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Functional role of the HIV-1 Rev exon 1 encoded region in complex formation and *trans*-dominant inhibition

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Abstract To study functional aspects of the exon 1 encoded region of the human immunodeficiency virus type 1 Rev protein, the viral Tev protein which exhibits low Rev activity but lacks the *rev* exon 1 encoded region was examined. Neither Rev–Tev heteromer complex formation nor inhibition of Rev by an export deficient Tev mutant was observed. Insertion of the *rev* exon 1 encoded region into the Tev mutant allowed it to oligomerize with Rev and act as a *trans*-dominant negative mutant. This showed that the exon 1 encoded region of Rev is essential for oligomerization and that oligomerization is a prerequisite for *trans*-dominant inhibition.

Key words: Human immunodeficiency virus type 1; Rev; Tev; Oligomerization; *Trans*-dominant inhibition

1. Introduction

The regulatory proteins of the human immunodeficiency virus type 1 (HIV-1) Tat and Rev are required for HIV gene expression and virus replication. The Tat protein stimulates and increases transcriptional elongation by binding to an RNA sequence located in the promoter region, long terminal repeat (LTR) [1,2]. The Tat protein is encoded by two exons, the first preceding the *env* gene and the second within the *env* gene. The *rev* gene consists of two exons partly overlapping the *tat* exons using different reading frames. The mRNAs encoding Tat and Rev are obtained by complete splicing of the viral pre-mRNA. The Rev protein promotes cytoplasmic expression of unspliced and incompletely spliced HIV-1 mRNAs encoding the structural HIV components [3–6]. Two functional domains necessary for function are encompassed within the exon 2 encoded region of Rev. The basic domain (amino acids (aa) 35–50) is responsible for the nuclear and nucleolar localization of Rev as well as specific binding of Rev to the RNA target sequence, Rev responsive element (RRE) [7–15]. The other essential domain (aa 75–83), called nuclear export signal (NES), signals active nuclear export of Rev [16–19]. Mutations of essential amino acids within this domain generate *trans*-dominant negative mutants [20–25]. It has been assumed that *trans*-dominant inhibition is caused by formation of mixed multimers consisting of wild type and mutant Rev [17,24,26]. Oligomerization appears to be critical for Rev function and studies of Rev mutants have indicated several amino acid residues encoded by exon 1 to be essential in this respect [24,27,28]. However, other studies have shown that additional regions of the molecule may be implicated [8,13,15,29,30]. The Tev protein, also called Tnv, is encoded by an alternatively spliced viral mRNA containing the sequences from *tat* exon 1, an internal *env* exon and *rev* exon 2 [31–33]. Accordingly, essential domains of both Tat and Rev are encompassed within the Tev protein. The Tat activity of Tev is similar to that of Tat, but the Rev activity has been found to be lower than that of wild type Rev [31,33].

The exon 1 encoded part of Rev is not present within the Tev protein. This provided the unique opportunity to examine its importance in mediating oligomer formation by inserting it into Tev. In addition, it allows one to investigate if complex formation is obligatory for *trans*-dominant inhibition. Therefore, the exploitation of the viral protein Tev instead of Rev mutants was an alternative strategy to analyze the function of the exon 1 encoded part of Rev.

2. Materials and methods

2.1. Plasmids

Plasmids encoding Tat and wild type/mutant Rev were kindly provided by M. Malim and B. Cullen [20,23]. The Rev mutant previously called Δ18/23 is here referred to as RevΔNES. The plasmid pcDNA1E7 containing the cytomegalo virus immediate early promoter and HIV-1/XHB2 *rev*, *env*, *nef* and 3′ LTR sequences, was a gift from J. Sodroski and R. Wyatt. The *rev* negative reporter plasmid pSVc21B containing the complete HIV sequence with a mutation in the *rev* gene was previously described [34]. The plasmid pNL1,4,6D7 encoding the *tev* cDNA sequence was generously provided by B. Felber [31]. The *tat* cDNA in pc*tat* was replaced by the *tev* cDNA using flanking *Sal*I and *Xho*I sites. The resulting plasmid was named pc*tev*. The plasmid pc*tevΔNES* was generated by combining the relevant parts of pc*tev* and pc*revΔNES* using the unique *Pst*I and *Bam*HI restriction sites. Insertion of *rev* exon 1 into the *tev* cDNA between the *env* exon and *rev* exon 2 was accomplished by a two step PCR approach. A fragment containing *tat* exon 1 and the *env* exon of *tev* was amplified from the pc*tev* plasmid while the other fragment was amplified from the pc*rev* plasmid. The two fragments were ligated and the resulting 985 bp fragment flanked by the *Sal*I and *Xho*I sites was cloned into the precut pc*tat* vector. The vector was called pc*tevNrev* and the expressed protein was called TevNRev. The pc*tevNrevΔNES* vector was made by the same procedure as pc*tevΔNES*. An overview of the different wild type and mutant proteins is shown in Fig. 1.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; Env, HIV-1 glycoproteins; LTR, long terminal repeat; NES, nuclear export signal; MAb, monoclonal antibody; CHO, Chinese hamster ovary cells; COS, African green monkey kidney cell line transformed by SV40

