trans-Dominant Inhibition of Human Immunodeficiency Virus Type 1 Rev Occurs through Formation of Inactive Protein Complexes

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The human immunodeficiency virus type 1 Rev protein controls expression of certain viral RNAs by binding to these RNAs in the nucleus. To investigate how dominant negative Rev mutants inhibit Rev function, we fused such mutants to hormone-dependent localization signals from the glucocorticoid receptor. Each was found to have fully potent inhibitory activity whether expressed in the nucleus or in the cytoplasm. Wild-type Rev colocalized with an inhibitory fusion protein, implying that the two proteins interact. The resulting complexes accumulated within nuclei in response to steroids but had no effect on expression of Rev-responsive mRNAs. A mutation known to block *in vitro* oligomerization of Rev abolished both complex formation and inhibitory activity of the mutant fusion proteins. Thus, *trans*-dominant inhibition of Rev does not require competition for nuclear substrates but may instead reflect the ability of a mutant to form nonfunctional complexes with the wild-type protein *in vivo*.

Replication of human immunodeficiency virus type 1 (HIV-1) requires expression of the virus-encoded transactivator protein Rev (reviewed in references 5, 8, and 9). Localized within the nuclei of infected cells, where it associates with nucleoli (10), Rev binds specifically to a region of RNA secondary structure known as the Rev response element (RRE) that is found in certain incompletely spliced HIV-1 transcripts (6, 11, 16, 27, 35, 39). Through a mechanism that is not fully understood, binding of Rev permits these nuclear transcripts to be exported to the cytoplasm (4, 12, 14, 15, 25), where they serve as mRNAs encoding virion structural proteins. Because these essential mRNAs remain confined to the nucleus when Rev is absent, HIV-1 proviruses that lack a functional *rev* gene are unable to produce infectious virions (13, 36-38). This finding suggests that therapies designed to inhibit Rev activity might be of value in treating HIV-1 infections.

One approach to blocking specific viral functions lies in the use of viral proteins containing dominant negative mutations. By definition, such mutants not only lack intrinsic wild-type activity but also inhibit function of the wild-type protein in *trans* (18). In some cases, inhibition occurs because the mutant competes for an essential substrate or cofactor that is available in limiting amounts; alternatively, for proteins that multimerize, a mutant may act by associating with wild-type monomers to form inactive mixed multimers. Several dominant negative mutants of Rev have been identified (23, 26, 28), and overexpression of one such mutant has been shown to inhibit HIV-1 replication in COS cells in a transient transfection assay (23).

Rev is 116 amino acids long and comprises at least two separate functional domains (19, 20, 23, 26, 31). The larger N-terminal domain (approximately residues 14 to 60) includes, but extends beyond, a distinctive arginine-rich tract at residues 35 to 50; sequences within this domain mediate specific binding of the RRE, provide the signals required for nuclear and nucleolar localization, and also are responsible for the tendency of Rev protein to oligomerize both on (7, 27)

and off (30, 40) the RRE *in vitro*. The second, more C-terminal domain (approximately residues 78 to 90) also is essential for biological activity, but its exact function has not yet been determined (26). Although mutations in either of these domains can inactivate Rev, only those in the C-terminal domain confer a dominant negative phenotype (20, 23, 26, 28). Because the dominant negative mutants retain the capacity for RRE binding, oligomerization, and nuclear localization, they could potentially act by competing for binding of nuclear transcripts that contain the RRE (11, 23, 24, 30, 39), by forming mixed multimers with wild-type Rev (27, 30, 40), or by some combination of these mechanisms.

In this report, we describe further studies of the mechanism by which dominant negative mutants inhibit Rev function. We find that competition for nuclear substrates is not required and that potent *trans* inhibition can occur even when the mutant protein is excluded from nuclei. Instead, our studies suggest that inhibitory activity depends at least in part on the ability of a mutant to form nonfunctional complexes with wild-type Rev *in vivo*. These complexes may arise through direct oligomerization of Rev inside the cell.

