

Analysis of Intracellular Trafficking and Interactions of Cytoplasmic HIV-1 Rev Mutants in Living Cells

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The HIV-1 Rev protein is an essential nuclear regulatory viral protein. Rev mutants that are able to block wild-type (WT) Rev activity *in trans* have been reported and used in antiviral approaches. Not only nuclear but also cytoplasmic Rev mutants were described and suspected to be transdominant by retaining WT Rev in the cytoplasm. To investigate their potential for cytoplasmic retention, we studied the localization, trafficking, and interactions of cytoplasmic Rev mutants containing mutations in the N-terminal multifunctional domain. Using a novel dual-color autofluorescent protein-tagging system, we found that coexpression of the nucleolar blue-tagged WT Rev protein together with green-labeled cytoplasmic Rev mutants did not result in the retention of WT Rev in the cytoplasm but, on the contrary, in colocalization of the mutants to the nucleolus. A combination of mutations abolished the interaction with WT Rev, defining two domains important for Rev protein interaction. The identified domains were also essential for specific Rev responsive element (RRE) RNA binding and nuclear retention. Inactivation of the nuclear export signal shifted the steady-state distribution of the mutants from the cytoplasm to the nucleus, indicating their capability for nucleo-cytoplasmic shuttling. The cytoplasmic mutants were not transdominant compared to the nuclear mutant RevM10BL. These results emphasize that efficient oligomerization with WT Rev combined with RRE-specific RNA binding are prerequisites for effective transdominance.

INTRODUCTION

The Rev protein is an essential factor for replication of the human immunodeficiency virus type 1 (HIV-1) (for detailed reviews, see Cullen, 1991; Parslow, 1993; Felber, 1997; Pavlakis, 1997). Rev promotes the transport, stability, and translation of unspliced and partially spliced HIV-1 mRNAs responsible for the production of the viral structural proteins. The effects of Rev on the posttranscriptional control of gene expression are mediated by the binding to a unique RNA stem-loop structure termed Rev responsive element (RRE) identified in the *env* coding region of HIV-1. Rev accumulates in the nucleolus but constantly shuttles between the nucleus and the cytoplasm. Two regions important for function have been defined. The N-terminal region, approximately residues 14–60, contains the specific RRE binding domain, an arginine-rich nuclear/nucleolar localization signal, and residues important for oligomerization. A C-terminal leucine-rich effector domain (NES) mediates nuclear export (for review, see Hope, 1997). Potential candidates like the eukaryotic initiation factor eIF-5A or CRM1 have been proposed as cellular shuttle factors (Bevec *et al.*, 1996; Fornerod *et al.*, 1997). Mutations in the Rev effector domain prevent Rev's export and cause a dominant nega-

tive phenotype. This appears to be mediated by the formation of heteromultimers between Rev and transdominant (TD) Rev, which results in the inhibition of the nucleocytoplasmic transport of Rev (Hope *et al.*, 1992; Stauber *et al.*, 1995; Szilvay *et al.*, 1995). Kubota *et al.* (1992) proposed that a Rev mutant lacking the N-terminal Arg-rich domain was transdominant by retaining WT Rev in the cytoplasm, thereby suggesting a new mechanism of transdominance. Visualizing the interaction of Rev mutants with WT Rev in the same cell is often hampered by the difficulties to raise mutant-specific antibodies. In addition, fixation and permeabilization procedures necessary for indirect immunofluorescence could give rise to artifacts changing protein localization (D'Agostino *et al.*, 1995; Szilvay *et al.*, 1997; Lee *et al.*, 1998). Thus we used a novel dual-color autofluorescent protein-labeling system (Stauber *et al.*, 1998) to study the trafficking and interactions of cytoplasmic mutants in the most natural environment, the living cell.

RESULTS

Rev14-GFP and Rev38-GFP are localized in the cytoplasm

To test if cytoplasmic Rev mutants can affect the localization of WT Rev in living cells, green fluorescent protein (GFP) was fused to Rev mutants that localize predominantly in the cytoplasm. pBRev14-GFP encodes a Rev mutant in which the amino acids 14–16 have been changed from RTV to EED (D'Agostino *et al.*,

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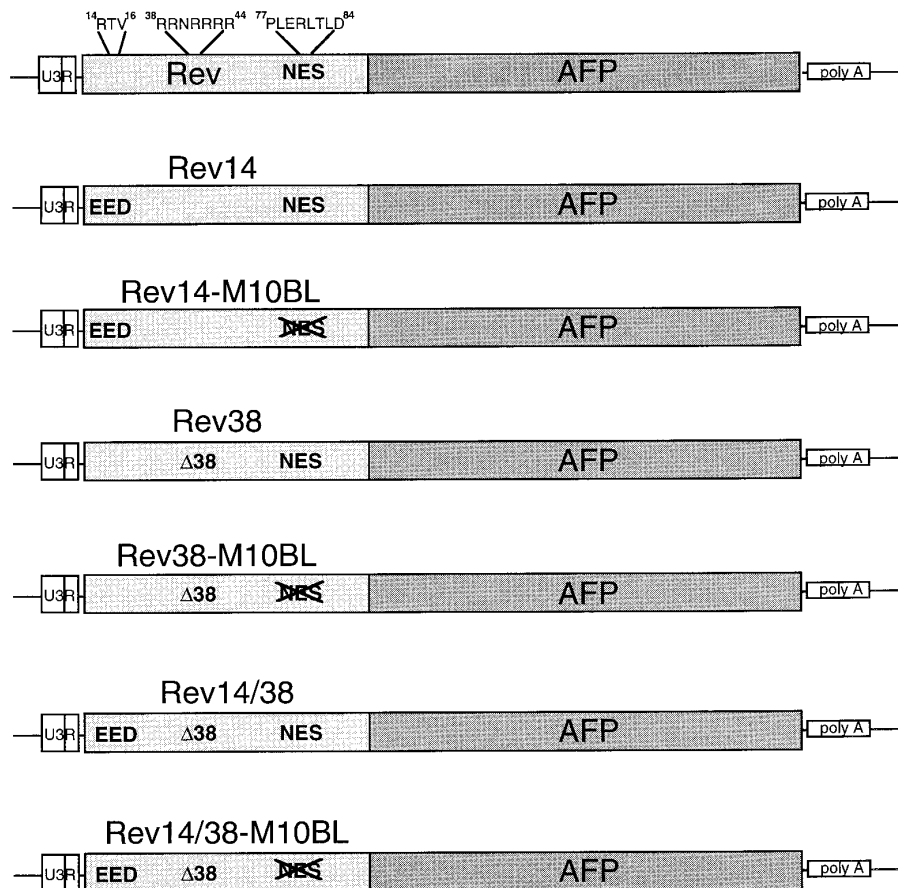


FIG. 1. Schematic representation of the plasmids coding for the different Rev-GFP or Rev-BFP fusion proteins. The constructs contain part of the HIV-1 5' LTR (U3 and part of the R region, up to nt +80) and the polyadenylation signal (poly A) of SV40. The GFP coding region is indicated by a shaded bar. In the constructs expressing Rev-BFP or Rev14M10BL-BFP, the GFP coding region is replaced by BFP. AFP indicates GFP or BFP.

