In Vivo HIV-1 Rev Multimerization in the Nucleolus and Cytoplasm Identified by Fluorescence Resonance Energy Transfer*

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Nuclear export of intron-containing human immunodeficiency virus type 1 RNA is mediated by the viral Rev protein. Rev is a nucleocytoplasmic transport protein that directly binds to its cis-acting Rev-responsive element RNA. Rev function depends on its ability to multimerize. The in vivo dynamics and the subcellular dependence of this process are still largely unexplored. To visualize and quantitatively analyze the mechanism of Rev multimeric assembly in live cells, we used high resolution in vivo fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching. By using two different dynamic FRET approaches (acceptor photobleaching and donor bleaching time measurements), we observed a strong Rev-Rev interaction in the nucleoli of living cells. Most interestingly, we could also detect Rev multimerization in the cytoplasm; however, FRET efficiency in the cytoplasm was significantly lower than in the nucleolus. By using fluorescence recovery after photobleaching, we investigated the mobility of Rev within the nucleolus. Mathematical modeling of the fluorescence recovery after photobleaching recoveries enabled us to extract relative association and dissociation constants and the diffusion coefficient of Rev in the nucleolus. Our results show that Rev multimerizes in the nucleolus of living cells, suggesting an important role of the nucleolus in nucleocytoplasmic transport.

The replication of the human immunodeficiency virus, type 1 (HIV-1), is regulated in a temporal manner by its viral mRNA expression. Control of HIV RNA expression is complex and involves the interplay of cis-acting viral transactivators and several cellular proteins. Rev is a viral trans-activator protein that controls the differential expression of viral proteins at the post-transcriptional level by allowing the accumulation in the cytoplasm of unspliced and singly spliced viral RNA containing the Rev-responsive element (RRE) (1–7). Rev interacts directly with a purine-rich stem-loop within RRE (6, 8), and through multimerization additional Rev molecules bind throughout the RRE. In the absence of Rev, the only viral RNA species that accumulate in the cytoplasm are multiply spliced and encode for the viral regulatory proteins. In the presence of Rev, the incompletely spliced mRNAs accumulate in the cytoplasm to serve as templates for viral structural protein synthesis or as viral genomes. Rev activity is therefore essential for virus replication.

Rev is a small protein of 116 amino acids or 18 kDa. Its cellular localization is nucleolar, but it has been shown to shuttle continuously between the nucleus and the cytoplasm (1, 9, 10). Nuclear localization is mediated by a short stretch of basic amino acids characterized by eight arginine residues located near the amino terminus of Rev that serves both as nuclear localization signal (NLS) and as an RNA binding domain (5, 11, 12). It is flanked on both sides by sequences that contribute to Rev oligomerization on the RRE but have no detectable role in RRE binding. The NLS of Rev has been reported to associate with importin β, as well as with B23, a protein involved in the nuclear import of ribosomal proteins (13, 14).

Nuclear export of Rev is mediated by its nuclear export signal, which is a leucine-rich domain between Leu-75 and Leu-84 located near the carboxyl terminus of the protein (15). Disruption of this leucine-rich domain yields proteins (e.g. RevM10) with a dominant negative phenotype that localize to the nucleolus, bind RRE, and yet lose their ability to exit from the nucleus (5, 9, 10, 16–20). The export receptor for the leucine-rich nuclear export signal of Rev has been identified as CRM1 (21–23).