MATERIALS AND METHODS

Plasmids. Detailed structures of the chloramphenicol acetyltransferase (CAT) reporter pDM128, the expression vectors pRSV-Rev, pRSV-Rev/GR, and ptk-Rev, and most mutant derivatives used in these studies have been reported elsewhere (19, 20). All gene fusions and mutations were verified by DNA sequencing.

Transfections and CAT assays. Transfections were performed by the calcium phosphate coprecipitation method using doubly CsCl-purified plasmids. CV1 cells were used for all CAT assays. COS7 cells gave modestly better morphology and transfection efficiency and so were used for immunolocalization and Western immunoblot studies, which did not involve simian virus 40-based plasmids.

In the standard cotransfection for CAT assay, each 10-cm plate of CV1 cells received the indicated amounts of Rev plasmids along with 1 μ g of pDM128, 0.25 μ g of the β -galactosidase expression vector pCH110 (Pharmacia), and

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sufficient pUC118 to maintain equal total DNA. Cells were washed once 18 h later and given fresh medium with or without 10^{-5} M dexamethasone (Dex) and then were harvested 36 to 40 h after transfection. Lysates were prepared by freeze-thawing three times in 0.25 M Tris (pH 7.5) followed by microcentrifugation for 5 min at 4°C , and aliquots of lysate were assayed colorimetrically for β -gal activity. Volumes that contained equal β -galactosidase activity were then assayed for CAT activity, which was quantitated by thin-layer chromatography and scintillation counting.

Immunofluorescence analysis. COS7 cells growing on glass coverslips in 3.5-cm culture plates were transfected with the amounts of plasmid specified in the figure legends; 18 h later, cells were washed once with Tris-buffered saline and given fresh medium with or without 10^{-5} M Dex. At 36 to 40 h after transfection, the cells were fixed and then stained with a specific primary antiserum followed by goat anti-rabbit immunoglobulin G as described previously (19). The Rev N-terminal antiserum (a gift from B. R. Cullen, Duke University) contained two rabbit antipeptide sera (each at a 1:800 dilution) directed against amino acids 1 to 20 and 27 to 50, respectively, of Rev (10). The Rev C-terminal antiserum (a gift from M.-L. Hammarskjöld and D. Rekosh, State University of New York at Buffalo) was a 1:1500 dilution of a rabbit serum that had been raised against an *Escherichia coli* fusion protein containing the C-terminal half of Rev. The glucocorticoid receptor (GR) antiserum (a gift from R. Sweet, Smith Kline & French; used at a 1:500 dilution) had been raised against the steroid-binding domain of mouse GR but cross-reacted with rat GR. CV1 cells contain no detectable endogenous GR (32).

Most immunolocalization studies were carried out under a triple-blind protocol, in which one investigator performed the transfections, a second added Dex to an unspecified subset of the plates, and a third stained and interpreted each plate without knowledge of the plasmids or hormone treatments used.

Western blots. Lysates of COS7 cell transfectants were prepared and analyzed as described previously (20), using the indicated antisera and ^{125}I -staphylococcal protein A (Amersham).

RESULTS

Quantitative assay for transactivation and *trans* inhibition under conditions of limiting Rev protein. We measure the activity of Rev by its ability to induce CAT enzyme expression from the HIV-1-derived reporter plasmid pDM128 in an internally controlled transfection assay (19, 20). Transcripts of pDM128 encode no known viral proteins, but they include a single intron that contains both the CAT coding sequence and the RRE. Because this intron is normally excised in the nucleus, CV1 cells transfected transiently with pDM128 alone express only trace CAT activity; cotransfection with a Rev expression plasmid, however, permits unspliced transcripts of pDM128 to enter the cytoplasm and so increases CAT expression. The extent of CAT induction depends in part on the Rev plasmid used (Fig. 1A). Under standard conditions, as little as 1 μg of pRSV-Rev, which transcribes a Rev cDNA by using the Rous sarcoma virus promoter, yields maximal (approximately 100-fold) CAT induction. By contrast, a related plasmid (ptk-Rev) containing the weaker herpes simplex virus thymidine kinase promoter yields CAT expression in direct proportion to the amount of Rev plasmid

