was constructed by ligation of the *Hin*dIII-*Bam*HI fragment of plasmid pH3Rev (27) into the *Hin*dIII-*Bam*HI site 5′ of the β-galactosidase gene. To obtain the correct reading frame, plasmid DNA was cleaved with *Bam*HI, the overhangs were filled using Klenow, and the DNA was religated.

**Functional analysis of *rev* mutants.** To examine the functional effects of the mutagenesis, the capacity of the mutant proteins to restore chloramphenicol acetyltransferase (CAT) expression in the heterologous assay system described previously (21) was utilized. COS-7 cells were transfected with 1 μg of plasmid pSVAR (21) along with 2 μg of pSV2neo, BL-SVRev, or one of the mutants, using the DEAE-dextran

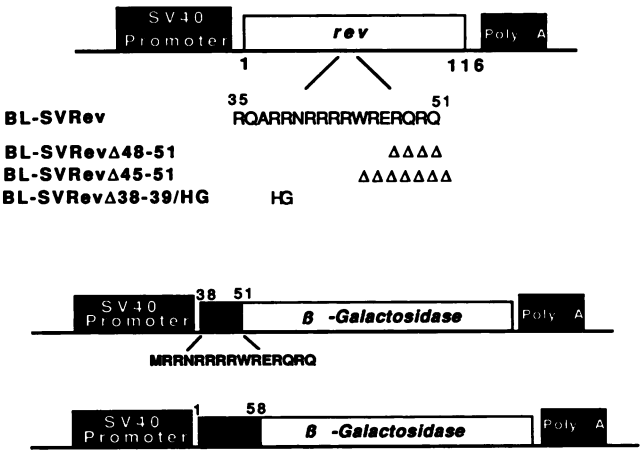


FIG. 1. Schematic representation of Rev expression plasmids (Top) and Rev/β-galactosidase fusion protein expression vectors (Bottom). Shown at the top are the mutants examined in this study. Δ, Deleted amino acids. The particular amino acid substitutions employed are indicated by the single-letter code.

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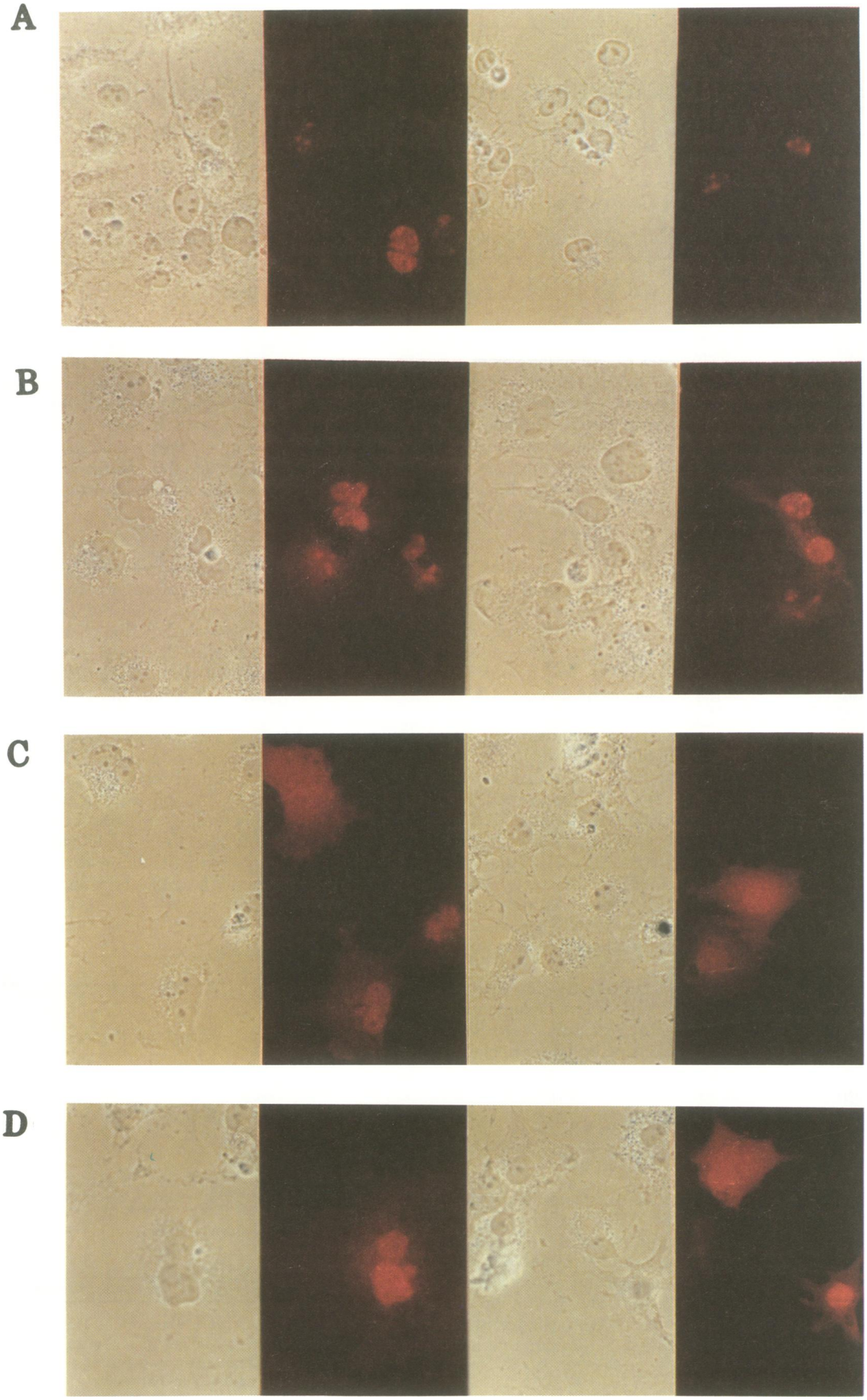