In vitro studies have shown oligomerization of Rev (5, 16, 24, 25); however, the actual in vivo subcellular localization and kinetic details of the Rev multimer complex formation are still largely unknown. The dynamic properties of Rev within the cell have a crucial role in its function. Moreover, the subcellular localization of these interactions is not explored. A detailed understanding of Rev multimerization requires the knowledge of timing and location of the different interactions in live cells. Therefore, we examined the molecular details of Rev multimerization and its kinetics inside living cells by fluorescence energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) measurements. The principle of FRET is based on the ability of a higher energy fluorophore molecule (donor) to transfer energy directly to a lower energy molecule (acceptor) when in close proximity. The presence of FRET indicates actual protein-protein interaction. The extent of FRET between a given donor and acceptor fluorescent molecule is dictated by the spectral parameter $R_0$, i.e. the distance at which...
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the FRET efficiency is 50%. In most cases, no FRET can be observed at distances greater than 10 nm. Therefore, FRET would occur with detectable efficiency only for those cases in which intimate interactions between the proteins were present. The mobility of a fluorescent protein can be assessed by using FRAP (26–29). In this technique, fluorescent molecules in a small region of the cell are irreversibly photobleached by using a high powered laser pulse; subsequent movement of surrounding nonbleached fluorescent molecules into the photobleached area is recorded by time-lapse microscopy. Mathematical modeling of the FRAP data enables us to extract information such as relative association and dissociation constants and the apparent diffusion coefficient (30, 31). FRET and FRAP are minimally invasive and enable us to study the actual dynamics and interactions of Rev inside living cells.

By using FRET, we demonstrate here that HIV Rev physically multimerizes inside living cells, and we visualize these interactions in specific subcellular compartments. We show that Rev multimerizes in the cytoplasm but forms a different complex than in the nucleus. By using FRAP and mathematical computation, we measured the diffusion coefficient of Rev in the nucleus and cytoplasm. Through the use of a Rev mutant that does not interact with the nucleolus and through mathematical modeling we extracted the apparent dissociation constant of Rev multimerization in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Plasmids—HeLa cells, human epithelial cells, were plated onto glass bottom microwell dishes (MatTek Corp., Ashland, MA) at 0.1 × 10^6 cells/plate and were cultured overnight in Dulbecco’s modified Eagle’s medium (Mediatec Inc., Herndon, VA) supplemented with 10% (v/v) fetal calf serum until 50% confluent. Cells were washed with phosphate-buffered saline and transfected using SuperFect® (Qiagen, Valencia, CA) transfection reagent according to the manufacturer’s manual and incubated overnight. Before imaging cells were washed with phosphate-buffered saline and complete medium without phenol red was added.

pBRev-BFP expresses the Rev protein fused to the blue fluorescent protein (BFP) emitting blue light (32). pRev-GFP and pTat-GFP plasmids produce fusion proteins of wild-type HIV-1 Rev and Tat, respectively, amino-terminally fused to the enhanced version of the green fluorescent protein (GFP) emitting green light (32).

Live Cell Imaging—Cells were grown in glass bottom microwell dishes; nucleoli manipulation was needed for imaging with an inverted microscope. All images were acquired with a laser-scanning confocal microscope (LSM 510; Carl Zeiss Inc., Thornwood, NY) equipped with an Axiovert 200 microscope (Zeiss) and a 40× 1.3 NA oil immersion Plan-Neofluar objective. Live cells were imaged in Dulbecco’s modified Eagle’s medium without phenol red, supplemented with 0.1% (v/v) fetal calf serum. For colocalization of BFP and GFP fusion proteins, BFP was excited with a multiphoton (Verdi/Mira 900, Coherent Inc, Auburn, CA) laser line at 780 nm and GFP with an argon laser line at 488 nm, and detection between 500 and 550 nm. The microwell dishes were separated by less than 10 nm. Consequently, one demonstra-

dation of FRET is an increase in donor fluorescence after photo-

bleaching the acceptor because the donor can no longer decay

Fluorescence Recovery after Photobleaching—Excitation of GFP was done at 488 nm and detection between 500 and 550 nm. The microwell dishes with overslip bottom were directly mounted onto an LSM 510 microscope. Live cells were imaged at 37°C (heating chamber, 2020 Technology Inc., Wilmingtom, NC) in Dulbecco’s modified Eagle’s medium without phenol red and supplemented with 10% (v/v) fetal calf serum. FRAP experiments were performed as follows: a 2-μm wide strip throughout the cell was photobleached; bleaching was completed in 200–600 ms, and recovery images were acquired every 0.4–1 s.

