Identification of *trans*-dominant HIV-1 rev protein mutants by direct transfer of bacterially produced proteins into human cells

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ABSTRACT

A synthetic *rev* gene containing substitutions which introduced unique restriction sites but did not alter the deduced amino acid sequence was used as a vehicle to construct mutations in *rev*. Insertion or substitution mutations within a domain of Rev resulted in proteins able to inhibit the function of Rev protein in *trans*. Rev function was monitored in a cell line, HLfB, which contained a *rev*⁻ mutant provirus. HLfB cells require the presence of *rev* for virus production, which was conveniently monitored by immunoblot detection of p24^{gag}. Trans-dominant mutants were identified after expression in bacteria and delivery into HLfB cells by protoplast fusion. In addition, the trans-dominant phenotype was verified by expression of the mutant proteins in HLfB cells after cotransfection. These studies define a region between amino acid residues 81 and 88 of *rev*, in which different mutations result in proteins capable of inhibiting Rev function.

INTRODUCTION

Replication of the human immunodeficiency virus, HIV-1, requires the regulated expression of viral genes (1–4). Regulation of proviral gene expression is achieved by the concerted action of both cellular and viral factors. The *tat* protein is a positive regulator of viral expression which controls the overall level of mRNA initiated from the viral long terminal repeat (LTR) promoter. *Tat* acts in conjunction with a *cis*-acting sequence in the R region of the LTR to mediate an accumulation of all viral transcripts (3, 5–13). The viral *rev* protein, a posttranscriptional positive regulator, influences the cytoplasmic levels of differentially spliced viral mRNAs. *Rev*⁻ mutant proviruses produce low levels of *gag-pol* and *env* mRNAs (14, 15) and the corresponding protein products. No virus is produced by such mutants (14–16). *Rev* promotes the transport from the nucleus into the cytoplasm, and the increased stability of a subset of viral mRNAs which share a *cis*-acting sequence element, the *rev*-responsive element (RRE) (3, 15, 17–22). As a consequence of *rev* action, there is increased expression of the *gag*, *pol*, and

env proteins (which are encoded by RRE-containing transcripts), leading to virus production. The *rev* gene product is a 19 kD phosphoprotein localized primarily in the nucleoli of *rev*-overproducing cells (17, 23–26).

Strategies designed to interfere with HIV-1 replication require the exploitation of viral-specific processes. One approach to this problem involves the introduction of *trans*-dominant mutant genes into susceptible cells. Intracellular expression of the mutant proteins would interfere with the function of the wild-type gene product, rendering an infecting virus phenotypically mutant. Mutations in viral genes often demonstrate a recessive phenotype, however, allowing complementation by wild-type alleles. *Trans*-dominant mutations are observed in the case of oligomeric proteins (27) or when the mutant saturates a necessary target site. In theory, cells producing *trans*-dominant mutant viral proteins should be refractory to superinfection by wild-type virus. This has been confirmed in the case of Herpes simplex virus (HSV) (28).

Trans-dominant mutations have been reported in a number of regulatory genes (29). Since HIV-1 *rev* mutants produce no virus, the *rev* gene provides an attractive target for antiviral intervention. We were interested in developing a panel of *trans*-dominant *rev* mutants which might provide a framework for the generation of useful antiviral agents. We elected to use bacterial expression as a central feature of a mutant screening procedure, since production in *E. coli* could simplify purification and biochemical characterization of the proteins. We report here on the synthesis of functional and mutant *rev* proteins in *E. coli* and the delivery of the proteins into indicator HeLa cell lines by protoplast fusion (30–33). Protoplast fusion had previously been used to detect the HTLV-1 transactivator protein, *tax* (34) after expression in *E. coli*. In preliminary experiments (Mermer *et al.*, unpublished data), we adapted this method to monitor the bacterial expression of functional HIV-1 transactivator (*tat*) protein with the indicator cell line HL3T1 (13). Since the technology proved very successful for *tat*, we decided to use the method for the analysis of *rev* and to extend it to the analysis of *trans*-dominant mutants. *Rev* mutants were constructed directly in bacterial expression plasmids, and the functional and variant *rev* proteins were

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expressed in *E. coli* cells. Protoplasts were prepared from these cells, and the bacterially expressed protein was analyzed in a functional assay after introduction into an indicator cell line by polyethylene glycol (PEG)-mediated protoplast fusion.