1995) fused to the complete GFP protein. This Rev mutant was chosen because it was reported to be cytoplasmic, although it still contained the complete Arg-rich region [resembling a classical nuclear localization signal (NLS)] and still bound to nuclei in an *in vitro* assay (D'Agostino et al., 1995). Likewise, the mutant Rev38-GFP is a hybrid between GFP and a Rev mutant in which the basic amino acids 38–44 (RRNR-RRR) were deleted (Fig. 1). This Rev mutant without the GFP-tag has been reported to be transdominant (Kubota et al., 1992). As shown previously, Rev-GFP localized almost completely to the nucleolus when expressed in human cells (Stauber et al., 1995) (see also Fig. 3B). In contrast, confocal laser scan microscopy (CLSM) of transfected HLTat cells demonstrated that both Rev-GFP mutants localized predominantly to the cytoplasm (Figs. 2A and 2B), although in some high-expressing cells, Rev14-GFP could also be detected at the nucleolus. Fixation of the cells led to the detection of Rev14-GFP at the nucleolus in most cells. We suggest that this is the result of the fixation procedure, demonstrating an advantage of the GFP technology to avoid artifacts caused by fixation. The localization of

the Rev-GFP mutants was similar in several other cell lines tested. Cotransfection of a plasmid encoding an RRE containing RNA did not cause detectable changes in protein localization. When tested in a Rev-dependent Gag expression assay, neither the presence of Rev14-GFP nor Rev38-GFP could stimulate the expression of Gag, verifying that both mutations resulted in nonfunctional Rev proteins (data not shown).

The Rev mutants are not defective in nuclear import but in nuclear retention

To interact with its RNA target, Rev has to be present in the nucleus. To investigate if the Rev mutants are able to enter the nucleus, we, in addition, inactivated the nuclear export signal (NES) in the mutants. Since Rev is a shuttling protein, the cytoplasmic steady-state localization can be caused by either lack of entry in the nucleus or by rapid nuclear export. We reasoned that blocking nuclear export should result in nuclear accumulation of the Rev mutants if they are capable of nuclear entry. In contrast, blocking nuclear export should not change their localization if the proteins always remain in the cytoplasm. The double mu-

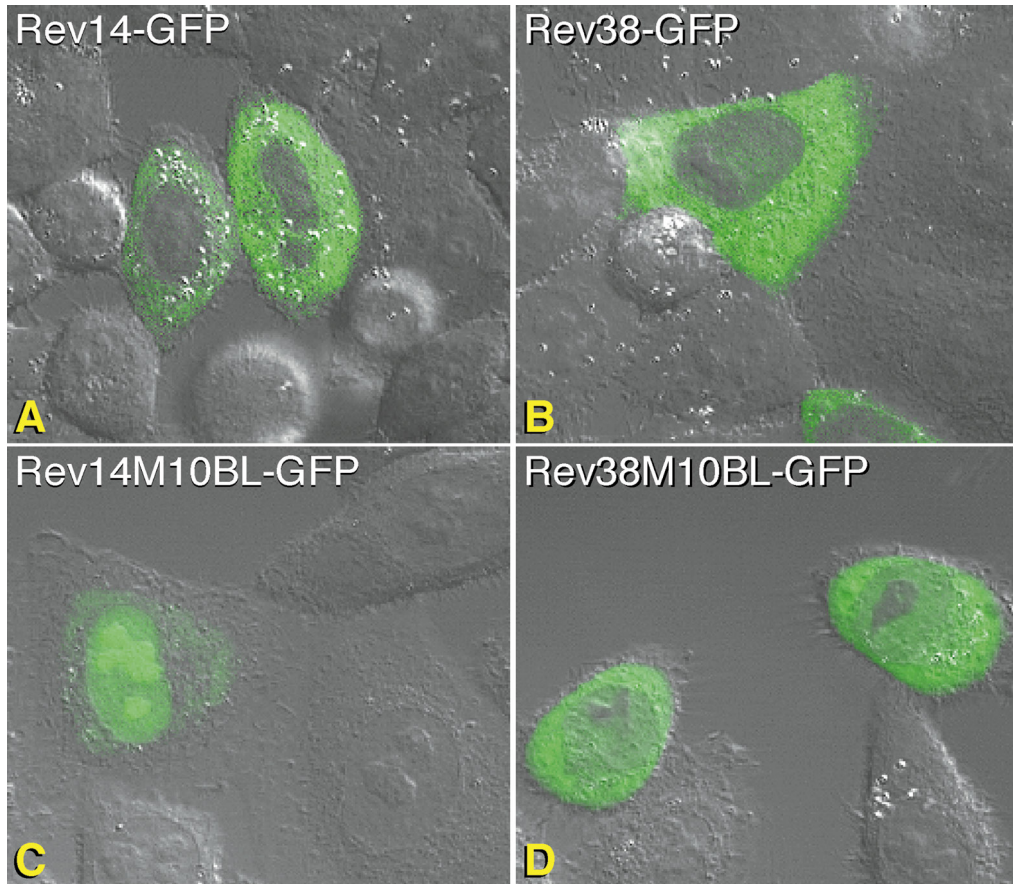


FIG. 2. Localization of Rev-GFP mutants in living cells. HLTat cells were transfected with 2 μg of the indicated plasmids, and 24 h later analyzed by confocal laser scan microscopy (CLSM). Rev14-GFP and Rev38-GFP localized in the cytoplasm (A and B). Inactivation of the nuclear export signal (M10BL) resulted in the predominant nuclear/nucleolar localization of the double mutants (C and D).

tants containing an inactivated nuclear export signal (M10BL) (see Fig. 1) were detected predominantly in the nucleus upon transient transfection (Figs. 2C and 2D). In contrast to Rev14M10BL-GFP, which accumulated at the nucleoli, Rev38M10BL-GFP was still excluded from the nucleoli. Similar localization of the double mutants without the GFP tag could be observed by indirect immunofluorescence using Rev-specific antibodies. Treatment of cells expressing Rev14-GFP or Rev38-GFP with leptomycin, an inhibitor of the Rev nuclear export pathway, resulted also in nuclear accumulation of the mutants (data not shown). These results suggest that a Rev mutant lacking the putative NLS is still able to enter the nucleus. The steady-state distribution, however, is predominantly cytoplasmic because the mutants are not efficiently retained in the nucleus but are readily exported.

WT Rev is not trapped in the cytoplasm by the Rev mutants but colocalizes the mutants to the nucleolus

The ability of tagging proteins with different-colored GFP mutants offered the opportunity to examine the interaction of Rev proteins in the same cell. If the

mutant Rev-GFP proteins were able to retain blue-tagged WT Rev in the cytoplasm by complex formation, it should result in colocalization of blue WT Rev-BFP in the cytoplasm.