All images were background-subtracted first. The recovery curve was obtained by computing the ratio of the integrated intensity over the bleached region divided by the integrated intensity over the full nucleus. Taking this ratio permits an accurate correction for photobleaching of the specimen over time as well as a correct immobile fraction computation (see also Lippincott-Schwartz computation of immobile fraction) (26). The recovery curve was then normalized so that the initial time point is 1.

Recovery curves were fitted using the three compartments kinetic reaction model of Carrero et al. (30) by using a double exponential fit. In this method, the organelle of interest is bleached all across its middle, separating it after bleaching into two unbleached compartments of approximately the same size and a thin bleached band. All experiments were done in the same manner, with a bleached area of the same width. No obvious immobile fluorophores were observed in the data, so the fit did not include an immobile fraction parameter. The second decay rates from the fit are in a relative scale (βd, in % s⁻¹, and βa, in % s⁻¹) and can therefore be compared. The diffusion coefficient was approximated by computing D from Carrero et al. (30) and multiplying it by (l − w) × V (where l is length of nucleus, and w is the width of bleach in μm in the vertical direction of the digitized image). This correction permitted determination of an absolute value for the diffusion coefficient, and all bleachings were done the same way, with the same number of bleaching iterations and width (i.e., 2 μm). However, excitation intensity levels changed from cell to cell.

RESULTS

Subcellular Localization and Colocalization of Rev in Living Cells—It has been shown biochemically that Rev multimerizes; however this multimerization has not been shown directly in living cells. To demonstrate interaction in mammalian cells, we studied the ability of wild-type Rev to relocalseize a cytoplasmic Rev mutant (RevM5) to the nucleoli by in vivo microscopy. Therefore, we produced fusion constructs of the two proteins with either the blue fluorescent protein (BFP) or the green fluorescent protein (GFP). This fluorescent pair has excitation and emission spectra that can be easily separated for detection with fluorescence microscopy. Rev-BFP localizes in the nucleoli (Fig. 1, top right panel). RevM5-GFP was found predominantly in the cytoplasm (Fig. 1, left bottom panel), in agreement with published data (5). RevM5 has two point mutations (R38D and R39L) in its RNA binding-site and is therefore unable to bind RRE RNA (5, 16). When Rev-BFP and RevM5-GFP were coexpressed, a significant fraction of RevM5-GFP was found in the Rev-BFP-containing nucleoli (Fig. 1, right bottom panel). Such colocalization of RevM5 with Rev in the nucleoli of living cells suggests interaction between the two proteins.

Visualization of Rev Multimerization in the Nucleoli of Living Cells by FRET—To demonstrate a direct molecular interaction between Rev molecules in living cells, we monitored FRET between BFP-tagged Rev and GFP-tagged Rev. The principle of FRET is based on the ability of a higher energy fluorophore molecule (donor) to transfer energy directly to a lower energy molecule (acceptor) with simultaneous quenching of the donor fluorescence when the donor and acceptor are separated by less than 10 nm. Consequently, one demonstration of FRET is an increase in donor fluorescence after photo-

bleaching the acceptor because the donor can no longer decay

(Mathworks, Inc., Natick, MA) and DIPimage (image processing toolbox for Matlab, Delft University of Technology, Delft, The Netherlands). The average FRET efficiency and its standard deviation were calculated from the FRET efficiencies of each individual cell. The standard Student’s t test was used to determine the statistical significance of the results.

Equation 1

\[ E = 1 - D_1/D_0 \]

where D is the mean intensity of the donor in the area where the acceptor was bleached, before (D0) and after (D1) acceptor bleaching.

The image and statistical analyses were performed with Matlab (Mathworks, Inc., Natick, MA) and DIPimage (image processing toolbox for Matlab, Delft University of Technology, Delft, The Netherlands). The average FRET efficiency and its standard deviation were calculated from the FRET efficiencies of each individual cell. The standard Student’s t test was used to determine the statistical significance of the results.