FIG. 1. Quantitative analysis of Rev transactivation and *trans* inhibition in CV1 cells. (A) Induction of CAT enzyme activity from reporter plasmid pDM128 by cotransfection of Rev expression vectors. Each plate of cells received 1 μg of pDM128. Western blot titrations indicate that pRSV-Rev yields at least 25-fold-higher Rev protein expression than does an equivalent amount of ptk-Rev (data not shown). (B) Inhibition of Rev function by dominant negative Rev mutants. Each plate received 1 μg of pDM128, 5 μg of ptk-Rev, and the indicated amounts of one of four mutant forms of pRSV-Rev. The structures of mutants M10, DN2, and DNA are given in Fig. 2A; fs58 is a recessive negative mutant produced by frameshift mutation at codon 58 (20).

transfected and so permits quantitation of Rev activity within an approximately linear range.

In this study, we assayed the ability of selected Rev mutants to block transactivation by ptk-Rev. Because efficient inhibition occurs only when mutants are present in excess (20, 23), these mutants were expressed as derivatives of pRSV-Rev. We initially tested three dominant negative mutants that had been characterized previously (20, 23): M10 and DN2 each contained a pair of missense mutations in the C-terminal domain, whereas DNA was produced by in-frame deletion of residues 78 to 114. As expected, cotransfection of each of these mutants along with 5 μg of ptk-Rev yielded a potent, dose-dependent inhibition of transactivation (Fig. 1B) that was not observed with wild-type Rev or with various N-terminal mutants (20; see below).

***trans*-dominant inhibition of Rev by mutant Rev proteins in the cytoplasm.** We have reported (19) that Rev can be placed under hormonal control by linking it to the steroid-binding domain of the rat GR. This domain lacks the DNA-binding and transcriptional regulatory activities of the intact GR but contains dominant localization signals that anchor it in the cytoplasm when steroids are absent and that transport it into the nucleus upon exposure to Dex (32). When fused to this GR domain, Rev undergoes similar hormone-dependent shifts in localization, with accompanying effects on Rev activity: the Rev/GR fusion protein is completely inactive when sequestered in the cytoplasm but produces transactivation within minutes after entering the nucleus.

To determine whether Rev/GR variants containing dominant negative mutations could act as inducible inhibitors of Rev, we prepared derivatives of the expression plasmid pRSV-Rev/GR that contained the mutations M10, DN2, and DNA, respectively (Fig. 2A). Four Rev/GR variants with mutations in the N-terminal Rev domain were used for comparison (Fig. 2A); we have previously shown that each of the latter mutations completely inactivates Rev and Rev/GR without affecting protein stability (19, 20). Cells

FIG. 2. *trans* inhibition of Rev by dominant negative Rev/GR mutants in the presence or absence of steroid. (A) Structures of wild-type and mutant Rev/GR fusion proteins. Rev/GR was created by fusing the full-length Rev cDNA sequence of HIV-1 strain BRU to codons 511 to 795 of the rat GR cDNA, with Asp replacing Glu at Rev position 116 (19). Unshaded rectangles denote approximate locations of the essential N-terminal and C-terminal domains in Rev. Missense mutations in the seven Rev/GR variants are indicated by transverse bars, with native and mutant amino acids (aa; in single-letter code) at each affected position listed at the left. Thus, R 38,39, G refers to substitution of Gly for Arg at positions 38 and 39. Δ78-114 denotes in-frame deletion of codons 78 to 114. (B and C) In

were first transfected with each Rev/GR mutant alone, and the subcellular localization of the fusion proteins was examined by indirect immunofluorescence in situ, using an antiserum specific for GR. All three C-terminal mutants showed a pattern of localization indistinguishable from that of wild-type Rev/GR: immunoreactive protein was confined almost exclusively to the cytoplasm of cells grown without steroid but was efficiently translocated into the nuclei and nucleoli of Dex-treated cells (Fig. 2B and C). As previously reported, the four N-terminal mutants showed this same hormone-dependent localization, except that MB2/GR and MB3/GR were selectively excluded from nucleoli (19, 20).