Coexpression of blue WT Rev-BFP together with Rev14-GFP or Rev38-GFP demonstrated that both mutants were still able to interact with WT Rev. However, the mutants did not trap WT Rev in the cytoplasm but, on the contrary, were translocated to the nucleolus by interaction with WT Rev (Figs. 3A and 3C). The presence of WT Rev-BFP in the same cell was verified by a UV-laser excited BFP (Figs. 3B, 3D, and 3F), whereas GFP was detected independently by excitation with an argon laser. The double mutant Rev14/38, combining the mutations 14 and 38 (see Fig. 1), did not colocalize to the nucleolus with WT Rev (Fig. 3E). Thus the combination of both mutations abolished the interaction with WT Rev. Our result suggested that two distinct regions of the Rev protein were important for efficient protein–protein interaction. However, we can not exclude the possibility that combining the mutations 14 and 38 could also induce drastic conforma-

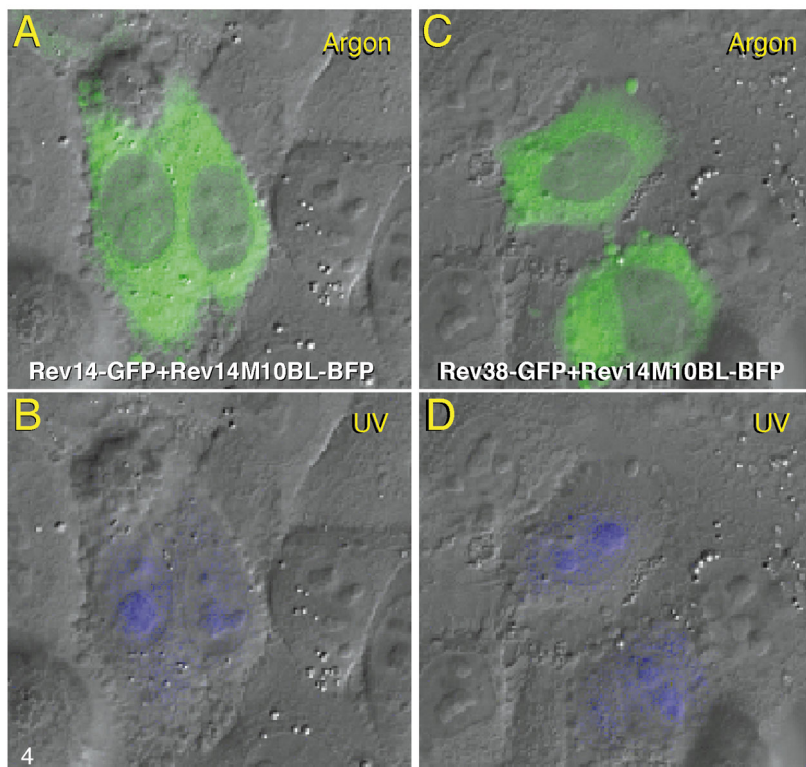
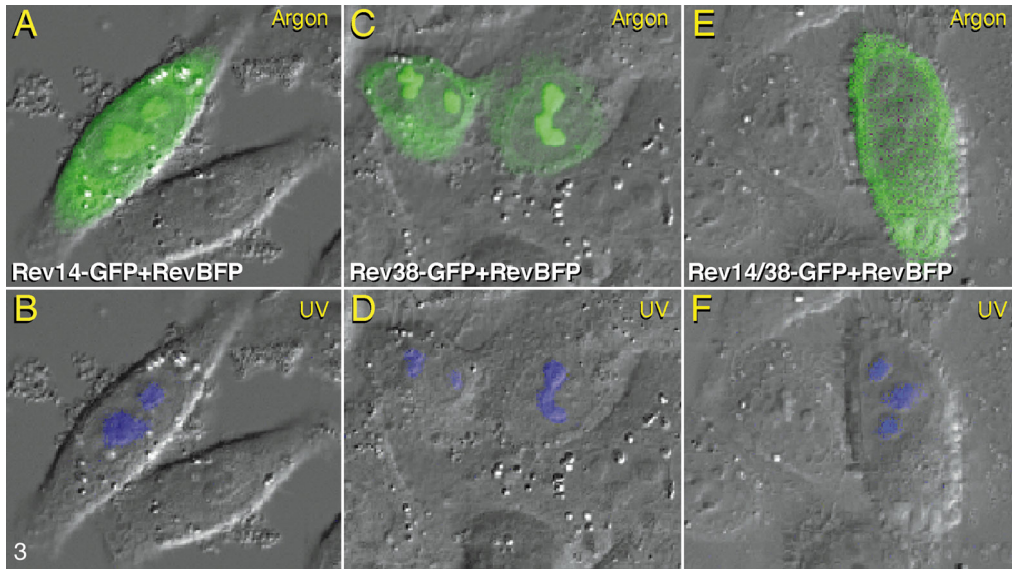


FIG. 3. Rev14-GFP and Rev38-GFP but not the double mutant Rev14/38-GFP interact with WT Rev-BFP in living cells. HLtat cells were transfected with 2 μ g of the indicated plasmids and analyzed by CLSM after 24 h. Coexpression of the blue-labeled WT Rev protein together with Rev14-GFP and Rev38-GFP resulted in their colocalization at the nucleolus (A and C). In contrast, Rev14/38-GFP was not colocalized to the nucleolus in the presence of WT Rev-BFP (E). The GFP-labeled proteins were detected using an argon laser (A, C, and E), whereas a UV-laser excited the WT Rev-BFP hybrid (B, D, and F).

FIG. 4. Rev14-GFP and Rev38-GFP are impaired in multimerization. HLtat cells were cotransfected with 2 μ g of plasmid DNA expressing the blue-tagged Rev14M10BL together with Rev14-GFP or Rev38-GFP. The tagged proteins were detected as described in Fig. 3. In contrast to the presence of WT Rev-BFP (Fig. 3), the cytoplasmic localization of the mutants (A and C) remained unchanged in the presence of Rev14M10BL-BFP (B and D).

tional changes in the double mutant Rev14/38, thus blocking the interaction with WT Rev. To control the possibility that the mutant Rev14/38 could no longer

interact with WT Rev because it was excluded from the nucleus, we constructed the mutant Rev14/38-M10BL, again inactivating the nuclear export signal. Following

transfection, this protein was detected in the nucleus, indicating that the mutant Rev14/38 is not a purely cytoplasmic protein but has the ability to shuttle (data not shown). The same protein interactions could be observed upon cotransfection of blue-tagged RevM10BL together with Rev14-GFP and Rev38-GFP, demonstrating that inactivation of the NES did not abolish Rev multimerization. In addition, cotransfection of the Rev-GFP mutants with untagged WT Rev or RevM10BL verified that the mutants were localized to the nucleolus via specific Rev–Rev protein interaction (data not shown).

Rev14-GFP and Rev38-GFP are impaired in multimer formation

Since Rev has to oligomerize on the RRE to function, we investigated whether the lack of the biological activity of the mutants was caused by impaired oligomerization. Homomultimer formation was studied by coexpressing blue-tagged Rev14M10BL-BFP, which localized primarily to the nucleolus (see Fig. 2C), together with Rev14-GFP. In contrast to coexpression with WT Rev-BFP (Figs. 3A and 3C) no significant green fluorescence was detected at the nucleolus (Fig. 4A). In addition, Rev38-GFP did not interact efficiently with Rev14M10BL-BFP (Fig. 4C). Again, excitation with the UV laser verified the presence of the blue Rev14M10BL-BFP at the nucleolus (Figs. 4B and 4D). These findings indicated that the mutants are impaired in oligomerization compared to WT Rev in live cells.