We have used this assay to identify three HIV-1 *rev* mutants which interfere with the function of wild-type *rev* in a *trans*-dominant manner. These findings have been confirmed by transient expression studies after transfection of the indicator cell line HLfB with eukaryotic vectors expressing these mutant proteins. A *trans*-dominant mutant in the HIV-1 *rev* gene in a location similar to the one identified here was recently reported by other investigators (35). These studies define a region of ten amino acid residues on *rev*, which is sensitive to a variety of changes resulting in a similar *trans*-dominant phenotype.

MATERIALS AND METHODS

Bacteria and plasmids

Plasmids were constructed in the *recA E. coli* strain DH5 α (Bethesda Research Laboratories). The *lon* protease mutant *E. coli* BU8049 (36) was used for expression of *rev*. The bacterial expression vector pUC12N (37, 38) contains an *Nco*I restriction site just upstream of the pUC12 polylinker, at the beginning of the *lac* open reading frame. The *Lac* phenotype of DH5 α transformants was assessed on *Lac* indicator media containing X-gal. A synthetic *rev* gene (*srev*) was constructed by ligation of six pairs of complementary oligonucleotides with an average length of 60. The correct arrangement of the oligonucleotide cassettes was insured by providing complementary, single-stranded cohesive ends. The full-length 360-bp product, which contained an *Nco*I cohesive end at the beginning of the *rev* open reading frame, was gel-purified and ligated to pUC12N which had been digested with *Nco*I and *Hind*III. Since the insertion of the synthetic gene maintained the open reading frame into *lac*, the appropriate recombinant plasmid was expected to confer a *Lac*⁺ phenotype on DH5 α . Plasmid DNA from primary *Lac*⁺ transformants was purified by retransformation of DH5 α , and a recombinant with the appropriate insert, named pUCsrev α , was identified by DNA sequencing. To construct the *srev* expression plasmid pUCsrev, pUCsrev α was digested with *Eco*RI and religated after repair synthesis at the *Eco*RI cohesive ends. The resulting four base-pair insertion generated a termination codon at the end of the *srev* gene. pUCsrev encoded a *rev* protein with one additional amino acid residue at the carboxyl terminus which had no apparent effect on *rev* function. Mutants in the *srev* gene were constructed by oligonucleotide substitution or insertion. The 12-mer palindromic linker used to construct mutant BL, GGAAGATCTTCC, was inserted at the *Hpa*I site of plasmid pUCsrev. The SL mutant was constructed by insertion of the linker TCCCCGCGGGGA at the *Hpa*I site. To construct the HS mutant, an oligonucleotide containing the three point mutations (Figure 1B) was substituted for the *Hpa*I-*Spe*I fragment in pUCsrev. The mutations were confirmed by DNA sequencing. For expression in HeLa cells, the expression vector LdK3pA was used. LdK3pA is a derivative of pL3pA (17) that contains the HIV-1 LTR promoter from the KpnI to *Hind*III site (–524 to +80) and the Simian virus 40 polyadenylation site. This vector allows for the expression of genes inserted into the polylinker between the HIV-1 LTR promoter and the Simian virus 40 polyadenylation signal. Like the bacterially expressed proteins, the mutant *rev* proteins expressed in these vectors had one additional amino acid at the carboxy terminus as compared with the *rev* protein of the infectious molecular HIV-1 clone HXB2.