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(33, 38),
by nonradiative transfer of energy to the acceptor. The range over which FRET between a donor (BFP) and an acceptor (GFP) occurs, varies in a sensitive manner and is dictated by the spectral parameter $R_0$, i.e. the distance at which the FRET efficiency is 50%. $R_0$ for the BFP-GFP pair is 49–52 Å (40, 41). Due to the sixth power dependence on distance, the FRET efficiency with this donor-acceptor pair is <10% beyond 72 Å. In most cases, no FRET can be observed at distances greater than 100 Å. Therefore, FRET between Rev-BFP and Rev-GFP occurs only if true molecular interactions between the respective proteins were present.

A first set of FRET experiments was performed in live HeLa cells. We created fusion constructs of Rev with either the BFP or the GFP. The functionality of the Rev fusion constructs has been shown earlier (32). HeLa cells were transfected with these constructs, and corresponding FRET efficiencies were determined by the acceptor photobleaching technique (33–35, 37). In Fig. 2, the first acquired image ($D_A$) is the fluorescence intensity distribution of the donor Rev-BFP directly excited by multiphoton laser at 780 nm. The second image ($A_D$) represents the fluorescence intensity distribution of the acceptor Rev-GFP excited at 488 nm. The acceptor fluorophore was subsequently photodestroyed in part of the field by repeated scanning with the 488 nm laser line, thereby eliminating energy transfer. Then a second donor fluorescence image ($D_D$) was taken. An increase in donor fluorescence intensity is expected in the region of acceptor photobleaching only in the case of FRET. This is apparent in the nucleolus of the cells co-expressing Rev-BFP and Rev-GFP, demonstrating oligomerization of Rev proteins in the nucleolus of living cells (Fig 2A). As negative control for the FRET experiments, cells expressing Rev-BFP and Tat-GFP were used (Fig. 2B). As expected, no FRET could be observed with these proteins because Tat and Rev do not interact despite their presence in the same location. These data demonstrate for the first time true molecular oligomerization of Rev in the nucleolus of intact cells. To determine the extent of FRET, the FRET efficiency was calculated at each pixel. For statistical analysis, the average FRET efficiency from $n$ cells analyzed was plotted as a box plot distribution showing the lower quartile, median, and the upper quartile (Fig. 2C). Cells transfected with wild-type Rev-BFP and Rev-GFP show an average FRET efficiency of 0.25 ± 0.05 that is significant higher ($p < 0.01$, Student’s $t$ test) than the control Tat-BFP with Rev-GFP (0.02 ± 0.03). We also measured the FRET efficiencies of the same pairs in fixed cells; there was no significant difference in FRET efficiency between fixed or live cells.

A second independent assessment of FRET between Rev-BFP and Rev-GFP in cells was made from donor bleaching kinetics (Fig. 3). In FRET, direct transfer of donor energy to the acceptor provides an additional decay mechanism for the donor, thus reducing the probability of donor decay via photobleaching and resulting in a consequential increase in the photostability of the donor. Accordingly, donor bleaching time will increase. For this intention, BFP is a good fluorophore because it bleaches easily, and an increase in bleaching time can be clearly observed. The donor bleaching time was monitored by repetitive scanning with the two-photon 780 nm laser line. Repetitive excitation of BFP results in bleaching of the fluorophore. The kinetics of BFP bleaching in cells coexpressing Rev-BFP and Rev-GFP were compared with the bleaching of BFP in cells expressing Rev-BFP and Tat-GFP, a pair that does not result in FRET (Fig. 3A). Rev-BFP bleaches slower (i.e. more photostable) when it is associated with Rev-GFP, demonstrating FRET between Rev-BFP and Rev-GFP. This is also apparent from the following experiment. GFP was photobleached in part of the nucleolus of cells expressing both Rev-BFP and Rev-GFP or Rev-BFP and Tat-GFP, thus destroying potential FRET. We compared the rate of donor (BFP) bleaching in the region of the nucleolus where GFP was photodestructured with the rate of donor bleaching in the GFP-bleached region of the same nucleolus. To do this, the ratio of the donor fluorescence in the GFP-bleached region versus the GFP-bleached region was plotted over time (ratios were normalized to 1 for the initial time point). If FRET existed in the nucleolus, an increase in the ratio would be observed over time, reflecting the difference in donor photostability between the two regions (Fig. 3B). In the Rev-BFP- and Rev-GFP-containing nucleoli, the ratio increases over time, whereas the ratio stays 1 in the Rev-BFP- and Tat-GFP-containing nucleoli. These FRET experiments support the conclusion that Rev multimerizes in the nucleolus of cells.