Rev14-GFP and Rev38-GFP have lost WT Rev RNA binding specificity

Specific binding to the Rev response RNA element (RRE) is essential for Rev function. To characterize the RNA binding properties, the Rev-GFP fusion proteins were purified after expression in *Escherichia coli* and tested in a bandshift assay in the presence of radio-labeled RRE RNA. Figure 5A illustrates that purified Rev (lanes 2–4) and Rev-GFP (lanes 5–7) were able to interact with RRE containing RNA to form specific complexes. The altered mobility of the complexes was caused by the higher molecular weight of Rev-GFP compared to Rev. Rev38-GFP showed no RNA binding activity (lanes 11–13). In contrast, Rev14-GFP bound efficiently to the RNA, but the observed complexes displayed a different mobility (lanes 8–10). We therefore tested the mutants for specific binding to the Rev primary binding site, present within hairpin loop IIa of the RRE. We found that in contrast to Rev or Rev-GFP, Rev14-GFP still bound efficiently to the RRE Δ 12S mutant, which lacks the primary Rev binding site (Fig. 5B, lanes 5–7). We reasoned that mutant Rev14-GFP has lost, at least partially, WT Rev-specific RRE binding and interacts with RNA nonspecifically.

Rev14-GFP and Rev38-GFP are not transdominant

Cotransfections of the Rev-GFP mutants together with WT Rev-GFP at a plasmid DNA ratio of 4:1 did not result in significant suppression of WT Rev activity as compared to cotransfection with the transdominant mutant RevM10BL (plasmid DNA ratio 2:1) (Fig. 6). Quantitation of the GFP signal produced from the cotransfected GFP expression plasmid pCMV-GFP-sg25 showed equal transfection efficiencies. The fluorescence resulting from the expression of the low amount of transfected Rev-GFP plasmids was 32-fold lower than the signal emitted by the coexpressed unfused GFP and could not affect the results. In addition, equal expression levels of the different Rev mutants were verified by Western blot analysis. Cotransfection of the untagged Rev mutants together with WT Rev also did not result in significant inhibition of WT Rev activity (data not shown). This finding suggests that the interaction of the Rev mutants with WT Rev off the RRE can not always be simply extrapolated to Rev complex formation on the RRE *in vivo*.

The Rev mutants appear to be impaired in complex formation with WT Rev on the RRE *in vitro*

To address the formation of heteromultimers on the RRE *in vitro*, we took advantage of the different mobility of the RNA–protein complexes formed by recombinant Rev and Rev-GFP hybrids. Competition experiments were performed using varying amounts of Rev and Rev-GFP mutant proteins (Fig. 7). Heteromultimers containing both Rev and Rev-GFP mutants should migrate differently compared to homomultimers formed exclusively by Rev or Rev-GFP mutants, respectively. We observed a weak formation of intermediate-size complexes by mixing Rev protein with Rev14-GFP (Fig. 7B, marked with arrows). However, the nonspecific RNA binding activity of the mutant Rev14-GFP did not allow us to distinguish clearly between mutant–WT Rev protein interaction or the binding of Rev14-GFP to RNA regions unoccupied by WT Rev. Only the mutant Rev38, which displayed no RNA binding activity, allowed us to study exclusively protein–protein interaction. As illustrated in Fig. 7A (lanes 2–5), we could not detect intermediate complexes of Rev and Rev38-GFP under our experimental conditions. This suggests that Rev mutants lacking RRE-specific RNA binding and proper oligomerization appear not to participate efficiently in complex formation on the RRE and are thus not transdominant.

DISCUSSION

In our study, we investigated the potential of cytoplasmic Rev mutants (see Table 1) to be transdominant by trapping WT Rev in the cytoplasm as suggested by Ku-

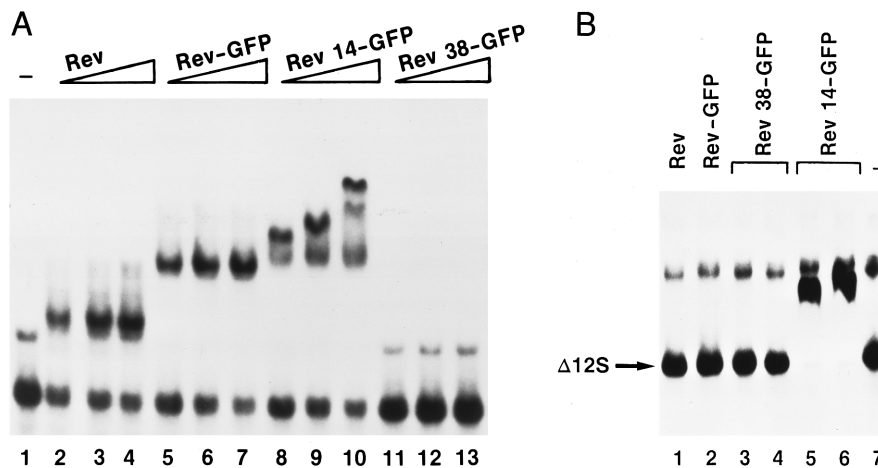


FIG. 5. (A) RRE RNA binding activity of the Rev-GFP mutants. The Gel retardation assay was performed incubating purified Rev proteins with ^{32}P -labeled RRE RNA. Lane 1, free RNA probe. In lanes 2–4, 5, 10, or 20 pmol of recombinant Rev or Rev-GFP proteins, respectively, were used. Lanes 5–7 contained 3.4, 6.8, or 13.6 pmol of Rev-GFP protein, respectively. Rev14-GFP protein concentrations were 5.3, 10.6, and 21.2 pmol (lanes 8–10). Rev38-GFP was used at a concentration of 4.7, 9.4, and 18.8 pmol (lanes 11–13). (B) Rev14-GFP is defective in WT Rev RRE binding specificity. The RNA binding assay was performed in presence of ^{32}P -labeled RRE Δ 12S RNA lacking the primary Rev binding site. Recombinant protein concentrations were: Lane 1, 20 pmol of Rev; lane 2, 13.6 pmol of Rev-GFP; lanes 3 and 4, 18.6 and 28 pmol of Rev38-GFP, respectively; lanes 5 and 6, 21.2 and 32 pmol of Rev14-GFP, respectively; lane 7, free RNA probe.

bota *et al.* (1992) and Furuta *et al.* (1995). Our approach, which was based on the change of the intracellular localization of the labeled Rev proteins, demonstrated

that the cytoplasmic Rev mutants were unable to retain WT Rev in the cytoplasm but, on the contrary, were transported to the nucleolus via interaction with WT Rev.

