Co-expression of a trans-dominant negative mutant of the human immunodeficiency virus type 1 (HIV-1) Rev protein affects the Rev-dependent splicing pattern and expression of HIV-1 RNAs

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Trans-dominant negative mutants of the human immunodeficiency virus type 1 (HIV-1) regulatory protein Rev inhibit the function of wild-type Rev in a dose-dependent manner. This was previously shown to be caused by nuclear retention of the wild-type protein. In the present work, further analysis of the trans-dominant negative effect was performed using cotransfection experiments with different constructs encoding HIV-1 Rev and viral structural proteins together with a plasmid encoding a trans-dominant negative Rev mutant. Thus, one species of pre-mRNA was transcribed from the reporter plasmids. This pre-mRNA was then either spliced or exported by Rev as unspliced RNA for translation of the HIV structural proteins. An immunofluorescence assay and Western blot analysis were used for analysis of protein expression. In situ hybridization was applied for labelling of unspliced mRNA in transfected cells, and RNase protection analysis was used to determine the relative amount of unspliced versus spliced mRNAs. The experiments confirmed that the trans-dominant negative mutant inhibited nuclear export of unspliced mRNA. It was, in addition, demonstrated for the first time that the trans-dominant negative mutant also affected a Rev-dependent regulatory step connected with viral pre-mRNA splicing. As a consequence, proteins expressed from unspliced and singly spliced HIV mRNAs decreased while there was an increase in protein products encoded by spliced and alternatively spliced mRNAs.

Introduction

The cytoplasmic expression of unspliced and incompletely spliced human immunodeficiency virus type 1 (HIV-1) mRNAs containing the information for the structural HIV components is dependent upon Rev at one or several steps between transcription and translation (Sodroski et al., 1986; Haseltine, 1991; Cullen, 1992; Green, 1993). The Rev-dependent mRNAs are characterized by two types of cis-acting sequences, a single Rev-responsive element (RRE) (Dayton et al., 1988, 1989; Malim et al., 1989a, 1990) and several cis-acting repressive sequences (CRS) (Rosen et al., 1988; Emerman et al., 1989; Cochrane et al., 1991; Maldarelli et al., 1991; Schwartz et al., 1992). These sequences are removed in the completely spliced HIV mRNAs that encode the regulatory proteins Tat, Rev and Nef. The spliced HIV mRNAs, therefore, do not require Rev for cytoplasmic appearance and translation. It is generally agreed that Rev is necessary for nuclear export of unspliced and singly spliced HIV mRNAs (Emerman et al., 1989; Felber et al., 1989; Hammarskjöld et al., 1989; Malim et al., 1989a; Kalland et al., 1991; Malim & Cullen, 1993; Fischer et al., 1994; Bray et al., 1994), but it is still not known whether Rev acts at other levels of post-transcriptional regulation. A number of studies have indicated that Rev also regulates the efficiency of mRNA splicing (Chang & Sharp, 1989; Lu et al., 1990; Kjems et al., 1991a; Kjems & Sharp, 1993; Stutz & Rosbash, 1994; Hammarskjöld et al., 1994; Favaro & Arrigo, 1997). This has, however, been opposed by other authors (Malim & Cullen, 1993; Fischer et al., 1994). There are reports demonstrating a Rev-dependent increase of viral mRNA stability (Felber et al., 1989; Malim & Cullen, 1993) and Rev has also been shown to promote mRNA polysomal loading in the cytoplasm (Arrigo & Chen, 1991; D’Agostino et al., 1992).
Site-directed mutagenesis of the 116 amino acid Rev protein has defined two functional domains in addition to shorter amino acid sequences and single amino acid residues which seem to be necessary for function. Mutations in a basic domain (amino acids 35–50) compromise nuclear and nucleolar accumulation and specific binding of Rev to the RRE RNA in vitro (Malim et al., 1989a; Cochrane et al., 1990; Hope et al., 1990; Olsen et al., 1990; Kjems et al., 1991a, b; Böhnlein et al., 1991; Malim & Cullen, 1991; Zapp et al., 1991). Oligomer formation of Rev molecules has been demonstrated both in vitro (Olsen et al., 1990; Malim & Cullen, 1991; Zapp et al., 1991) and in vivo (Hope et al., 1992; Bogerd & Greene, 1993; Madore et al., 1994; Szilvay et al., 1997). The ability to form oligomers appears to be critical for Rev function and several amino acid residues in the N-terminal region of Rev have been demonstrated to be essential for oligomerization (Olsen et al., 1990; Malim & Cullen, 1991; Zapp et al., 1991; Hope et al., 1992; Szilvay et al., 1997; Stauber et al., 1998).