Rev Multimerizes in the Cytoplasm of Living Cells—The function of Rev is to transport single spliced and unspliced HIV RNA from the nucleus to the cytoplasm. As reported above and in earlier studies (32, 42, 43), Rev is localized in the nucleolus of cells; however, Rev mutants of the NLS localize mainly in the cytoplasm. These observations prompted us to determine whether Rev is able to oligomerize in the cytoplasm of cells. Therefore, we measured FRET between Rev38-BFP and Rev38-GFP. Rev38 has a mutation in its nuclear localization signal preventing Rev from exporting RNA from the nucleus to the cytoplasm. As reported above and in earlier studies (32, 42, 43), Rev is localized in the nucleolus of cells; however, Rev mutants of the NLS localize mainly in the cytoplasm. These observations prompted us to determine whether Rev is able to oligomerize in the cytoplasm of cells. Therefore, we measured FRET between Rev38-BFP and Rev38-GFP. Rev38 has a mutation in its nuclear localization signal and is mainly found in the cytoplasm (32). FRET results for cells transfected with Rev38-BFP and Rev38-GFP are shown in Fig. 4. The first acquired image ($D_A$) is the fluorescence intensity distribution of the donor Rev38-BFP excited by a two-photon laser at 780 nm. The second image ($A_D$) represents the fluorescence intensity distribution of the acceptor Rev38-GFP excited directly at 488 nm. The acceptor fluorophore (GFP) was subsequently photobleached in part of the field by repeated scanning with the 488-nm laser line, thereby eliminating energy transfer. Then a second donor fluorescence image ($D_D$) was taken. FRET is apparent in the cytoplasm of the cells, as seen by an increase in donor fluorescence intensity in the region of acceptor photobleaching, demonstrating a Rev complex in the cytoplasm (Fig. 4A, $D_1$). As a control for the FRET experiments, cells expressing Rev38-BFP and free GFP were used (Fig. 4B).
As expected, no FRET could be observed with these proteins, as they do not interact despite their presence in the same location. The FRET efficiency from \( n \) cells analyzed was plotted as a box plot distribution showing the lower quartile, median, and the upper quartile (Fig. 4C). These results demonstrate a direct molecular multimerization of Rev in the cytoplasm of intact cells.

Analysis of in Vivo Rev-GFP Interaction Kinetics and Mobility by FRAP—The nucleolus has been suggested as an integral part required for Rev function (44). Therefore, it is important to understand the mobility properties of Rev in the nucleolus. To analyze further the in vivo characteristics of Rev in the nucleolus, we studied its mobility by using FRAP-based experiments. In these experiments, the GFP in a small area of a cell is rapidly photobleached by using a high intensity laser pulse. The movement of unbleached molecules from the neighboring areas into the photobleached region is then recorded by time-lapse microscopy as the recovery of fluorescence in the photobleached area (26, 27, 29, 31). Intracellular mobility is influenced by specific and nonspecific interactions, catalytic activity, and diffusion. Therefore, the FRAP results reflect the sum of these three activities. Rev-GFP was photobleached in a small 2-μm strip extending through the nucleolus of cells (Fig. 5A). As a reference, we measured the fluorescence recovery rate of free GFP that diffuses freely within the nucleoplasm. The recovery curves were fitted by a single exponential, and the respective half-lives are given in Table I. The calculated \( t_{1/2} \) for