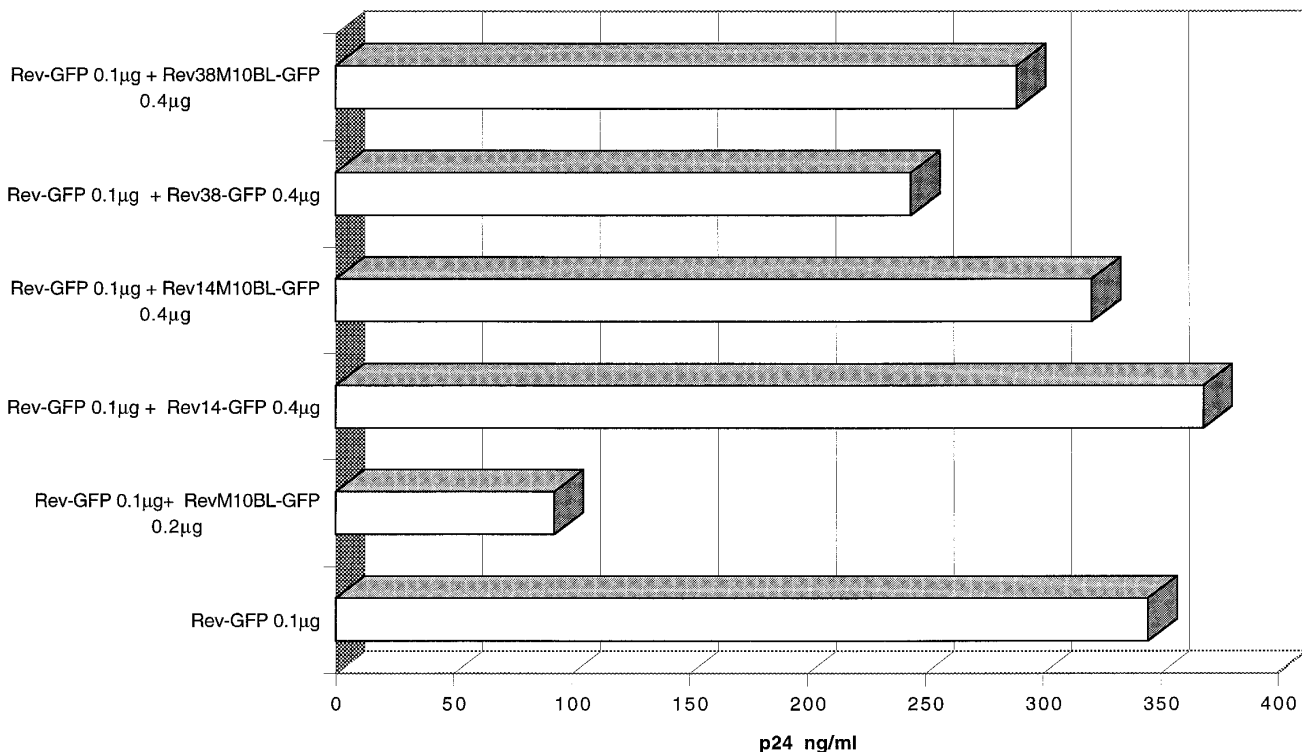


FIG. 6. The mutant Rev-GFP fusion proteins are not transdominant. 239 cells were transfected with 0.1 μg of pBrev-GFP alone or in combination with different amounts of the indicated mutant Rev-GFP expression plasmids, together with 1 μg of pL3tat, 1 μg of the *gag*-expression vector pB37R, and 2 μg of pCMV-GFPsg25. 48 h later the cells were harvested and intracellular Gag production was measured by a p24^{gag} antigen capture assay. Duplicate 293 plates were used and the results were averaged. Similar results were obtained in two independent experiments. Transfection efficiencies were controlled quantitating GFP as described in Materials and Methods.

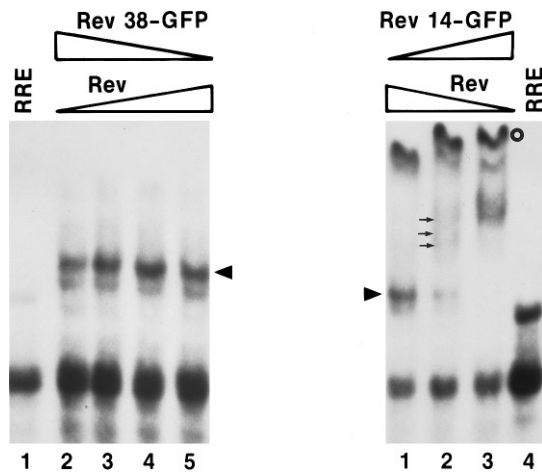


FIG. 7. *In vitro* assay to address complex formation of Rev14-GFP and Rev38-GFP with WT Rev on the RRE. Complexes of recombinant WT Rev with 32 P-labeled RRE RNA were performed for 15 min and subsequently different amounts of Rev38-GFP or Rev14-GFP were added. (A) Lane 1, free RNA probe; lane 2–4, 5, 10, and 20 pmol of WT Rev were combined with 20, 10, and 5 pmol of Rev38-GFP, respectively. Lane 5, RNA incubated with 20 pmol of WT Rev. (B) Lanes 1–3, 5, 10.6, and 21.6 pmol of Rev14-GFP were combined with 20, 10, and 5 pmol of Rev, respectively. Arrows mark the formed intermediate complexes. Lane 1, free RNA probe. Triangle, Rev-complexes; arrows, intermediate Rev/Rev14-GFP complexes; circle, Rev14-GFP complexes.

Thus we suggest that the reported inhibitory effect of Rev38 on viral replication (Furuta *et al.*, 1995) may be mediated by an unknown mechanism, e.g., competition for Rev binding cofactors as suggested in a study on the HTLV-I Rex protein (Katahira *et al.*, 1995). Alternatively, inhibition of virus replication in stable Rev38-expressing cell lines may be an effect not related to the properties of the Rev38 mutant.

Several *in vitro* and *in vivo* studies have indicated that mutations in the N-terminal multifunctional region of Rev impair multimerization, although other studies proposed that deletion of the Arg-rich domain does not affect oligomerization (Malim *et al.*, 1989; Olsen *et al.*,

1990; Zapp *et al.*, 1991; Madore *et al.*, 1994; Szilvay *et al.*, 1997; Thomas *et al.*, 1998). Colocalization of the single, but not of the double, mutants from the cytoplasm to the nucleolus via protein interaction in our study suggested that two domains are important for Rev oligomerization in live cells. Thus the mutants are able to interact with WT Rev because one domain is supplied by WT Rev and the other is presented by the Rev mutants. Efficient homooligomerization, on the other hand, appears to be impaired because the mutants are lacking the second interaction domain. The study of Thomas *et al.* (1997, 1998) indicated that the N-terminal part of Rev displays a protein interaction interface composed of two α -helices contacting via hydrophobic residues. The mutations described in this report disrupt either the integrity of helix 1 (Rev14) or helix 2 (Rev38) and are in agreement with this model. Specific oligomerization of the Rev mutants off the RRE was not only observed with WT Rev but also with RevM10BL, demonstrating direct protein interaction with a mutant Rev protein lacking the NES. This finding supports the proposed mechanism for transdominance caused by the formation of inactive heteromultimers between Rev and RevM10BL in the nucleus (Hope *et al.*, 1992; Stauber *et al.*, 1995; Szilvay *et al.*, 1995).

The development of autofluorescent proteins with different excitation and emission spectra allows for direct observation of protein interactions in living cells (Stauber *et al.*, 1998). Since GFP was shown to dimerize at high protein concentrations and the structure of BFP is similar to GFP (Palm *et al.*, 1996), it is important to include an untagged interaction partner as a control (e.g., unlabeled WT Rev in this report) to assure the specificity of the protein interaction. In our study, the GFP-tagged double mutant Rev14/38 no longer interacted with BFP-tagged WT Rev, underlining that we were examining specifically Rev–Rev interactions.