Rev was originally defined as a nuclear and nucleolar protein (Cullen et al., 1988). Subsequent studies revealed that the subcellular localization is more dynamic (Kalland et al., 1994b) implying that Rev is a nucleocytoplasmic shuttle protein (Kalland et al., 1994a; Meyer & Malim, 1994; Richard et al., 1994). The leucine-rich activation domain of HIV-1 Rev was the first discovered example of an amino acid motif that signals active nuclear export (Meyer & Malim, 1994; Szilvay et al., 1994). The leucine-rich activation domain of HIV-1 Rev has been described (Kalland et al., 1994b) implying that Rev is a nucleocytoplasmic shuttle protein (Kalland et al., 1994a; Meyer & Malim, 1994; Richard et al., 1994). The plasmid pGL2 has the HXB2-sequence from 804–1711 cloned into the HindIII site of pGEM-7f (+) (Promega). The reporter plasmid pDM128 provided by T. Hope contains the CAT gene flanked by HIV intron sequences and the 5’ and 3’ splice sites from the env gene. Most of the sequence for env exon 2 is deleted (Hope et al., 1990). The plasmid used for generation of the probe protecting spliced and unspliced RNA from the reporter plasmid pDM128 was kindly provided by Y. Huang and G. C. Carmichael (Huang & Carmichael, 1997).

**Methods**

- **Plasmid constructs.** The plasmid pCDNA1E7 containing the CMV immediate early promoter and HIV-1/XH22 rev, env, nef and 3’ LTR sequences, was a gift from J. Sodroski and R. Wyatt. The CMV rev plasmids were gifts from B. Cullen and M. Malim (Malim et al., 1989b). The plasmid Δ18/23 encodes a trans-dominant negative Rev mutant where amino acid residues 75 and 86 were mutated and the intervening sequence was deleted. This plasmid is referred to as TD rev throughout this study and the protein expressed from this vector is called TD Rev. The plasmid Δ12/14 encodes a Rev mutant where amino acid residues 100–112 are deleted. This Rev mutant has wild-type activity. The plasmid pSVc21 containing the HIV-1 HX2C2 provirus was a gift from W. A. Haseltine. The plasmid pNL14.6D7 containing the 10D cDNA with the HIV-1 LTR promoter was generously provided by B. Felber (Benko et al., 1990). The vector pGL2 has the HXB2-sequence from 1084–1711 cloned into the HindIII site of pGEM-7f (+) (Promega).

- **Cell lines and transfection.** COS cells were seeded into 35 mm wells or 100 mm plates 1 day prior to transfection, grown to 60–70% confluence and transfected by the lipofectamine procedure of Gibco BLR using 7 µl/50 µl lipofectamine per 35 mm well/100 mm plate. The different amount of plasmid DNA used in each experiment is indicated above each lane in the RNase protection and Western blot figures.

- **Monoclonal antibodies.** The anti-Rev MABS 8E7 and 1G7 have been described (Kalland et al., 1994b). In short, MAB 8E7 (IgG2a) binds to the activation domain of Rev and consequently does not recognize TD Rev. MAB 1G7 (IgG2b) binds to an epitope in the C-terminal region of Rev that is deleted in the Rev mutant Δ12/14. The anti-Rev MAB 8E7 was also used to detect the Tev/Tnv protein. For detection of gp160/120 (Env) the anti-gp120 MAB ADP327 (IgG1) from C. Thiria and C. Buck and supplied by H. C. Holmes, Medical Research Council, London, UK, was used (Thiria et al., 1989). For Western blot analysis the anti-gp120 MAB T7 kindly provided by L. Åkerblom, was also used (Åkerblom et al., 1990).