Each chimeric protein was then tested for *trans*-inhibitory activity in the presence or absence of Dex (Fig. 2D and E). Control assays confirmed that wild-type pRSV-Rev/GR could induce CAT expression from pDM128 only in the presence of Dex (lane 3) but that Dex treatment did not affect the constitutive activity of ptk-Rev (lane 4) or the ability of unfused DN2 to block Rev (lane 5). Wild-type Rev/GR, as well as some N-terminal mutants, had a modest inhibitory effect when expressed along with Rev in the nuclei of Dex-treated cells (Fig. 2E, lanes 6 to 10); this may resemble the squelching phenomena observed in other systems (33). None of the latter constructs had detectable inhibitory activity in the cytoplasm (Fig. 2D, lanes 6 to 10). By contrast, all three C-terminal mutants were strongly inhibitory (lanes 11 to 13), with two mutants (DN2/GR and DNΔ/GR) producing effects comparable to that of unfused DN2. Remarkably, however, the inhibitory effect of these three mutants was not hormone dependent: efficient *trans*-dominant inhibition occurred even when the mutant proteins remained anchored in the cytoplasm.

This surprising observation was confirmed when we compared dose-response curves for the inhibitory Rev/GR mutants in the presence or absence of Dex. Figures 3A and B illustrate that hormonal treatment had only minor and inconsistent effects on inhibitory activity: each dominant negative fusion protein was as potent in the cytoplasm as in the nucleus. Moreover, the dose-response curve for cytoplasmic DNΔ/GR (open triangles) was essentially identical to that of the most potent unfused mutant (dotted line), making it unlikely that partial proteolysis or the leakage of small amounts of the fusion protein into nuclei could account for this inhibition. Detailed titration in the absence of Dex (Fig. 3C) confirmed that the response to ptk-Rev was not affected by excess wild-type Rev/GR in the cytoplasm (squares) but was inhibited in a dose-dependent manner by cytoplasmic DN2/GR (circles). The effect of any given amount of cytoplasmic DN2/GR was inversely related to the expression of wild-type Rev. These data indicated that *trans* inhibition results from a specific antagonistic interaction that is dependent on the relative concentrations of wild-type and mutant

situ immunofluorescence localization of DN2/GR, using anti-GR antibodies. COS7 cells were grown either without (B) or with (C) 10^{-5} M Dex for 40 h after transfection. Identical results were obtained with DNΔ/GR and with M10/GR (data not shown). (C and D) *trans*-inhibitory activity of Rev/GR mutants in the presence or absence of Dex. CV1 cells were transfected with 1 μ g of pDM128 along with plasmids encoding the indicated proteins and then were grown in the absence (D) or presence (E) of Dex for 36 h prior to CAT assay. Rev denotes 5 μ g of ptk-Rev; remaining designations indicate 10 μ g of wild-type or mutant forms of pRSV-Rev or pRSV-Rev/GR. Data shown are from a single experiment that was repeated three times. Sham, pUC118 alone; No Rev, pDM128 alone.

FIG. 3. Dynamics of *trans* inhibition by Rev/GR mutants in the nucleus and cytoplasm in CV1 transfectants. (A and B) Cells were cotransfected with 1 μg of pDM128, 5 μg of ptk-Rev, and various amounts of wild-type or mutant forms of pRSV-Rev/GR or pRSV-Rev and were then grown in the presence (filled symbols) or absence (open symbols) of 10^{-5} M Dex for 40 h prior to CAT assay. Relative transactivation denotes the level of CAT expression as a fraction of that induced by ptk-Rev alone, after subtraction of the trace activity found in sham-transfected cells. Data in panels A and B are from the same study. (C) Cells received 1 μg of pDM128 and various amounts of ptk-Rev, either alone or in combination with the indicated amounts of pRSV-Rev/GR or pRSV-DNA/GR. CAT expression was determined 36 h after transfection. All cells were grown without Dex.

proteins and further implied that this interaction could take place in either the nucleus or the cytoplasm.