Analysis of the RRE–RNA binding specificity revealed that not only multimerization but also RNA binding was

TABLE 1
Properties of Rev-GFP Mutants

Protein	Localization	Multimerization ^a	Shuttling	RRE binding ^b	Transdominance
Rev-GFP	Nucleolus	+	+	+	–
RevM10BL-GFP	Nucleolus	+	–	+	+
Rev14-GFP	Cytoplasm	+	+	–	–
Rev14M10BL-GFP	Nucleus/nucleolus (cytoplasm)	+	–	n.d.	–
Rev38-GFP	Cytoplasm	+	+	–	–
Rev38M10BL-GFP	Nucleus/ (cytoplasm)	+	–	n.d.	–
Rev14/38-GFP	Cytoplasm	–	+	n.d.	n.d.
Rev14/38M10BL-GFP	Nucleus/ (cytoplasm)	–	–	n.d.	n.d.

^a Heteromultimerization with WT Rev *in vivo*.

^b Rev-specific RRE binding *in vitro*. n.d., not determined.

affected in the mutants. Rev14 was still able to bind to RNA but lost the specificity of WT Rev for the RRE structure. This indicated that the Arg-rich region is necessary but not sufficient for RRE-specific RNA binding. It also underlined the importance of addressing not only RRE RNA but WT Rev-specific RRE RNA binding (i.e., by using a RRE RNA lacking the primary Rev binding site) to fully characterize Rev mutants. Our results indicate that residues around amino acid 14 are important to maintain a distinct protein conformation, which allows the positively charged Arg-rich domain to recognize specifically the RRE structure. This structure is also essential for the nucleolar localization of Rev, possibly by binding to 5S RNA, which at least partially mimics the RRE structure (Lam *et al.*, 1998).

Although the described mutants were still able to interact with WT Rev off the RRE, additional inactivation of the NES in the mutants did not result in a transdominant phenotype as compared to RevM10BL (Mermer *et al.*, 1990; Stauber *et al.*, 1995). We reasoned that to form a biologically active complex on the RRE, Rev has to oligomerize correctly using both high-affinity interaction domains. When using our *in vitro* assay, we did not find efficient complex formation on the RRE between the mutants and WT Rev which supports this assumption. Alternatively, because the mutants are impaired in RRE-specific RNA binding, the correct specificity can be prerequisite for active heterocomplex formation on the RRE. Thus Rev mutants that lack the NES but are still able to oligomerize efficiently with WT Rev and retain RRE-specific RNA binding activity (e.g., RevM10BL or RevM10) (Benko *et al.*, 1990; Malim *et al.*, 1992) remain so far the best candidates for an anti-HIV-1 gene therapy approach (Nabel *et al.*, 1994).

Several groups have proposed that cytoplasmic Rev mutants are biologically inactive because they are excluded from the nucleus (Berger *et al.*, 1991; Hamerschmid *et al.*, 1994). By inactivating the NES in the studied mutants, we could demonstrate their competence for nuclear entry. Nuclear accumulation after leptomycin treatment and complex formation of the mutants with the nonshuttling nucleolar protein RevM10BL also support this finding. Since Rev38 is lacking the putative NLS of Rev (Cochrane *et al.*, 1990; Kubota *et al.*, 1989), we are currently investigating the importance of the Arg-rich domain for nuclear entry or nuclear retention in the context of Rev.

In summary, we have used a two-color GFP-tagging system to detect protein-protein interactions in living cells. The two-color approach is straightforward, does not require subsequent modifications, and may therefore be applied widely. With this assay, we demonstrated that the analyzed cytoplasmic Rev mutants were unable to retain WT Rev in the cytoplasm but colocalized with WT Rev to the nucleolus. In addition,

our findings suggest that two domains are important for efficient Rev oligomerization. The integrity of the defined domains is also essential for nucleolar accumulation and RRE-specific RNA binding, underlining the fact that the RNA binding and oligomerization domains of Rev highly overlap. The cytoplasmic localization of the mutants is not caused by their inability to enter the nucleus but by their impaired nuclear retention. No or unspecific RNA binding together with impaired multimerization explain why the mutants are biologically inactive and not transdominant.

MATERIALS AND METHODS

Cells and transfections

For microscopic and functional studies the human cell lines HLTat and 293 were used (Stauber *et al.*, 1995). Cells were transfected and cytoplasmic extracts were prepared as described (Stauber *et al.*, 1995). To assay for Rev activity, the production of HIV-1 Gag protein was quantitated as described (Stauber *et al.*, 1995). In experiments using 293 cells, Tat protein was provided *in trans* by cotransfection with the Tat-expression plasmid pL3tat (Felber *et al.*, 1990). To control for transfection efficiency, 2 μ g of the GFP-expressing plasmid pCMV-GFPsg25 (Stauber *et al.*, 1998) was cotransfected. The GFP signal was quantitated in a Cytofluor II plate reader (Perseptive Biosystems) equipped with at 485/20 nm excitation and 530/30 nm emission filter as described (Stauber *et al.*, 1998).

Recombinant plasmids

pBsrev or pBsrevM10BL produces Rev or RevM10BL, respectively (Stauber *et al.*, 1995). pB37R expresses *gag* in a Rev-dependent manner under the HIV-1 5' LTR promoter (Stauber *et al.*, 1995). pCMV-GFPsg25 and pCMV-BFPsg50 express enhanced versions of the GFP emitting green or blue light, respectively, under the control of the CMV early promoter (Stauber *et al.*, 1998). pBrev-GFP was constructed by replacing the *NheI*-*Bst*BI DNA fragment of pBsrev-GFP (Stauber *et al.*, 1995) by the *NheI*-*Bst*BI DNA fragment from pCMV-GFPsg25. pBrev14-GFP was generated by replacing the *Afl*II-*Hind*III DNA fragment of pBrev-GFP by the corresponding DNA fragment from pBsrev14-16EED (D'Agostino *et al.*, 1995). In the plasmid pBrev14M10BL, the *Hind*III-*NheI* fragment of pBrev14-GFP was replaced by the corresponding fragment of pBsrevM10BL. pBsrevM10BL encodes a transdominant Rev in which the nuclear export signal is inactivated (Stauber *et al.*, 1995). To obtain the mutant pBrev38-GFP, the amino acids 38-44 were deleted by single-stranded site-directed mutagenesis as described (Schwartz *et al.*, 1992). To clone pBrev38M10BL-GFP, the *Hind*III-*Bgl*II fragment of pBsrevM10BL-GFP was substituted by the corresponding fragment from pBrev38-GFP, and the WT GFP was subsequently replaced with the mutant GFP from pCMV-GFPsg25 by exchanging the

NheI–*Bst*BI DNA fragment. In the double mutant pBrev14/38-GFP, the *Hind*III–*NheI* DNA fragment in the plasmid pBrev14-GFP was replaced with the corresponding fragment from pBrev38-GFP. To generate the mutant pBrev14/38M10BL-GFP, the same strategy was used to replace the *Hind*III–*NheI* DNA-fragment from pBrev14-GFP with the DNA sequence from pBrev38M10BL-GFP. The constructs pBrev14M10BLN and pBrev38M10BLN encode the mutant Rev proteins Rev14M10BL and Rev38M10BL, respectively, without the GFP tag. To generate these plasmids, the constructs pBrev38M10BL-GFP and pBrev14M10BL-GFP were linearized with *Nco*I, treated with Klenow enzyme, and religated.