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2.2. Cell lines and transfections

Chinese hamster ovary (CHO) cells or COS-7 cells were seeded into 35 mm wells 1 day prior to transfection, grown to 60–70% confluence and transfected by the lipofectamine procedure of Gibco BLR using 5 μl lipofectamine per 35 mm well. The amount of plasmid per well varied from 50 to 200 ng for pSVc21B, 1–2 μg for the *tev* and *rev* plasmids and 500 ng pcDNA1E7.

2.3. Monoclonal antibodies (MAbs) and immunofluorescence analysis

The anti-Rev MAb 8E7 (IgG2a) and the anti-Tat MAb 1D9 (IgG1) also recognizing the Tev protein have been described [35,36]. The MAb 8E7 did not recognize the ΔNES mutants. The MAb binding epitopes are schematically outlined in Fig. 1. For detection of gp160/120 (Env) the anti-gp120 MAb ADP327 (IgG1) supplied by H.C. Holmes was used [37]. The immunofluorescence assays were performed as previously described using goat isotype specific secondary antibodies conjugated with FITC or Texas red (Southern Biotechnical) [17].

2.4. Western blot analysis

COS cells in 35 mm wells were collected in 150 μl of lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$ and 0.5% NP40) 48 h after transfection. The samples were analyzed by Western blot using the ECL detection system (Amersham) as previously described [17]. Pre-stained molecular weight standards (Bio-Rad) and a recombinant Rev protein (Intracel) were included in the experiments.

3. Results

3.1. Comparison of Rev, Tev and TevNRev

Immunofluorescence analysis of CHO and COS cells transfected with the different *rev* and *tev* containing plasmids demonstrated a similar intra-cellular distribution of the Rev, Tev and TevNRev proteins localizing to the nucleoli, nucleoplasm and the cytoplasm (not shown). As the RevΔNES protein, also the TevΔNES and TevNRevΔNES localized to the nucleus with extensive nucleolar accumulation (not shown). The Rev activity of Tev was reported either to be low or undetectable [31,33]. In this study, however, the Rev activity of Tev was easily assessed using the *rev* negative provirus construct.

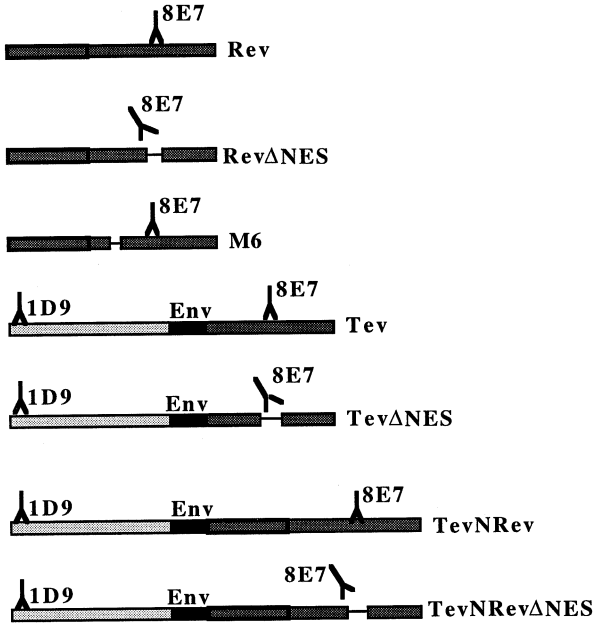

Fig. 1. The Rev and Tev wild type and mutant proteins schematically outlined. The epitopes of the MAbs 8E7 and 1D9 are indicated. The drawings are not to scale.