Mutation of the oligomerization domain abolishes *trans*-inhibitory activity. To act as a dominant inhibitor, a mutant must retain one or more functional properties of the wild-type protein (18). Each inhibitory mutant in our series has an intact N-terminal Rev domain, a region known to provide signals for RRE binding, protein localization, and *in vitro* oligomerization. Because RRE-containing transcripts are absent from the cytoplasm of cells that express a dominant negative Rev mutant (23), our finding that such mutants can act in the cytoplasm suggested that neither RRE binding nor nuclear localization was essential. We therefore examined whether the known oligomerization signals of Rev (27, 30, 40) were necessary for dominant negativity.

For this purpose, two alternative triple-point mutations were introduced into DNAΔ/GR (Fig. 4A). One of these mutations (designated MA4) involved sequences within the N-terminal domain and was known to prevent Rev oligomerization *in vitro*; the second (MA1) involved nearby non-

essential sequences outside this domain (20, 40). Neither mutation affects localization of Rev/GR (20). Western blot analysis, using antisera specific for the N terminus of Rev, confirmed that each mutant was expressed at a level similar to that of Rev/GR and DNAΔ/GR (Fig. 4B). Whereas the MA1 mutant gave substantial *trans* inhibition of Rev in the cotransfection assay, however, DNAΔ/GR-MA4 proved to have little or no inhibitory activity (Fig. 4C). Similar results were obtained when the same mutations were introduced into DN2/GR: the MA4 mutation (but not MA1) completely abolished inhibitory activity (data not shown). Cytoplasmic *trans* inhibition thus required specific N-terminal sequences that have been implicated in Rev protein oligomerization; this finding suggested that inhibition might result from an interaction between the wild-type and mutant proteins themselves.

Wild-type Rev protein forms a complex with a *trans*-inhibitory mutant *in vivo*. We therefore sought direct evidence that these proteins interact *in vivo*, using an immunofluorescence colocalization assay. This assay made use of

FIG. 4. Evidence that dominant negative activity of cytoplasmic DNAΔ/GR is abolished by a mutation in the N-terminal Rev domain. (A) Structures of two N-terminal mutants of DNAΔ/GR, depicted as in Fig. 2A. (B) Western blot detection of Rev, Rev/GR, and DNAΔ/GR derivatives in transfected COS7 cells. Proteins were detected by using a primary antiserum directed against the N-terminal half of Rev, after fractionation on a denaturing 14% polyacrylamide gel. Positions of 14 C-labelled protein standards are indicated at the left. (C) CAT expression in CV1 cells transfected with 5 μg of ptk-Rev (Rev) and 1 μg of pDM128 either alone or in combination with 10 μg of the indicated derivatives of pRSV-DNAΔ/GR. Sham, pUC118 alone; No Rev, pDM128 alone.

FIG. 5. Colocalization of wild-type Rev with the dominant negative fusion protein DNA/GR. (A) DNA/GR is not detected by an antiserum against the C-terminal portion of Rev. The Western blot was prepared by using the same protein extracts depicted in corresponding lanes of Fig. 4B. Positions of ^{14}C -labelled protein standards are indicated at the left. (B and C) Hormone-dependent localization of Rev in the presence of DNA/GR. COS7 cells were transfected with 10 μg of each plasmid indicated and then were grown for 36 to 40 h in the absence (B) or presence (C) of Dex. Indirect immunofluorescence staining was performed in situ, using a primary antiserum specific for C-terminal sequences that are found in Rev but not in DNA/GR. Each pattern shown here was identified under triple-blind conditions in two to six plates of cells from two or more independent experiments and typified the vast majority of stained cells in each plate. DNA/GR was confirmed by using anti-GR antibodies in three additional pairs of plates (one of which was cotransfected with ptk-Rev), but no

the fact that DNA/GR lacks the sequences recognized by an antiserum directed against the C-terminal half of Rev (Fig. 5A). Thus, when cells that coexpress Rev and DNA/GR are stained with this antiserum, only the location of the wild-type protein is observed. We reasoned that if Rev tended to bind the dominant negative mutant, then the presence of excess DNA/GR in the nucleus or cytoplasm might perturb localization of Rev in a hormone-dependent fashion.