The constructs pBrev-BFP, pBrevM10BL-BFP, and pBrev14M10BL-BFP were constructed by replacing the *NheI*–*Bst*BI DNA fragments from the plasmids pBrev-GFP, pBsrevM10BL-GFP, and pBrev14M10BL-GFP, respectively, with the corresponding fragment from the plasmid pCMV-BFPsg50.

For bacterial expression, the genes coding for WT and mutant Rev-GFP were cloned into the expression vector pET3a (Novagen), in which expression is under the control of the bacteriophage T7 *phi*10 promoter. pET3a plasmid DNA was digested with *Nde*I, treated with Klenow enzyme, and subsequently digested with *Bam*HI. To generate compatible DNA ends for cloning, the Rev-GFP or mutated Rev-GFP coding sequences were amplified by PCR using appropriate primers. The amplified DNA fragments were digested with *Bam*HI and ligated into the modified pET3a vector, resulting in the plasmids pET3aRev-GFP, pET3aRev14-GFP, and pET3aRev38-GFP. The construct for the expression of the untagged WT Rev protein has been described elsewhere (Mermer et al., 1990). Plasmids were verified by sequence analysis as described previously (Stauber et al., 1995).

Microscopy of living cells and image analysis

Cells were seeded into coated 50-mm glass-bottom microwell dishes (MatTek) or Lab-Tek chamber slides (Nunc), cultured in phenol red free DMEM (10% FCS) and transfected after 24 h. One day later, the cells were analyzed by a Zeiss LSM 410 Micro System in the confocal mode. For GFP excitation, an argon/krypton laser at 488-nm wavelength was used, whereas a UV laser at 364-nm wavelength was used to excite BFP. Emitted fluorescence was detected with a 510- to 540-nm band-pass filter for GFP or with a 420-nm cutoff filter for BFP, respectively. Normaski images were made using a 543-nm green laser and appropriate polarized lenses.

Indirect immunofluorescence

Transfected cells were fixed, permeabilized, and incubated with a polyclonal rabbit anti-Rev antiserum (diluted 1:100 in PBS) as described earlier (Stauber et al., 1995).

Appropriate secondary rhodamine conjugated anti-IgG antibodies (diluted 1:200 in PBS) were added for 1 h, and cells were analyzed by microscopy. Extensive washings with PBS were performed after each step throughout the procedure.

Leptomycin treatment

HLtat cells transfected with the indicated plasmid were treated after 24 h with leptomycin (6 nM) for 30 min and examined by fluorescence microscopy without fixation.

Bacterial protein expression and purification

The untagged Rev protein was expressed and purified as described previously (Benko et al., 1990). To produce WT and mutant Rev-GFP proteins, the pET3a expression constructs were transformed into the *E. coli* strain BL21(DE3), which expresses T7 RNA polymerase under the control of the *lac* repressor. Cells were grown to a OD_{660nm} of 0.6, and subsequently gene expression was induced by adding IPTG to a final concentration of 0.5 mM. Three hours later the cells were harvested and lysed by sonication.

Rev-GFP was purified from inclusion bodies after solubilization in 7 M urea and 20 mM Tris-HCl (pH 8.9), applied on a Mono Q column, and eluted with 300 mM NaCl. After repetition of chromatography on a Mono Q column, Rev-GFP was refolded by rapid dilution into 10-fold excess of 20 mM Tris-HCl (pH 8.9), subjected to another Mono Q step without urea, and eluted with 350 mM NaCl.

Rev14-GFP and Rev38-GFP were purified by a three-step procedure. Crude cell extracts containing 20 mM Tris-HCl (pH 7.5) and 800 mM ammonium sulfate were applied on a phenyl-Sepharose column. The column was washed with a reverse gradient of ammonium sulfate and proteins were eluted with 20 mM Tris-HCl (pH 8.5). Fractions containing Rev14-GFP or Rev38-GFP were subsequently applied on a Mono Q column as described above. Fractions from the Mono Q column were dialyzed against 20 mM Tris-HCl (pH 8.5) and applied on a cellulose phosphate column. Recombinant Rev14-GFP or Rev38-GFP were eluted with 400 mM NaCl. The purified Rev-GFP proteins showed one major band on a denaturing polyacrylamid gel with an apparent molecular weight close to 43 kDa.

RNA binding assay

In vitro transcription and RNA binding assay were performed as described (Benko et al., 1990). The HIV-1 env gene sequence (nt 7352--7417) encoding the Rev response element was inserted as an *Xho*I–*Hind*III PCR fragment into pBluescriptKS+ (Stratagene), resulting in the plasmid pKSR72S. Plasmid DNA was linearized at the *Hind*III site and transcribed *in vitro* to generate RRE RNA (72 nt). Plasmids pGEMRRE330 and pGEM RRE Δ 12S was used to

generate RRE330 RNA and Δ 12SRRE (Benko et al., 1990). The Δ 12SRRE RNA lacked the high-affinity Rev binding site stem-loop IIa.