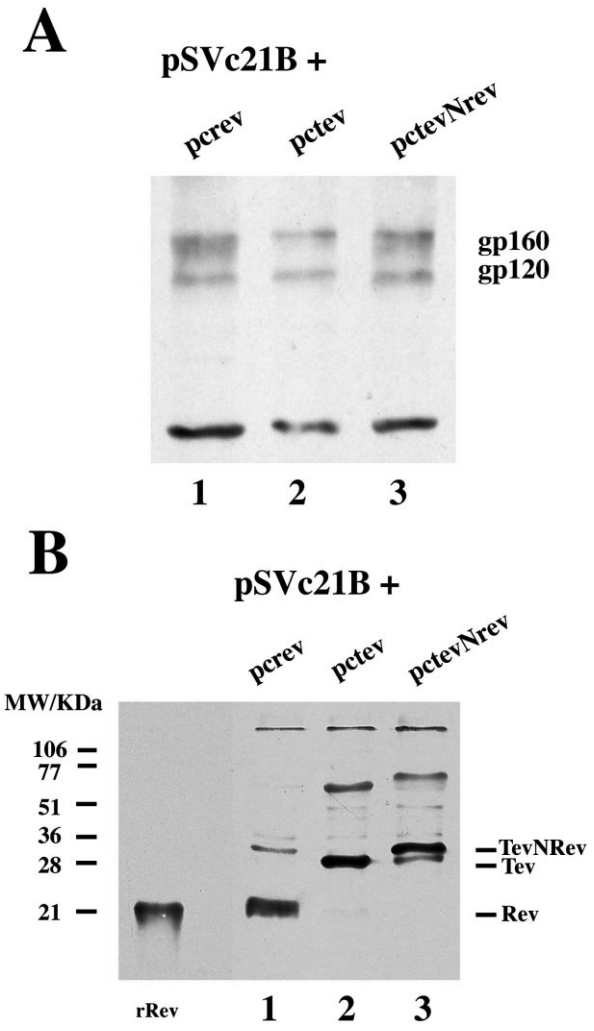

Fig. 2. Rev function of wild type Rev, Tev and TevNRev. Western blot analysis of lysates from COS cells co-transfected with the *rev*$^-$ proviral construct pSVc21B together with pc*rev* (lanes 1), pc*tev* (lanes 2) and pc*tevNrev* (lanes 3). A: The samples were separated on a 7.5% SDS–PAGE. Detection of gp160/120 by the anti-gp120 MAb ADP327. B: The same samples as in A were separated by 15% SDS–PAGE. The anti-Rev MAb 8E7 was used as primary antibody. Recombinant Rev (25 ng) was applied in the lane to the left. The migration of Rev, Tev and TevNRev proteins is indicated to the right.

Cells transfected with pc*rev* and pc*tevNrev* produced a similar amount of the Env proteins, whereas less was produced in cells transfected with pc*tev* (Fig. 2A, lanes 1–3). Comparable amounts of Rev, Tev and TevNRev were expressed in the cells co-transfected with pSVc21B and pc*rev*, pc*tev* or pc*tevNrev*, respectively (Fig. 2B, lanes 1–3).

3.2. Oligomerization between M6 and ΔNES mutants

The observed low Rev activity of the Tev protein may be caused by its inability to oligomerize. In order to test this assumption, an in vivo oligomerization assay was used. The assay employs co-expression of the nuclear import defective Rev mutant M6 and other Rev mutants comprising an intact nucleolar localization signal. Oligomerization between M6 and these mutants is then demonstrated by nucleolar import of M6 [27]. In cells expressing M6 only, the mutant was localized to the cytoplasm with a diffuse nucleoplasmic staining

in some cells always excluding the nucleoli (Fig. 3A). To examine if oligomerization between the Tev and Rev proteins takes place, the M6 plasmid was co-transfected into CHO cells together with pc*tevΔNES* or the control plasmid pc*revΔNES*. As previously described, co-expression of RevΔNES with M6 directed M6 to the nucleoli and the nucleoplasm (Fig. 3B). Co-expression of M6 with TevΔNES did not change the cytoplasmic localization pattern of M6. TevΔNES was detected in the nucleoli and the nucleoplasm while M6 localized to the cytoplasm in the same cells (Fig. 3C–F). Accordingly, no interaction between M6 and TevΔNES had occurred. However, nucleolar localization of M6 was observed when expressing TevNRevΔNES with M6 though less efficient as when RevΔNES was used. This was reflected in the number of transfected cells with nucleolar localization of M6 and the remains of cytoplasmic M6 in the same cells (Fig. 3G–J).