- **Western blot analysis.** COS cells in 35 mm wells were collected in 150 µl of lysis buffer 48 h after transfection (Szilvay et al., 1995). The samples were separated by 5–7.5% or 15% SDS–PAGE for detection of gp160/120 or Rev respectively. The separated proteins electrophoretically transferred to nitrocellulose (0.22 µm) were subjected to immunodetection by the appropriate antibodies as previously described using the ECL detection system (Amersham) (Szilvay et al., 1995). Pre-stained molecular mass standards (Bio-Rad) and recombinant Rev protein (Intracel) were used as standards. The films were scanned using a Microtek Scannaker IIX flatbed scanner and the figures were created using the program Adobe Photoshop version 3.0.

- **Immunofluorescence.** For the immunofluorescence assay cells were seeded onto 12 mm coverslips in 24-well plates 24 h after transfection. The next day the coverslips were washed with PBS and fixed in 4% formaldehyde followed by permeabilization in ice-cold methanol. The coverslips were stored in methanol at −20 °C. The assays were performed as previously described (Szilvay et al., 1995). For double labelling, isotype-specific secondary antibodies conjugated with FITC or Texas Red (Southern Biotechnology) were used. The samples were...
trans-dominant inhibition of Rev

Fig. 1. Western blot analysis of lysates from COS cells in 35 mm wells transfected with the indicated amount of plasmid DNA. (A) Detection of gp160/120 by the anti-gp120 MAb T7. (B) Detection of wild-type Rev by the anti-Rev MAb 8E7. Purified recombinant Rev protein (25 ng) was applied in the lane at the far left. Lysates from the same experiment were used in (A) and (B); 15 µl samples of cell lysates corresponding to 7–5% of the total amount of lysates were loaded into the wells in (A) while 20 µl samples of cell lysates corresponding to 10% of the total amount were loaded into the wells in (B). The SDS–PAGE [7–5% in (A), 15% in (B)] and the blotting procedures were done in parallel.

viewed at a magnification of 400 × using a Nikon Microphot-SA with epifluorescence. Microscopic images were captured by a colour camera (Hamamatsu 3 chips cooled CCD camera with a Hamamatsu C5810 controller unit) and transferred by a frame grabber to a Macintosh Power PC where the images were stored digitally. The figures were created using Adobe Photoshop version 3.0.

Detection of p24 in the culture medium of COS cells transfected with pSVc21. Cell culture medium from COS cells transfected with the proviral clone pSVc21 was collected 40 h after transfection. After addition of Triton X-100, the samples were stored at −20 °C. The p24 ELISA test was performed as previously described (Sundquist et al., 1989).

In situ hybridization. Digoxigenin-labelled RNA probes were generated using an in vitro transcription kit (Boehringer Mannheim). The intron-probe was transcribed from a plasmid containing a 2006 bp fragment of the HIV-1 genome (nt 6127–8133 in GenBank locus HIVHXB2CG) using SP6 RNA polymerase. Cells were transfected and fixed as for the immunofluorescence assay. The hybridization protocol was performed as described (Bøe et al., 1998). After hybridization the RNA probe was labelled using a rhodamine-conjugated anti-digoxigenin Fab fragment. Simultaneous detection of Rev or gp160/120 was performed using the anti-Rev MAb 8E7 or the anti-gp120 MAb ADP327, respectively, as primary antibodies, biotin conjugated antibodies as secondary antibodies and then FITC-conjugated streptavidin (Pierce). The cells were examined as described using a confocal laser scanning microscope (Bøe et al., 1998) Pseudo-colouring of images was performed using a Macintosh computer and Adobe Photoshop version 3.0.

RNase protection assay. COS cells were seeded in 10 cm tissue culture plates 1 day prior to transfection. Transfections were then carried out using 50 µl lipofectamine with 2.5 µg pDM128, 5 µg pCRrev and 10 µg TD rev. Total cellular RNA was isolated as described by Chomczynski & Sacchi (1987) 48 h after transfection. RNase protection was performed.