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**Fig. 2. Oligomerization of Rev in the nucleolus of living cells as measured by FRET.** A, cells expressing Rev-BFP and Rev-GFP were analyzed for FRET by confocal microscopy using the photobleach method. This method is based on the principle that energy transfer is eliminated when the acceptor is bleached, thereby yielding an increase in donor fluorescence. Intensity images of Rev-GFP (A₀ and A₁) and Rev-BFP (D₀ and D₁) in the nucleoli of a cell are shown. D₀ indicates the fluorescence intensity distribution of Rev-BFP directly excited at 780 nm with a 2-photon laser. A₀ indicates the fluorescence intensity distribution of Rev-GFP excited at 488 nm. Rev-GFP was subsequently photobleached in a specific segment of the nucleolus (white dotted box), thereby eliminating energy transfer. Then a second donor fluorescence image (D₁) was taken. A₁ indicates the fluorescence intensity distribution of Rev-GFP after photobleaching. D₁ indicates the fluorescence intensity distribution of Rev-BFP after photobleaching of the acceptor (Rev-GFP). B, as negative control the intensity maps of Rev-BFP (D₀ and D₁) and Tat-GFP (A₀ and A₁) in the nucleoli of a cell were also determined by the same method. C, quantification of FRET efficiencies. The indicated number of cells (n) were analyzed in three different experiments, and the calculated FRET efficiency is given as box plot distribution. The box has lines at the lower quartile, median, and upper quartile values. Bars at the end of the boxes indicate 1.5 times the inter-quartile range.
with the nucleolus, (ii) Rev-Rev interactions, and (iii) Rev diffusion within the nucleolus. To discriminate between these three interactions, we performed FRAP experiments with a Rev mutant (RevM5-GFP) that was not able to perform one of these three actions, namely the binding to the nucleolus. In contrast to wild-type Rev, RevM5 does not localize in the nucleoli, as shown in Fig. 1. However, RevM5-GFP concentrates in the nucleolus only if wild-type Rev is present, suggesting an interaction between the two proteins. Therefore, the recovery kinetics of FRAP measurements from RevM5-GFP in the nucleolus reflects the sum of (i) RevM5-Rev interactions and (ii) RevM5 diffusion within the nucleolus. The half-life for RevM5-GFP in the Rev-BFP-containing nucleolus is 61.7 ± 28.9 s, three times faster than Rev-GFP (Fig. 5D). For comparison, a similar area extending through the cytoplasm of cells expressing only RevM5-GFP was photobleached (Fig. 5C). The RevM5-GFP in the cytoplasm recovered rapidly and uniformly with a half-life of 1.67 ± 0.44 s. The difference in recovery kinetics between Rev-GFP and RevM5-GFP reflects the interaction of Rev with the nucleolus.

Analyzing the mobility of the proteins by computing their half-life and effective diffusion coefficient offers little information in terms of interaction reactions of the proteins. Mathematical modeling of the FRAP data enabled us to extract the diffusion constant and the relative association and dissociation constants (Table I). The FRAP recoveries from nucleolar Rev-GFP and RevM5-GFP and from cytoplasmic RevM5-GFP were much better fitted with the double exponential than the single exponential, strongly suggesting a contribution of interaction in the recovery kinetics. There was no difference in the double exponential or single exponential fits for free-GFP FRAP recoveries. The FRAP recovery curves were fitted by using the three compartments kinetic reaction model of Carrero et al. (30) by using a double exponential fit, and \( k_a \), \( k_d \), and the diffusion coefficient \( D \) were extracted (Table I). The association and decay rates from the fit are in relative scale (\( k_a \) in \( \%s^{-1} \) and \( k_d \) in \( \%/s^{-1} \)) and can therefore be compared between one and the other. The diffusion coefficient was obtained by computing \( D \) from Carrero et al. (30) and multiplying it by \((l - w) \times 1/4 \) (where \( l \) is length of nucleus, and \( w \) is the width of bleach in \( \mu m \) in the vertical direction of the digitized image). This correction permitted us to have an absolute value for the diffusion coefficient. The computation of the diffusion coefficient was very reliable because changing the width of the bleach rectangle resulted in the same diffusion coefficients for the same sample (data not shown). However, \( K \) depends on the size of the bleach and is therefore a relative value only valid to compare the binding strength of the different proteins analyzed with the same experimental setup. Our data demonstrate that in the nucleolus Rev-GFP (\( D = 0.2036 \pm 0.0695 \mu m^2/s \)) and RevM5-GFP (\( D = 0.3410 \pm 0.1901 \mu m^2/s \)) diffuse very slowly within the nucleolus. As reference, the reported diffusion coefficients of free GFP are also given (45, 46). The relative amount of nuclear unbound protein versus bound is similar for both Rev-GFP and RevM5-GFP as given by \( K \) (1.0–1.5 \( \% \)). This is also apparent from their steady state localizations as both Rev-GFP and RevM5-GFP (when in the presence of Rev-BFP) are concentrated in the nucleolus to the same extent (Fig. 1, right upper and lower panel). In the cytoplasm, RevM5-GFP moves five times slower than free GFP. One hypothesis is that RevM5-GFP is a bigger protein complex than free GFP, confirming the ability of RevM5-GFP to multimerize in the cytoplasm or that RevM5-GFP is obstructed in its movement within the cytoplasm. In addition the \( k_d \) for RevM5-GFP in the cytoplasm is about 10 times lower than for free GFP, suggesting interaction of RevM5-GFP in the cytoplasm.