3.3. Trans-dominant inhibition of Rev by ΔNES mutants

A large amount of Env proteins and very little of the Rev protein are expressed from the vector pcDNA1E7 (containing the HIV *rev* and *env* genes). It was previously shown that co-expressed RevΔNES reduced the amount of Env proteins. Concurrently, the amount of Rev protein increased showing that inhibition of Rev leads to enhanced splicing of the pre-mRNA [38]. Accordingly, increase of the Rev protein encoded by spliced mRNA enabled an assessment of the bona fide *trans*-dominant inhibitory effect distinguishing it from toxic effects or a competition between wild type and mutant Rev for target RNA which may also inhibit the expression of gp160/120 (Env). This assay was used to examine if TevΔNES executed a *trans*-dominant negative effect when co-expressed with Rev. For comparison, cells co-transfected with pcDNA1E7 and p*revΔNES* were included. There was a clear inhibition of expressed Env protein (gp160/120) by RevΔNES (Fig. 4A, lanes 1 and 2). Concurrently, more Rev was produced (Fig. 4B, lanes 1 and 2). There was, however, neither decrease of gp160/120 (Fig. 4A, lane 4) nor increase of Rev (Fig. 4B, lane 4) by co-expressed TevΔNES. No significant

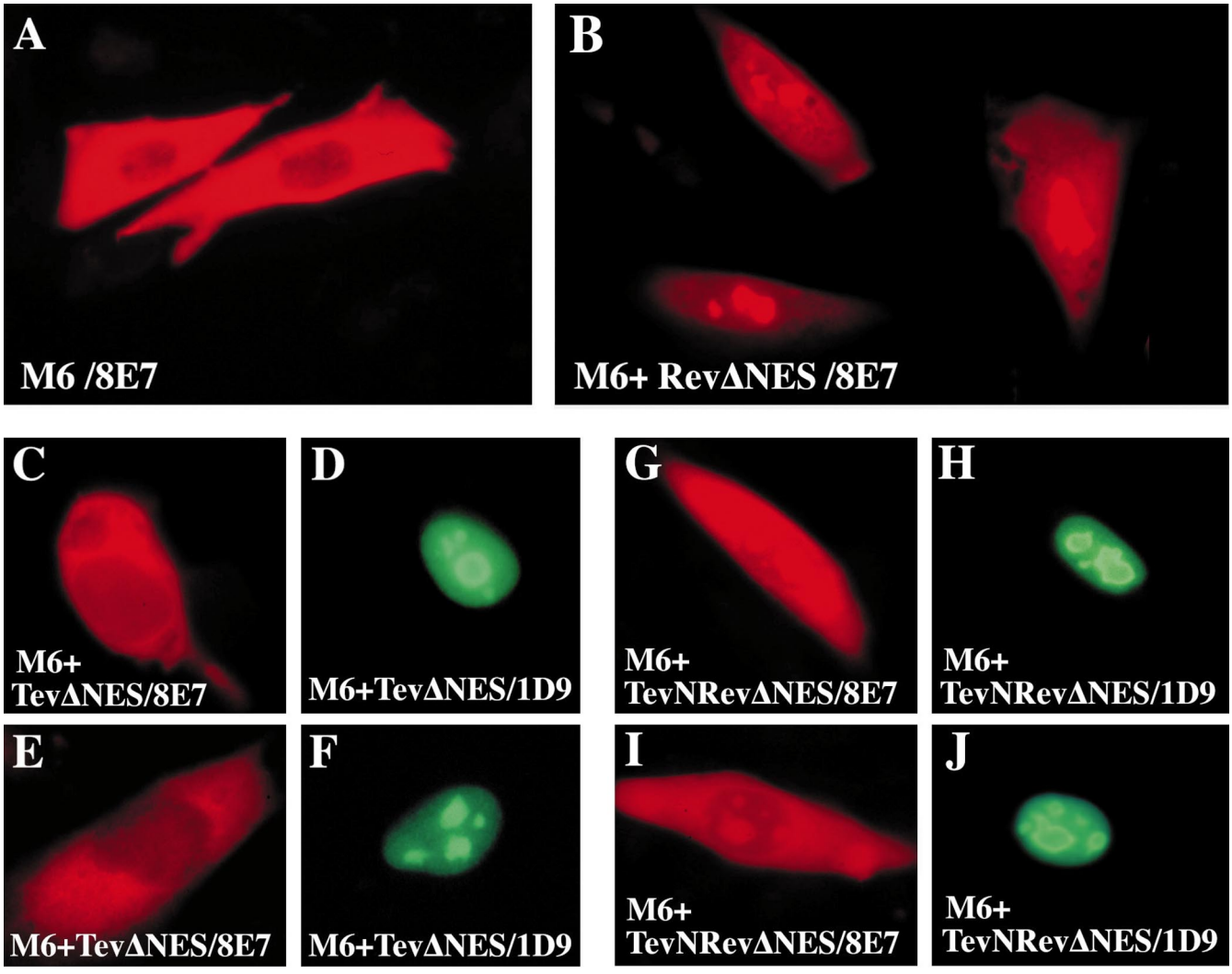

Fig. 3. In vivo oligomerization assay. Immunofluorescence analysis of the Rev mutant M6 expressed alone or together with the different ΔNES mutants in CHO cells. M6 is detected by the anti-Rev MAb 8E7 combined with Texas red labeled anti-mouse IgG2a, while TevΔNES and TevNRevΔNES are detected by the anti-Tat MAb 1D9 combined with FITC-labeled anti-mouse IgG1. A: M6 localizes to cytoplasm in expressing cells. B: M6 localizes to the nucleus and nucleoli when co-expressed with RevΔNES (not recognized by MAb 8E7). C and D, E and F: Double labeling of M6 and co-expressed TevΔNES. M6 localizes to the cytoplasm while TevΔNES localizes to the nucleus and nucleolus in the same cells. G and H, I and J: Double labeling of M6 and co-expressed TevNRevΔNES. M6 localizes to the cytoplasm, nucleus and nucleoli in cells co-expressing TevNRevΔNES which is exclusively in the nucleus.