As illustrated in Fig. 5B and C, this proved to be the case. Cells were cotransfected with pRSV-DNA/GR and ptk-Rev in the same relative amounts used for CAT assays; to eliminate bias, immunofluorescence analysis was performed under a triple-blind protocol (see Materials and Methods). As expected, no immunoreactive protein was detectable in cells receiving pRSV-DNA/GR alone (data not shown). Cells transfected with ptk-Rev alone stained less intensely overall, and in a somewhat different pattern, than those receiving pRSV-Rev: in ptk-Rev transfectants, no nucleoplasmic staining could be detected, and the prominently stained nucleoli were usually accompanied by distinct staining of the perinuclear cytoplasm. Irrespective of the plasmid used, Dex treatment had no effect on the pattern of staining in cells that expressed only Rev.

In cells that coexpressed DNA/GR in excess, however, Rev acquired a hormone-dependent pattern of localization (Fig. 5B and C). When steroids were absent, most of the immunoreactive protein remained in the cytoplasm, though significant levels of nucleolar staining also were detected. Upon exposure to Dex, however, essentially all of the protein accumulated within nuclei, where it could be found throughout the nucleoplasm as well as in nucleoli. This hormone-dependent translocation of unfused Rev was detected under triple-blind conditions in all six pairs of samples cotransfected with pRSV-DNA/GR and was apparent in the vast majority of immunoreactive cells from each sample. By contrast, no such effect was detected in cells that expressed Rev together with the oligomerization-defective mutant DNA/GR-MA4.

Taken together, these data indicate that the inhibitory mutant DNA/GR interacts with wild-type Rev to form stable heteromeric complexes within the cell, that the localization of these complexes is hormone dependent, and that complex formation can be at least partially inhibited by a mutation (MA4) in the Rev oligomerization signals. The functional consequences of the MA4 mutation (Fig. 4C) further suggest that the capacity to form such complexes may be essential for dominant negative activity.

DISCUSSION

Oligomerization of Rev. Recent studies have demonstrated that bacterially synthesized Rev protein tends to oligomerize in vitro and in vivo. Chemical cross-linking and gel filtration data indicate that Rev exists predominantly as a tetramer (29, 40), and there is evidence that higher-order multimers can form in vitro even in the absence of RNA (17). Mutations that prevent oligomerization eliminate Rev activity, but the basis of this loss of activity remains controversial. While some studies indicate that oligomerization may be a prereq-

staining was detected by using the Rev C-terminal antibody in cells transfected with pRSV-DNA/GR alone (three plates) or in sham-transfectants (four plates). Conditions of photographic printing differ somewhat to compensate for differences in overall fluorescence intensity; magnification is similar in all panels.

uisite for RRE binding (30, 40), others suggest that monomers of Rev can bind the RRE and subsequently oligomerize on the RNA, and that it is this substrate-dependent oligomerization which is essential for function (7, 27). Either of these mechanisms (or a combination of the two) could account for the ability of a single RRE to bind up to eight Rev monomers simultaneously (7, 16). Significantly, both RRE-dependent and RRE-independent oligomerization appear to be mediated by the same sequences in Rev, implying that the two phenomena are closely related: both can be blocked by N-terminal mutations (particularly at residues 14 to 26), but neither is affected by mutations in the C-terminal domain (27, 30, 40).

Our studies now provide evidence that wild-type and dominant negative forms of Rev interact to form mixed multimers in vivo. In particular, we have used an immunofluorescence colocalization assay to demonstrate that Rev forms a complex with the dominant negative Rev fusion protein DNA/GR. The interaction between these two proteins was observed in the absence of any known RNA substrate and was strong enough to affect the subcellular distribution of Rev: complexed with an excess of DNA/GR, Rev tended to be retained in the cytoplasm when steroids were absent but was translocated efficiently to the nucleus upon exposure to Dex (Fig. 5B and C). The specificity of interaction was confirmed by our finding that a mutation (MA4) known to prevent in vitro oligomerization of Rev (40) also abolished colocalization.