FIG. 2. Indirect immunofluorescence of COS cells expressing mutant Rev proteins. Shown are COS cells expressing authentic Rev (A), RevΔ48-51 (B), RevΔ45-51 (C), and RevΔ38-39/HG (D).

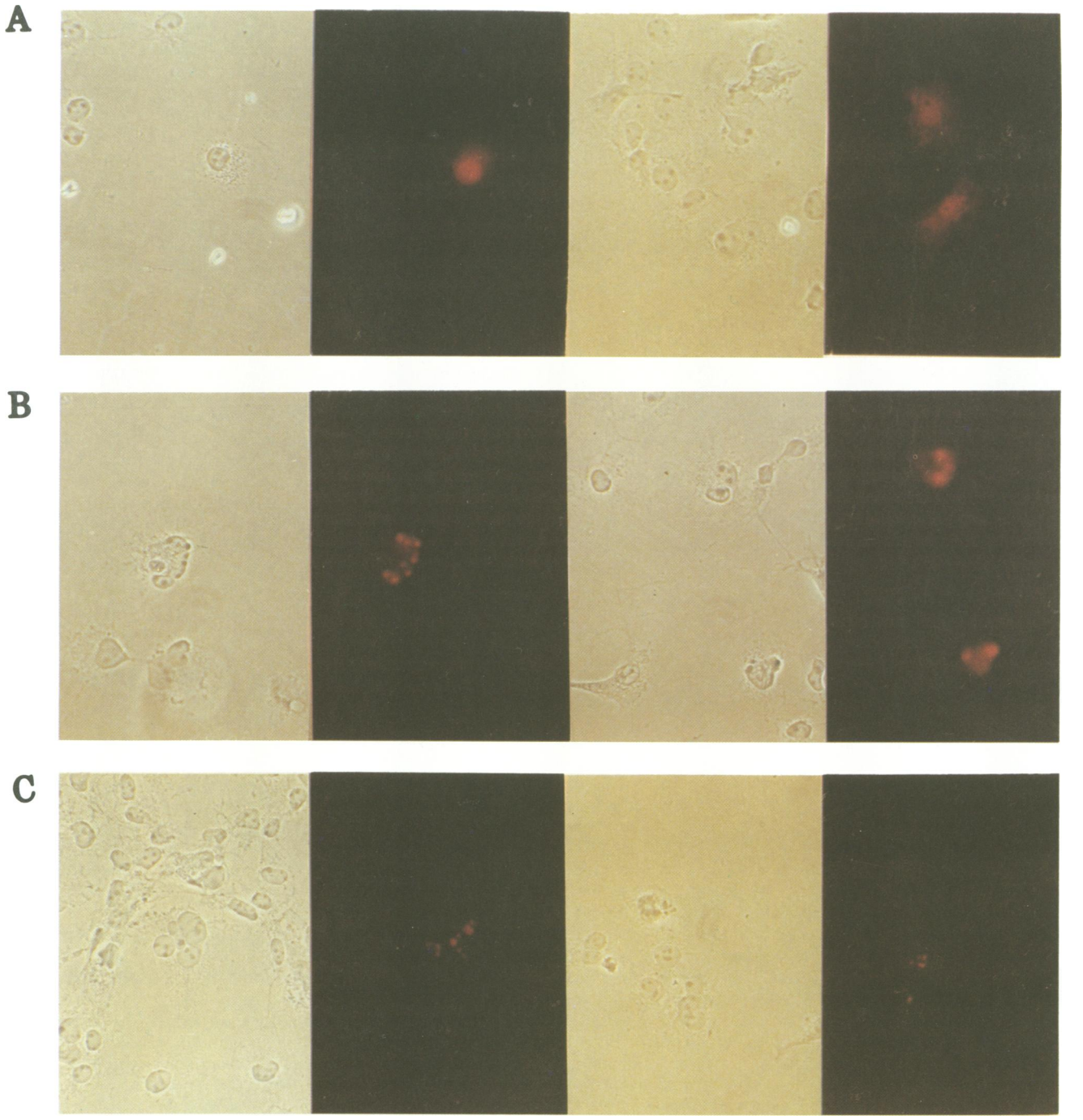


FIG. 3. Indirect immunofluorescence of COS cells expressing Rev/β-galactosidase fusion proteins. COS-7 cells were transfected with plasmid Rev38-51/β-galactosidase (A) or Rev1-58/β-galactosidase (B and C), and indirect immunofluorescence was done at 48 h posttransfection. Localization of recombinant proteins was by incubation with either anti-β-galactosidase antibody (A and B) or with anti-Rev antibody (C).

protocol previously described (5). Cells were harvested at 48 h posttransfection, and CAT assays were performed (9).

**Indirect immunofluorescence of COS cells expressing mutant Rev proteins.** COS-7 cells were plated at a density of $10^5$ cells per 35-mm-diameter dish 24 h before transfection. Cells were transfected with 1 μg of plasmid DNA by the DEAE-dextran transfection procedure as described previously (5). At 48 h posttransfection, cells were washed with phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde in PBS (pH 7.8) for 30 min at room temperature, washed for 5 min in PBS plus 10 mM glycine, incubated for 5 min with PBS-glycine, incubated with 25 mM glycine for 30 min, again washed with PBS-glycine, and finally blocked with 3% goat serum (Vector Laboratories) in Western antibody buffer (1% bovine serum albumin in PBS [pH 6.5], with 0.5 M NaCl and 0.05% Tween 20) for at least 1 to 5 h at room temperature. After being blocked, antibody was diluted in Western antibody buffer to a concentration of 1:300 and added to the

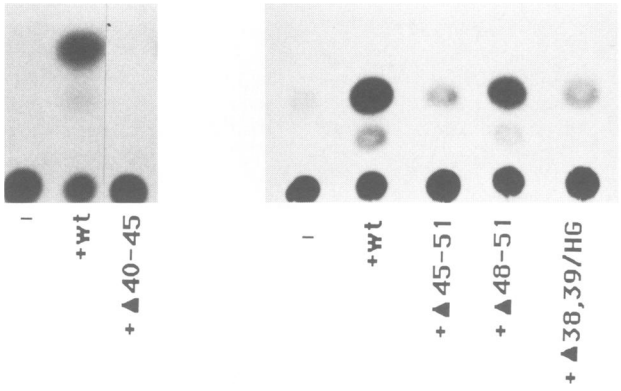


FIG. 4. Functional analysis of Rev mutants. COS-7 cells were transfected with pSVAR along with one of the following plasmids: pSV2Neo (lane −), BL-SVRev (lane +wt), BL-SVRevΔ40-45 (lane Δ40-45), BL-SVRevΔ48-51 (lane Δ48-51), BL-SVRevΔ45-51 (lane Δ45-51), or BL-SVRevΔ38-39/HG (lane Δ38,39/HG). At 48 h after transfection, cells were harvested and CAT assays were performed as outlined previously (9). After autoradiography, assays were quantitated by liquid scintillation. Units of activity are percent conversion per min as follows: pSV2neo, 0.17; BL-SVRev, 1.74; BL-SVRevΔ45-51, 0.29; BL-SVRevΔ48-51, 1.27; BL-SVRevΔ38-39/HG, 0.39.