Recovery of Rev-GFP in the nucleolus (197 ± 61 s) was very high compared with that of free GFP (0.11 ± 0.06 s), suggesting that Rev-GFP is associated with high affinity binding sites within the nucleolus. Here we anticipate that the recovery of Rev-GFP in the nucleolus reflects a mix of (i) Rev interactions...
DISCUSSION

The multimerization of HIV-1 Rev is a key event for the nucleocytoplasmic transport of incompletely spliced and unspliced viral mRNAs encoding the viral structural proteins. Although the three-dimensional structure of Rev has not yet been determined, sequence analysis and data from circular dichroism, nuclear magnetic resonance, and Raman spectroscopy studies strongly suggest that the multimerization region of Rev are two separate α-helices. These two helices contact each other over a limited region and may actually form a single interaction area in the functional protein structure. Random mutational analysis of Rev revealed that the residues that participate in multimerization reside in the amino terminus of the protein, in regions flanking the basic domain responsible for RNA binding and nuclear localization (16, 39). Recently, the amino acids important for these intermolecular interactions in vitro have been identified (24, 25, 47, 48). Although some information is available on the in vitro interaction, little is known about its spatial and temporal behavior in vivo. To explore multimerization of Rev directly inside living cells, we exploited modern microscopy techniques (FRET and FRAP) by using BFP and GFP fusion proteins. This fluorescent pair has excitation and emission properties favorable for FRET and has already been used successfully in cells (49–54).

The most widely used approach for monitoring FRET is by measuring the acceptor emission upon exciting the donor. Analyzing FRET reliably by this method is often difficult because of complications caused by bleed through of donor fluorescence in the acceptor channel as well as unintended excitation of acceptor during donor excitation. However, these effects can be moderated by different algebraic correction methods (55, 56). We used an alternative and more straightforward approach to detect FRET between Rev molecules in living cells by photobleaching the acceptor (33–37). The advantage of acceptor photobleaching is that correction issues are alleviated, and increases in donor fluorescence cannot be related to acceptor

![Figure 4: Multimerization of Rev38 in the cytoplasm of living cells as measured by FRET.](image-url)
FIG. 5. **Fluorescence recovery after photobleaching of Rev-GFP and RevM5-GFP in living cells compared with free-GFP.** HeLa cells expressing Rev-GFP (A), free-GFP (B), or RevM5-GFP (C) were imaged by confocal fluorescence microscopy. A 2-μm-thick line was bleached across the width of the cell nucleus, and images were gathered during the course of recovery. D, quantitative analysis of FRAP demonstrates that the FRAP rate of Rev-GFP is slower than RevM5-GFP in the presence of Rev-BFP.
bleed through because the acceptor fluorescence was destroyed. However, when using this method for measuring FRET in live cells, the bleaching speed is critical because during bleaching the proteins of interest may have exchanged with the unbleached area. Therefore, this real time FRET method is not suitable for free diffusible proteins in living cells. FRAP experiments showed that the mobility of Rev in the nucleolus was very slow (Fig. 5A); therefore, we measured FRET between Rev-BFP and Rev-GFP by using the photobleach method in live cells. These fusion proteins have been shown earlier to be fully functionally active (32). There was no difference in FRET efficiency between live cells or fixed cells. Measuring FRET in live cells resulted in the detection of true molecular interactions existing in vivo in specific cellular locations with great resolution. In similar conditions, the FRET efficiency between Rev-BFP and Rev-GFP was significantly higher than the negative control Tat and Rev, clearly demonstrating a multimerization of Rev in the nucleoli of living cells.