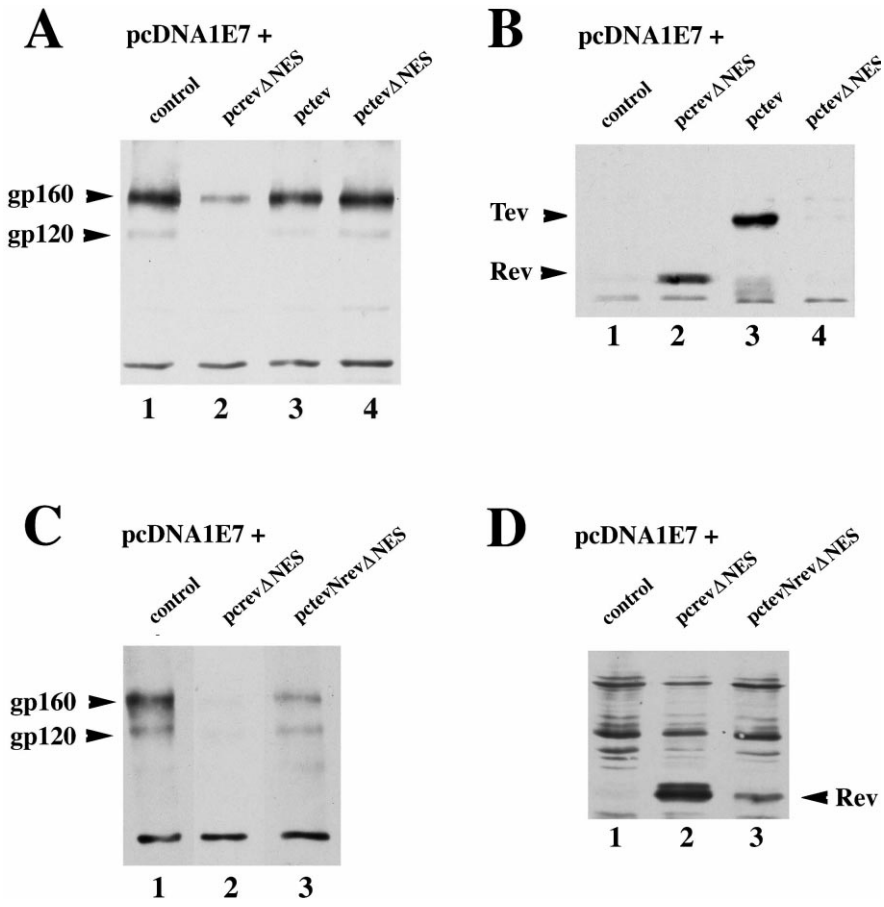

Fig. 4. Inhibition of the Rev activity by ΔNES mutants. Western blot analysis of lysates from COS cells co-transfected with pcDNA1E7 and the plasmids indicated above the lanes. The samples were separated on 7.5% SDS–PAGE (A and C) for detection of gp160/120 by the anti-gp120 MAb ADP327 or 15% SDS–PAGE (B and D) for detection of Rev by MAb 8E7. A: Lane 1, gp160/120 expressed from pcDNA1E7 encoding *rev* and *env*. Lane 2, inhibition of gp160/120 production by co-expressed RevΔNES. Lane 3, little effect of co-expressed Tev. Lane 4, no inhibition by co-expressed TevΔNES. B: Rev expression in the same samples as in A. Lane 1, Rev expressed from pcDNA1E7. Lane 2, increase of Rev caused by co-expressed RevΔNES. Lane 3, detection of co-expressed Tev, no increase of Rev. Lane 4, no increase of Rev by co-expressed TevΔNES. C and D: The inhibitory effect of TevNRevΔNES compared to that of RevΔNES. The *trans*-dominant inhibitory effect of TevNRevΔNES is shown as decrease of gp160/120 (comparing lanes 1 and 3 in C) and increase of Rev (comparing lanes 1 and 3 in D).

inhibition of gp160/120 production was demonstrated by co-transfection with pc*tev* (Fig. 4A,B, lanes 3). On the other hand, Western blot analysis of COS cells co-transfected with pcDNA1E7 and pc*tevNrevΔNES* showed that also the ability to *trans*-dominantly inhibit Rev was achieved by the insertion of the *rev* exon 1 encoded part into the Tev mutant (Fig. 4C,D). The inhibition was demonstrated as downregulation of Env proteins with an accompanied increase of wild type Rev (Fig. 4C,D, comparing lanes 1 and 3). This experiment also included control cells co-transfected with RevΔNES for comparing the effect of the two ΔNES mutants (Fig. 4C,D, lanes 2).

4. Discussion