These results are compatible with the view that intracellular complexes form through a direct association of N-terminal Rev domains similar to that observed in vitro (30, 40). We cannot, however, be certain that the two phenomena are identical. It remains to be determined, for example, whether specific cellular proteins or RNAs are also present within these complexes and whether such cellular factors might be involved in Rev function. Owing to the design of the colocalization assay, we could not determine whether Rev can form similar complexes with wild-type Rev/GR. However, the finding that overexpression of cytoplasmic Rev/GR does not reduce transactivation by unfused Rev (Fig. 2D and E and 3C) suggests that complexes comprising only wild-type monomers may be more labile than those containing a dominant negative mutant, at least outside the nucleus. By preventing interactions of the C-terminal domain with cellular proteins, dominant negative mutations might conceivably strengthen or prolong the linkage between Rev monomers in a cellular milieu. The present results demonstrate that stable heteromeric Rev complexes can form within cells; additional studies will be needed to determine the composition of these complexes and the requirements for their formation.

Mechanism of *trans*-dominant inhibition of Rev. The C-terminal mutations that confer dominant negativity affect neither the subcellular localization of Rev nor its ability to bind the RRE. On the basis of these observations, it has been proposed that such mutants (perhaps in oligomeric form) might interfere with Rev function by competing for a limiting supply of RRE-containing transcripts in the nucleus (11, 23, 24, 30, 39). The results of the present study, however, argue against this view. In particular, we find that the effect of a dominant negative mutant is entirely independent of its location within the cell: whereas Rev/GR itself can transactivate only after entering the nucleus, each *trans*-inhibitory fusion protein that we tested was as potent in the cytoplasm as in the nucleus (Fig. 2), and two were as potent as their unfused counterparts.

This finding implies that *trans* inhibition does not require

competition for nuclear transcripts or any other uniquely nuclear event. While it is possible that repression could occur through different mechanisms in the nucleus and cytoplasm, the virtually identical dose-response curves obtained in the presence or absence of Dex (Fig. 3) argue that a single process is involved. Some reports have suggested that Rev can alter the stability or translational efficiency of RRE-containing mRNAs in the cytoplasm (2, 14, 22), but such effects could not readily account for our results, as we detect no activity of wild-type Rev/GR in the cytoplasm.

Our data suggest a different interpretation. We have shown that Rev forms a heteromeric complex with the dominant negative protein DNA/GR and that the intranuclear concentration of these complexes is much higher in the presence of Dex than in its absence (Fig. 5B and C). If Rev remained active within these complexes, hormone treatment would be expected to enhance transactivation under conditions in which Rev is limiting; on the other hand, if the complexes competed effectively for RRE binding, transactivation would tend to decline after Dex treatment. In practice, however, neither an increase nor a decrease is observed (Fig. 3A and B): the complexes appear functionally inert.

This finding suggests that dominant negative mutants inactivate Rev directly by forming stable but nonfunctional complexes with the wild-type protein. This model requires no assumption as to the nature of the complexes, but it is consistent with the notion that cellular Rev forms multimers similar to those observed in vitro. If such complexes also incorporate cellular cofactors that are necessary for Rev activity, the sequestration of these cofactors within nonfunctional complexes would tend to produce a further inhibitory effect on transactivation. This, in turn, might account for the ability of cytoplasmic DNA/GR to block transactivation completely (Fig. 2D and E) even when a small proportion of wild-type Rev gains access to the nucleus (Fig. 5B and C). The finding that complexes containing a dominant negative mutant are biologically inactive may indicate that two or more intact C-terminal domains must be juxtaposed in order to induce RNA export. Any residual Rev activity observed when cells express a mixture of mutant and wild-type monomers could then be attributed to the proportion of wild-type complexes that form. Under this model, the capacity to form complexes is essential for *trans* inhibition, a view supported by our finding that a mutation (MA4) which inhibits Rev/Rev interaction in vivo and in vitro abolishes dominant negative activity. Consistent with this model, a multimerization-defective Rev mutant that reportedly has undiminished RRE binding activity (27) shows a recessive negative phenotype (23).

We conclude that the dominant negative phenotype of C-terminal Rev mutants reflects their ability to form nonfunctional complexes with the wild-type protein and perhaps with unidentified cellular factors as well. It remains to be determined whether a similar mechanism underlies the inhibition of Rev by dominant negative mutants of the Rex protein from human T-cell leukemia virus type I (3, 34).

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