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REFERENCES

- Benko, D. M., Robinson, R., Solomin, L., Mellini, M., Felber, B. K., and Pavlakis, G. N. (1990). Binding of trans-dominant mutant Rev protein of human immunodeficiency virus type 1 to the cis-acting Rev-responsive element does not affect the fate of viral mRNA. *New Biol.* **2**(12), 1111–1122.
- Berger, J., Aepinus, C., Dobrovnik, M., Fleckenstein, B., Hauber, J., and Bohnlein, E. (1991). Mutational analysis of functional domains in the HIV-1 Rev trans-regulatory protein. *Virology* **183**(2), 630–635.
- Bevec, D., Jaschke, H., Oft, M., Woehl, T., Himmelspach, M., Pacher, A., Schebesta, M., Koetznitz, K., Dobrovnik, M., Csonga, R., Lottspeich, F., and Hauber, J. (1996). Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF-5A. *Science* **271**, 1858–1860.
- Cochrane, A. W., Perkins, A., and Rosen, C. A. (1990). Identification of sequences important in the nucleolar localization of human immunodeficiencyvirus Rev: Relevance of nucleolar localization to function. *J. Virol.* **64**, 881–885.
- Cullen, B. R. (1991). Human immunodeficiency virus as a prototypic complex retrovirus. *J. Virol.* **65**, 1053–1056.
- D'Agostino, D. M., Ciminale, V., Pavlakis, G. N., and Chieco-Bianchi, L. (1995). Intracellular trafficking of the human immunodeficiency virus type 1 Rev protein: Involvement of continued rRNA synthesis in nuclear retention. *AIDS Res. Hum. Retroviruses* **11**, 1063–1072.
- Felber, B. K. (1997). Regulation of mRNA expression in HIV-1 and other retroviruses. In "mRNA Metabolism and Post-Transcriptional Gene Regulation," pp. 323–340. Wiley-Liss, New York.
- Felber, B. K., Drysdale, C. M., and Pavlakis, G. N. (1990). Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. *J. Virol.* **64**, 3734–3741.
- Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leusin-rich nuclear export signals. *Cell* **90**, 1051–1060.
- Furuta, R. A., Kubota, S., Maki, M., Miyazaki, Y., Hattori, T., and Hatanaka, M. (1995). Use of a human immunodeficiency virus Type 1 Rev mutant without nucleolar dysfunction as a candidate for potential AIDS therapy. *J. Virol.* **69**, 1591–1599.
- Hammerschmid, M., Palmeri, D., Ruhl, M., Jaksche, H., Weichselbraun, I., Bohnlein, E., Malim, M. H., and Hauber, J. (1994). Scanning mutagenesis of the arginine-rich region of the human immunodeficiency virus type 1 Rev trans activator. *J. Virol.* **68**(11), 7329–7335.
- Hope, T. J. (1997). Viral RNA export. *Curr. Biol.* **4**, 335–344.
- Hope, T. J., Klein, N. P., Elder, M. E., and Parslow, T. G. (1992). trans-dominant inhibition of human immunodeficiency virus type 1 Rev occurs through formation of inactive protein complexes. *J. Virol.* **66**(4), 1849–1855.
- Katahira, J., Ishizaki, T., Adachi, A., Yamamoto, K., and Shida, H. (1995). Effects on translation initiation factor eIF-5A on the functioning of human T-cell leukemia virus type I Rex and human immunodeficiency virus Rev inhibited trans dominantly by a Rex mutant deficient in RNA binding. *J. Virol.* **69**, 3125–3133.
- Kubota, S., Furuta, R., Maki, M., and Hatanaka, M. (1992). Inhibition of human immunodeficiency virus type 1 Rev function by a Rev mutant which interferes with nuclear/nucleolar localization of Rev. *J. Virol.* **66**, 2510–2513.
- Kubota, S., Siomi, H., Satoh, T., Endo, S., Maki, M., and Hatanaka, K. (1989). Functional similarity of HIV-I rev and HTLV-I rex proteins: Identification of a new nucleolar-targeting signal in rev protein. *Biochem. Biophys. Res. Commun.* **162**, 963–970.
- Lam, W.-C., Seifert, J. M., Amberger, F., Graf, C., Auer, M., and Millar, D. P. (1998). Structural dynamics of HIV-1 Rev and its complexes with RRE and 5S-RNA. *Biochemistry*. In press.
- Lee, S., Neumann, M., Stauber, R. H., Pause, A., Zhou, S., Linehan, W. M., Pavlakis, G. N., and Klausner, R. (1998). Transcription dependent nuclear/cytoplasmic trafficking of the von Hippel-Lindau tumor suppressor protein linked to the green fluorescent protein. Manuscript in preparation.
- Madore, S. J., Tiley, L. S., Malim, M. H., and Cullen, B. R. (1994). Sequence requirements for Rev multimerization in vivo. *Virology* **202**(1), 186–194.
- Malim, M. H., Böhnlein, S., Hauber, J., and Cullen, B. R. (1989). Functional dissection of the HIV-1 Rev trans-activator derivation of a trans-dominant repressor of Rev function. *Cell* **58**, 205–214.
- Malim, M. H., Freimuth, W. W., Liu, J., Boyle, T. J., Lysterly, H. K., Cullen, B. R., and Nabel, G. J. (1992). Stable expression of transdominant Rev protein in human T cells inhibits human immunodeficiency virus replication. *J. Exp. Med.* **176**, 1197–1201.
- Mermer, B., Felber, B. K., Campbell, M., and Pavlakis, G. N. (1990). Identification of trans-dominant HIV-1 rev protein mutants by direct transfer of bacterially produced proteins into human cells. *Nucleic Acids Res.* **18**, 2037–2044.
- Nabel, G. J., Fox, B. A., Post, L., Thompson, C. B., and Woffendin, C. (1994). A molecular genetic intervention for AIDS effects of a trans-dominant negative form of Rev. *Hum. Gene Ther.* **5**(1), 79–92.
- Olsen, H. S., Cochrane, A. W., Dillon, P. J., Nalin, C. M., and Rosen, C. A. (1990). Interaction of the human immunodeficiency virus type 1 Rev protein with a structured region in env mRNA is dependent on multimer formation mediated through a basic stretch of amino acids. *Genes Dev.* **4**, 1357–1364.
- Palm, G. J., Zdanow, A., Gaitanaris, G. A., Stauber, R. H., Pavlakis, G. N., and Wlodawer, A. (1996). The structural basis for spectral variations in green fluorescent protein. *Nat. Struct. Biol.* **4**, 361–365.
- Parslow, T. C. (1993). Post-transcriptional regulation of human retroviral gene expression. In "Human Retroviruses" (B. R. Cullen, Ed.), pp. 101–136. IRL Press, Durham, NC.
- Pavlakis, G. N. (1997). The molecular biology of Human Immunodeficiency Virus Type 1. In "AIDS: Biology, Diagnosis, Treatment and Prevention" (V. T. DeVita, S. Hellman, and S. A. Rosenberg, Eds.), Vol. 4, pp. 45–74. Lippincott-Raven Publishers, Philadelphia.
- Schwartz, S., Campbell, M., Nasioulas, G., Harrison, J., Felber, B. K., and Pavlakis, G. N. (1992). Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. *J. Virol.* **66**(12), 7176–7182.
- Stauber, R., Gaitanaris, G. A., and Pavlakis, G. N. (1995). Analysis of trafficking of Rev and transdominant Rev proteins in living cells using Green Fluorescent Protein fusions: Transdominant Rev blocks the export of Rev from the nucleus to the cytoplasm. *Virology* **213**, 439–449.
- Stauber, R. H., Horie, K., Carney, P., Hudson, E. A., Tarasova, N. I., Gaitanaris, G. A., and Pavlakis, G. N. (1998). Development and applications of enhanced green fluorescent protein mutants. *BioTechniques* **24**, 462–471.
- Szilvay, A. M., Brokstad, K. A., Boe, S.-O., Haukenes, G., and Kalland, K. H. (1997). Oligomerization of HIV-1 Rev mutants in the cytoplasm and during nuclear import. *Virology* **235**, 73–81.

- Szilvay, A. M., Brokstad, K. A., Kopperud, R., Haukenes, G., and Kalland, K. H. (1995). Nuclear export of the human immunodeficiency virus type 1 nucleocytoplasmic shuttle protein Rev is mediated by its activation domain and is blocked by transdominant negative mutants. *J. Virol.* **69**(6), 3315–3323.
- Thomas, S. L., Hauber, J., and Casari, G. (1997). Probing the structure of the HIV-1 Rev trans-activator protein by functional analysis. *Protein Eng.* **10**, 103–107.
- Thomas, S. L., Oft, M., Jaksche, H., Casari, G., Heger, P., Dobrovnik, M., Bevec, D., and Hauber, J. (1998). Functional analysis of the human immunodeficiency virus type 1 Rev protein oligomerization interface. *J. Virol.* **72**, 2935–2944.
- Zapp, M. L., Hope, T. J., Parslow, T. G., and Green, M. R. (1991). Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: A dual function for an arginine-rich binding motif. *Proc. Natl. Acad. Sci USA*