cells. Cells were incubated at 4°C overnight. The following day, cells were washed twice with PBS containing glycine for 10 min each and then incubated in the dark with rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G secondary antibody (diluted 1:100 in Western antibody buffer plus 1% goat serum) with gentle shaking at room temperature for 1 h. Cells were subsequently washed three times with PBS containing glycine. Cover slips were mounted, and cells were viewed with a light, fluorescence microscope.

## RESULTS AND DISCUSSION

**Identification of sequences involved in nuclear localization.** The Rev protein has been previously shown to be localized to the nucleus (6, 18), with marked accumulation in what appeared to be nucleoli (18). Using deletion analysis coupled with Rev/β-galactosidase fusion proteins, previous work has shown that amino acids 40 to 45, encoding the sequence NRRRRW, are sufficient to confer localization to the nucleoplasm (18). To determine whether a distinct nucleolar determinant is also present, site-directed mutagenesis (15) was used to alter the basic amino acids on either side of the nuclear localization signal (18) (Fig. 1). Plasmid DNAs harboring the desired mutation were transfected into COS cells, and the subcellular localization of Rev was determined by indirect immunofluorescence (Fig. 2). A Rev mutant lacking the amino acids RQRQ which flank the nucleoplasm localization sequence localized to the nucleus and maintained nucleolar accumulation (Fig. 2B). However, extension of this deletion to include amino acids WRE, thereby removing the sequence WRERQRQ, produced a protein that localized to the nucleoplasm but gave clear nucleolar exclusion (Fig. 2C). A similar phenotype was observed with RevΔ38-39/HG in which amino acids 38 and 39, both encoding arginines, were converted to His and Gly, respectively (Fig. 2D). While studies show that nuclear localization signals often share the common feature of being highly basic (3, 14, 18), comparison of the three Rev mutants described above indicates that charge alone is not the determinant for localization to the nucleolus.