We demonstrate oligomerization of Rev in the cytoplasm using Rev38, a mutant of the NLS (32). In this mutant the amino acids 38–44 comprising the NLS are deleted therefore preventing binding to RNA (32). We used this mutant to study the multimerization of Rev in the cytoplasm, because wild-type Rev is located predominantly in the nucleolus and only very little Rev-BFP or Rev-GFP can be found in the cytoplasm. It has been proposed earlier that dimerization of Rev is required for nuclear import (57). These observations are in favor of a model by which Rev moves back and forth between the nucleus and cytoplasm as a multimeric complex. Future studies will focus on the mechanistic details and function of the cytoplasmic Rev in more detail.

The measurement of the efficiency of FRET in different subcellular compartments also offers an interesting tool to understand the interface of protein-protein interaction inside the cell. A lower FRET efficiency in the cytoplasm of Rev-Rev suggests a different complex. Furthermore, we measured oligomerization occurring in cells that do not express the RRE RNA. Therefore, the results also demonstrate that RRE is not required to form Rev complexes in the nucleolus and cytoplasm.

The mobility of Rev in the different cellular compartments is not presently known. Therefore, we studied the mobility of Rev-GFP in the nucleolus by FRAP. We demonstrate that the mobility of Rev-GFP is very slow within the nucleolus. These data imply that Rev-GFP is attached to affinity binding sites within the nucleolus. The nucleolus is the site of ribosomal RNA transcription, and the fact that Rev concentrates in the nucleolus suggests that the nucleolus may be essential for Rev function. Rev is required for export of viral mRNA to the cytoplasm (1, 5), a process essential for virus replication (5, 58). It has been suggested previously that HIV-1 RNA is trafficking through the nucleoli of cells, giving the nucleolus a critical role in HIV-1 RNA export (59). Therefore, it is important to study the mobility and affinity kinetics of Rev within the nucleolus. Our data demonstrate that in the nucleolus Rev-GFP diffuses very slowly. This is not surprising as the nucleolus is an aggregate of macromolecules including the rRNA genes, precursor rRNAs, mature rRNAs, rRNA-processing enzymes, small nuclear ribonucleoproteins, ribosomal protein subunits, and partly assembled ribosomes forming a large granular structure. The measured diffusion coefficient of Rev in the nucleolus is comparable with the reported nucleolar proteins (31, 60). In the cytoplasm, however, Rev-M5-GFP is moving at rates much faster than in the nucleolus but slower than free GFP, suggesting the existence of a multimeric complex in the cytoplasm as confirmed by FRET or interaction with cellular structures. In addition, our k_d measurements suggest that in the cytoplasm Rev-M5-GFP is bound to a cellular structure. It would be interesting to study the functionality of this phenomenon in more detail.

Although several in vitro studies have suggested that Rev multimerization is facilitated by interaction with RRE (16, 39, 61–63), it has remained unclear whether multimerization occurs prior to or subsequent to RRE binding. Comparison of the dissociation constant of Rev-GFP and Rev-M5-GFP in the nucleolus when bound to Rev-BFP revealed that the interaction of Rev-GFP with the nucleolus is stronger than its multimerization. However, although this is not a direct confirmation, the data are indeed consistent with the hypothesis that RNA helps multimer formation in vivo. Study of the in vivo interaction of Rev with RRE and with the cellular transport machinery will permit us to address this issue.

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