Fig. 2. Immunofluorescence of COS cells transfected with the plasmids indicated. (A, B) Cells cotransfected with the control plasmid pgL2. Double labelling of Env and Rev using the anti-gp120 MAb ADP327 and the anti-Rev MAb 8E7 combined with FITC-labelled anti-mouse IgG1 and Texas Red-labelled anti-mouse IgG2a. The cytoplasmic Rev staining of the two cells in B is excluded from the Golgi apparatus where the staining of the Env proteins is most abundant (marked with arrows). (C, D, E, F) Cells cotransfected with the TD rev plasmid. Double labelling of Env and wild-type Rev as in (A) and (B). (G, H) Cells cotransfected with the A12/14 rev plasmid. Double labelling of Env and Rev with the anti-gp120 MAb ADP327 and the anti-Rev MAb 1G7 combined with FITC-labelled anti-mouse IgG1 and Texas Red-labelled anti-mouse IgG2b. Cells stained by both the anti-gp120 MAb and the anti-Rev MAb are marked with arrowheads in (A), (E), (F) and (G). In order to demonstrate double labelling of gp120 and wild-type Rev when possible, cells from different fields of vision were selected in order to create the panels.
using the RPA III kit from Ambion. Probes were generated by in vitro transcription using T3 RNA polymerase in the presence of [α-32P]UTP. As template for the in vitro transcription a plasmid provided by Y. Huang and C. C. Carmichael was used (Huang & Carmichael, 1997). Labelled probes were hybridized to target RNA in the hybridization buffer supplied in the kit at 43 °C for 4 h. After digestion with RNase A and T1 the protected fragments were resolved on a 5% acrylamide–8 M urea gel and visualized by autoradiography. The 415 nt probe protected 185 nt of spliced mRNA and 397 nt of unspliced mRNA (Huang & Carmichael, 1997). The films were scanned using an Agfa Snap Scan 600 flatbed scanner using the program Color-It version 3.0 for Macintosh Power PC. Adobe Photoshop version 3.0 was used when designing the figure.

Results

Trans-dominant negative inhibition of Rev-dependent Env expression is not caused by toxic nucleolar Rev overexpression

COS cells transfected with pcDNA1E7 containing the rev and env genes express substantial amount of gp160/120 and very little Rev protein (Szilvay et al., 1995). It was previously shown that cotransfection with TD rev inhibits production of gp160/120 from pcDNA1E7 and that this inhibition was apparently caused by nuclear retention of the wild-type Rev protein encoded by pcDNA1E7. However, a large amount of TD rev plasmid was used in these experiments. Such high levels of TD Rev expression also induced disruption of the nucleolar structure in most of the expressing cells (Szilvay et al., 1995). Similar observations have been reported for cells overexpressing the wild-type protein (Nosaka et al., 1993). The trans-dominant negative effect might therefore be caused by overaccumulation of Rev and TD Rev in the nucleus and could in reality be a cytotoxic effect. Initial experiments over-expressing wild-type Rev or TD Rev with pcDNA1E7 verified these suspicions. Above a certain level, overexpression of wild-type Rev in trans caused downregulation of Env expressed from pcDNA1E7 (not shown). In order to distinguish between the toxic and a genuine trans-dominant negative effect, the concentration of TD rev was titrated down to levels where normal nucleolar structure of the transfected cells was maintained. This was accomplished using 1 µg of TD rev per 35 mm plate of COS cells. An increasing ratio of TD Rev to wild-type Rev was then achieved by a successive reduction of cotransfected pcDNA1E7.

Hence, 1-0 µg of the rev plasmids pcrev or TD rev was cotransfected with decreasing concentrations of pcDNA1E7. To the samples which received no rev plasmid, 1 µg of the control plasmid pGL2 was added.

Addition of 1-0 µg pcrev did not significantly change the levels of gp160/120 compared to those expressed from pcDNA1E7 with pGL2 (Fig. 1 A; compare lanes 1–3 with lanes 4–6). When TD rev was added, a marked decrease of gp160/120 production was observed (Fig. 1 A; compare lanes 1–3 with lanes 7–9).