**Rev protein contains a nucleolar targeting signal.** We predicted, on the basis of the above results, that the nucleolar targeting sequence consisted of at least the two mutated regions. To test this hypothesis, a fusion protein was constructed by joining the peptide MRRNRRRRWRERQRQ to the amino-terminal end of *E. coli* β-galactosidase. β-galactosidase ($M_R$, 116,000), when expressed in mammalian cells, will normally remain in the cytoplasm (23). As shown in Fig. 3A, the Rev38-51/β-galactosidase fusion protein localized in the nucleoplasm but gave no nucleolar staining. Localization to the nucleus is likely conferred by the NRRRRW motif, in agreement with previous findings (18). The lack of nucleolar accumulation with this fusion protein suggests that Rev amino acids 38 to 51, although required for nucleolar localization, are not sufficient. To examine the possibility that the nucleolar determinant extends further toward the amino terminus, a hybrid protein was made consisting of amino acids 1 to 59 of Rev joined to β-galactosidase. As shown in Fig. 3B, this Rev1-58/β-galactosidase protein did localize to the nucleolus. The capacity of the fusion protein to accumulate in the nucleolus indicates that the amino-terminal portion of Rev does contain a nucleolar localization signal. Hence, the association of Rev with the nucleolus cannot be ascribed to experimental artifact.

**Functional significance of nucleolar localization for Rev function.** To assess the importance of nucleolar localization for Rev function, the ability of the mutant Rev proteins to restore gene expression directed by plasmid pSVAR (21) was determined. Previous studies using the pSVAR vector (21) demonstrate that sequences located within the coding region of the HIV *env* gene inhibit the expression of heterologous genes when present in *cis* and that the repressive effects of these sequences can be relieved by coexpression of Rev in *trans*. Thus, expression of the CAT gene linked to these HIV proviral sequences mimics the regulation observed with the HIV *gag* and *env* genes themselves (10, 11) and therefore permits the simple demonstration of Rev function. As shown in Fig. 4, cotransfection with plasmids BL-SVRev or BL-SVRevΔ48-51 was able to restore CAT gene expression, whereas plasmids BL-SVRevΔ45-51 and BL-SVRevΔ38-39/HG, encoding nonnucleolar forms of the protein, were found to have markedly reduced function.

These experiments favor a model whereby the localization of Rev in the nucleolus plays an important role in its ability to regulate HIV gene expression. Of interest, the human T-cell lymphotropic virus type I Rex protein, which is thought to function in a manner analogous to Rev (12, 13, 22) and indeed can substitute for Rev in some instances (20), is also localized in the nucleolus (24). However, comparison of the amino acid sequences of Rex and Rev reveals that, notwithstanding a basic stretch of amino acids, there exists little homology between the nucleolar determinants of these two proteins and implies that different sequence elements can confer nucleolar targeting. Localization could be conferred by a secondary structure within these domains. Alternatively, given that the nucleolus is not separated by any membranous structure from the surrounding nucleoplasm, the capacity to concentrate in the nucleolus could reflect the ability of Rev to interact with a component unique to this structure. Thus, this hypothesis would eliminate the need for a distinct nucleolar localization signal since only the ability to bind to any of the nucleolar-specific components or factors concentrated in this region need exist. This concept is strengthened by the observation that Tat (1, 26, 28),

another key regulatory protein of HIV, is also found predominantly in the nucleolus (23) but lacks any homology with Rex or Rev. The identification of three different nucleolar proteins that lack any significant amino acid sequence homology suggests that properties other than the capacity of a short stretch of amino acid sequence to bind to one particular protein confers nucleolar localization.

The suggestion that Rev regulates export of viral structural mRNAs from the nucleus to the cytoplasm, taken together with the results reported here, strongly suggests that nucleolar constituents may be involved in this export process. Indeed, earlier models have hypothesized that certain RNAs are transported from nucleus to cytoplasm via their association with ribosomal subunits which are synthesized in the nucleolus (4). Moreover, recent studies indicate that some nucleolar proteins shuttle from the cytoplasm to the nucleolus (2), proving that exchange between these two subcellular compartments exists. Future efforts should shed light on the intriguing role of the nucleolus in the HIV life cycle.

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