In this study functional properties of the exon 1 encoded region of Rev were examined by comparing the activities of the natural viral protein Tev lacking the *rev* exon 1 encoded region with an artificial Tev protein where the missing Rev region was inserted (Fig. 1). The Rev activity of wild type Tev was, according to previous studies, found to be lower than that of Rev [31], while the activities of TevNRev and Rev were similar (Fig. 2). It should be kept in mind that the amount of viral pre-mRNA available for Rev regulation in the cells expressing the two Tev proteins may have been higher than in the cells co-transfected with pc*rev* since transcription from the LTR promoter is enhanced by Tat and Tev [31]. There was, however, a clear difference in Env expression when comparing the cells transfected with pc*tev* and pc*tevNrev* suggesting an increase in the Rev activity caused by the exon 1 encoded region present in TevNRev.

It was found that the export deficient Tev protein neither oligomerized with Rev M6 nor *trans*-dominantly inhibited Rev. Both these functions were gained by inserting the exon 1 encoded part into the Tev mutant. The inhibition of TevNRevΔNES was not as efficient as the inhibition by RevΔNES (Fig. 4C,D, comparing lanes 2 and 3). Also, the nuclear import of M6 by co-expressed TevNRevΔNES was not as conspicuous as when RevΔNES was co-expressed (Fig. 3). The observed differences in activity may be caused by an impaired folding of the exon 1 encoded region of TevNRev. It was not possible to ensure correct folding of the Rev moiety other than to consider the observed antibody recognition and the rise in Rev activity that both are indications of native conformation. Nevertheless, the results demonstrated a correlation between the ability of ΔNES mutants to *trans*-dominantly

inhibit Rev function and the ability of such mutants to oligomerize with Rev. These experiments therefore confirmed the previous mutation studies suggesting that it is the exon 1 encoded N-terminal region that mediates oligomerization of Rev molecules [24,27,39]. Furthermore, it was shown that heteromeric complex formation indeed is required for the *trans*-dominant negative inhibition of Rev by ΔNES mutants.

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