Cotransfection of pcDNA1E7 with TD rev is followed by increased expression of wild-type Rev

Almost no Rev was detected by Western blot analysis of COS cells transfected with pcDNA1E7 and the control plasmid pGL2 (Fig. 1 B, lanes 1–3). However, when TD Rev was co-expressed, the amount of wild-type Rev protein increased substantially (Fig. 1 B, lanes 7–9). Concurrently, the levels of

Table 1. Mean number of cells positive with anti-gp120 and anti-Rev MAbs per microscope field of view at 100× magnification

Microscope fields of view with the maximum number of positive cells were selected.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>No. of cells stained by:</th>
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<tr>
<td></td>
<td>anti-gp120</td>
</tr>
<tr>
<td>pcDNA1E7 (µg)</td>
<td>18</td>
</tr>
<tr>
<td>pGL2 (µg)</td>
<td>25</td>
</tr>
<tr>
<td>Δ12/14 (µg)</td>
<td>21</td>
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<td>TD (µg)</td>
<td>11</td>
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<tr>
<td>0.25</td>
<td>17</td>
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<td>0.50</td>
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<td>0.25</td>
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<td>0.50</td>
<td>8</td>
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* Most fields of view were negative; only fields with positive cells were selected.
NA, Not applicable (since 8E7 binds to Δ12/14 and 1G7 binds to TD).
the Env proteins were reduced (Fig. 1A). There was a dose-response effect of pcDNA1E7 concentration on Rev expression when co-expressed with TD Rev while approximately the same amount of wild-type Rev was detected in the three cultures receiving 1 µg TD rev (Fig. 1B, lanes 4–9). Indirect immunofluorescence analysis of transfected COS cells verified the expression patterns found in the Western blot experiments. The majority of the cells transfected with pcDNA1E7 was strongly labelled with the anti-gp120 MAb in contrast to very few cells with detectable wild-type Rev (Fig. 2A, B; Table 1). Selected cells labelled with both the anti-gp120 MAb and the anti-Rev MAb 8E7 are marked with arrowheads in Fig. 2. The cytoplasmic Rev staining was excluded from the Golgi apparatus where the staining for gp120 was most dominant, demonstrating the specificity of the Rev staining (marked with arrows in Fig. 2A, B). When cotransfecting pcDNA1E7 together with TD rev, the fraction of Env-expressing cells decreased and the staining in each of the positive cells was reduced also (Fig. 2C, E; Table 1). At the same time there was a significant increase in the amount of detectable wild-type Rev (Fig. 2D, F; Table 1). As a control experiment the mutant Δ12/14 Rev, which has wild-type activity, was cotransfected with pcDNA1E7. This Rev mutant affected neither the expression of gp160/120 nor the wild-type Rev level. Wild-type Rev was detected using the anti-Rev MAb 1G7, which does not recognize Δ12/14 Rev (Fig. 2G, H; Table 1).

**Upregulation of proteins encoded by spliced and alternatively spliced mRNAs using the proviral clone pSVc21 and TD rev**

Immunofluorescence and Western blot analysis using the proviral construct pSVc21 demonstrated that more wild-type Rev was expressed from this vector than from the subgenomic vector pcDNA1E7 (not shown). Consequently, low concentrations of pSVc21 (50–250 ng) had to be used in order to induce the trans-dominant negative effect by 1 µg of TD rev. Fig. 3 shows the results of an experiment using 10 and 5 ng of pSVc21 together with 1 µg of TD rev. The amounts of viral structural proteins p24 Gag and gp160/120 Env encoded by unspliced and single-spliced mRNA, respectively, were reduced when TD Rev was present (Fig. 3A, B). At the same time, increased levels of wild-type Rev and the Tev/Tnv protein were detected by the MAb 8E7 (Fig. 3A). Lysates of cells transfected with pcrev, pNL1,4,6D7 or pcDNA1E7 were analysed on 15 or 7–5% SDS–PAGE, respectively, together with the pSVc21 samples in order to demonstrate the migration of the Rev, Tev/Tnv and Env proteins (Fig. 3A, lanes 5, 6 and 7).

**In situ hybridization detecting unspliced mRNA in COS cells transfected with pcDNA1E7 and the TD mutant**

COS cells cotransfected with 0.5 µg of pcDNA1E7 and 1 µg of the control plasmid pGL2 or 1 µg of TD rev were double labelled using the intron probe to detect unspliced mRNA and the anti-gp120 MAb or the anti-Rev MAb 8E7 to detect the respective viral proteins. Similarly to the immunofluorescence assay, a large amount of the Env proteins was detected in the cells transfected with pcDNA1E7 and pGL2 (Fig. 4A). Unspliced mRNA was mainly localized to the cytoplasm in these cells (Fig. 4B). Very few of the cells expressed detectable levels of Rev (Fig. 4C). When the cells were cotransfected with pcDNA1E7 and TD rev, the amount of Env proteins was notably decreased (Fig. 4E) with a
corresponding increase of wild-type Rev (Fig. 4G). The staining pattern of unspliced mRNA in the cells cotransfected with TD rev using the intron probe was changed in two aspects (Fig. 4F, H). First, the overall intensity of the intron staining was lower. Secondly, in the cells with detectable levels of the intron probe the staining was evident, particularly in the nucleoplasm. Fig. 5 displays cells cotransfected with pcDNA1E7 and TD rev viewed at higher magnification (1000 ×). Rev was detected in the nucleoli and in the nucleoplasm, whilst the unspliced mRNA was found in the nucleoplasm. The pattern of the nucleoplasmic staining of Rev and mRNA was similar. No accumulation of Rev or mRNA at the nuclear membrane or in the nuclear periphery was evident.

Effect of TD Rev on the ratio of spliced to unspliced mRNA in a Rev-dependent system analysed by an RNase protection assay

A probe protecting the exon sequences in pcDNA1E7 or pSVC21 would also protect TD rev mRNA sequences. Therefore, the construct pDM128 was used since most of the rev exon 2 sequence is deleted in this construct (Hope et al., 1990). The probe protecting the 3’ intron/exon junction sequences of the mRNA transcribed from this vector did not hybridize to TD rev mRNA or rev mRNA (Fig. 6, lane 1).
Furthermore, the use of a different genetic system revealed that increased splicing caused by TD Rev inhibition of Rev is a characteristic of Rev-dependent mRNAs and not an artefact connected with specific constructs. Fig. 6 shows the results of an RNase protection assay of COS cells transfected with pDM128 alone (lane 2), pDM128 and pcrev (lane 3), and pDM128, pcrev and TD rev (lane 4). Consistent with Huang & Carmichael (1997), the ratio of unspliced to spliced mRNA detected in lane 2 was reversed in lane 3 where wild-type Rev was co-expressed.

Comparison of the ratios between unspliced and spliced mRNA in lanes 3 and 4 reveals a distinct shift towards spliced RNA in lane 4, where both TD rev and pcrev were cotransfected with pDM128 into COS cells.

**Discussion**

The ability of mutants of the activation domain of Rev to dominantly inhibit Rev activity in a dose-dependent manner is well established (Malim et al., 1989b; Venkatesh & Chinnadurai, 1990; Malim et al., 1991). Such mutants are deficient in their active export from the nucleus (Meyer & Malim, 1994; Szilvay et al., 1995; Wolff et al., 1995) and the basis for the trans-dominant inhibition was found to be the retention of wild-type Rev in the nucleus during co-expression (Stauber et al., 1995; Szilvay et al., 1995; Wolff et al., 1995). High expression levels of wild-type Rev or TD Rev often result in nucleolar fragmentation. This made us consider nucleolar dysfunction as the real basis for the trans-dominant negative effect of TD Rev. However, when lowering the amount of plasmid DNA encoding rev and TDrev, a distinction between the nucleolar dysfunction caused by overexpression of TD Rev protein and the trans-dominant negative inhibition of Rev function was apparent. By the use of plasmid constructs which provide rev in cis it was discovered that TD Rev not only downregulated the expression of structural proteins translated from the incompletely spliced mRNAs, but additionally and concomitantly upregulated the expression of wild-type Rev encoded by spliced mRNA.

When the proviral construct pSVC21 was used in cotransfection experiments with TDrev, not only the amount of Rev protein increased. Higher levels of the Tev/Tnv protein translated from an alternatively spliced HIV mRNA was observed also (D’Agostino et al., 1992; Salfeld et al., 1990). An interpretation of these observations may be that both the host cell splicing machinery and the nuclear export of intron-containing HIV mRNAs are targets for normal Rev activity. This assumption was confirmed using an RNase protection assay. It was shown that the relative amount of spliced versus unspliced viral mRNA was shifted towards spliced mRNA when Rev was inhibited by co-expressed TD Rev. Analysis of single cells cotransfected with pcDNA1E7 and TD rev using in situ hybridization demonstrated both nuclear confinement and a decrease of unspliced mRNA (Figs 4 and 5). Neither HIV RNA nor Rev accumulated at any peripheral site of the nucleus. Instead, intron-containing mRNA and Rev were distributed throughout the nucleoplasm in cells containing detectable amounts of unspliced mRNA. These findings suggest that the trans-dominant negative effect is executed in the nucleoplasm and not at the nuclear membrane. There are several feasible means by which Rev may increase the amount of unspliced viral mRNAs. The effect can be achieved indirectly by removing the viral pre-mRNA at the sites of transcription before splicing occurs. However, since not only unspliced HIV pre-mRNA, but also singly spliced HIV mRNAs, are subject to Rev regulation, it is obvious that Rev can act on an mRNA that has already been committed to a splicing pathway. This indicates an association of Rev with the splicing machinery. Furthermore, double labelling utilizing in situ hybridization and indirect immunofluorescence has shown that the HIV pre-mRNA and Rev preferentially localize to the periphery of nuclear speckles in regions of the weak SC-35 staining (Boe et al., 1998). These areas correspond to sites of active transcription and splicing (Wansink et al., 1993; Misteli et al., 1997). Oligomerization is essential for Rev function and complexes consisting of wild-type Rev and TD Rev have been demonstrated in the cytoplasm, during nuclear import and in the nucleus (Hope et al., 1992; Szilvay et al., 1997; Stauber et al., 1998). The nuclear retention of wild-type Rev by TD Rev was suggested to be caused by generation of export-deficient mixed multimers (Stauber et al., 1995; Szilvay et al., 1995; Wolff et al., 1995). Support for this assumption is that the nuclear export of the human T-lymphotropic virus type I protein Rex, which does not interact with Rev (Bogerd & Greene, 1993), is not affected by a trans-dominant negative Rev mutant (Stauber et al., 1995). However, inhibition of Rex function by a trans-dominant negative Rev mutant in an HIV-based system has been demonstrated (Solomin et al., 1990). These apparently opposing results concerning the Rex protein indicate that at least two different inhibitory mechanisms are mediated by TD Rev. One of these could be caused by competition by TD Rev for binding to the target RNA leading to nuclear confinement of the RRE RNA. Direct competition for the cellular cofactors CRM1 (exportin 1), eIF5a or hRip (Rab) seems unlikely since they have been shown to interact with the activation domain or NES, which is deleted in TD Rev (Ruhl et al., 1993; Bogerd et al., 1995; Ossareh-Nazari et al., 1997). However, the proposed formation of mixed multimers may cause imperfect binding to any of these factors or with other unknown cellular cofactors. Such defective interactions may take place at any of the steps along the shuttling pathway of Rev. One consequence might therefore be that Rev simply does not reach the nuclear targets for Rev regulation. Whatever the underlying mechanism of inhibition, the effect of trans-dominant inhibition is an increase of viral pre-mRNA splicing leading to higher levels of protein products from spliced and alternatively spliced mRNAs. The results therefore provide new evidence to support the assumption that Rev regulates the
splicing of viral pre-mRNA besides facilitating the export of singly spliced and unspliced viral mRNAs. The recent publication by Huang et al. (1999) confirms this. It was shown that RNA transport elements from intronless cellular and viral mRNAs act as polyadenylation enhancers and inhibitors of splicing. These elements were further demonstrated to functionally replace Rev and RRE in a Rev dependent expression system (Huang et al., 1999). These findings support the suggestion that Rev regulates more than the nuclear export step of viral mRNAs.

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