Cell lines

The HLfB cell line was generated by cotransfection of HeLa cells with the *rev*[–] provirus fB (14, 15, 39) and pSV2neo (40). Provirus fB contained a four bp insertion at the *Bam*HI site in *rev* (nt 8021). G418 resistant transformants were screened for the production of *env* and *gag* products after complementation of the *rev* defect by polyethylene glycol (PEG)-mediated fusion (9) with the *rev*-producing cell line HLtat-*rev* (Felber et al., unpublished). One high producer cell line, (HLfB 189–45), was used for the experiments reported here. This cell line was negative for virus production as determined by several methods including cocultivation with the indicator cell line H938 (3), electron microscopy, reverse transcriptase assays, and p24^{gag} antigen capture assay. High levels of virus were produced following the introduction of *rev* protein by PEG-mediated fusion with *rev*-producing cell lines.

Protoplasts

Protoplasts were prepared by a modification of published procedures (30,41). Transformants in *E. coli* BU8049 were grown in M9CA medium (42) containing 0.4% glycerol until the late logarithmic phase of growth. A quantity of cells equivalent to 10 O.D. units was sedimented and washed with 10 mM Hepes pH 7.5 at room temperature. Pellets were gently resuspended in 1.0 ml of 10 mM Hepes, 20% sucrose (HBS-20), which had been prewarmed at 37°C. Lysozyme digestion, at a concentration of 0.1 mg/ml, was carried out at 37°C for 12 minutes with slow shaking. 0.1 ml of 0.1M EDTA pH 8.0 was added slowly with shaking, and the suspension was incubated at 37°C for 8 minutes with slow shaking. Examination of the culture by phase contrast microscopy consistently revealed virtually 100% conversion to protoplasts with this protocol. Protoplasts were pelleted at 6,000 rpm in an Eppendorf centrifuge, resuspended in the same volume of HBS-20, and added slowly to 4.0 ml of phosphate buffered saline (PBS) containing 9% sucrose (PBS-9).

Complementation assay

For the complementation experiments shown in Figures 3, 4, and 5, 1.2×10^6 HLfB cells were plated in 60 mm plates. The following day, 5×10^9 protoplasts in 2.5 ml PBS-9 were added to the washed monolayer by centrifugation at 2000 rpm for 2 minutes in a Sorvall RT 6000 centrifuge. PEG-mediated fusion was performed as previously described (9). Briefly, the supernatant was aspirated and 1.5 ml of a 50% PEG solution in Dulbecco's modified Eagle medium (DMEM), prewarmed at 37°C, was added gently and allowed to incubate at room temperature for 1 min. The PEG solution was aspirated and the plates were washed twice with 5 ml of DMEM. Plates were incubated at 37°C with 5 ml DMEM containing 50 μ g/ml gentamycin. Washed cells were harvested the following day in 0.4 ml of 0.5 $\times$ RIPA buffer as described (15). For the protoplast competition experiment (Figure 4), proteins were concentrated by precipitation with methanol-acetone before immunoblot analysis. Quantitation of the immunoblot (Figure 4B) was performed with a radioanalytic imaging system and software from AMBIS (Automated Microbiology Systems, Inc.). To normalize for experimental variability, the results were expressed as p24/p55 ratio in Figure 4B. The ratio obtained in the presence of only functional *rev* was assigned the value of 100. Since *rev* was introduced into a small proportion of the cells in these experiments, the effect on the level of p55^{gag} production was small. Therefore, p55^{gag} can serve as an internal normalization standard in these experiments.

A

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atggcaggaagaagcggagacagcgcgaagagctcatcagaacagtcagactGatcaag
1  M A G R S G D S D E E L I R T V R L I K

cttctctatcaaagcaaccacctcccaatcccgaggggacGcgTcaggcGcgCaggaat
21  L L Y Q S N P P P N P E G T R Q A R R N

agaagGCGCCggtggagagagagacagagacagatccattcgatATCtgaacggatcctt
41  R R R R W R E R Q R Q I H S I S E R I L

ggcacttatctgggacgatctgcAgagcctgtgcctcttcagctaccaccgcttgagagG
61  G T Y L G R S A E P V P L Q L P P L E R

TtAactcttgattgtaacgaggattgtggaactAGtgggacAcagggTgtgggGTCacct
81  L T L D C N E D C G T S G T Q G V G S P

caaatattggtggaatctcctacagtaCtCgagtcaggaactaaagaGAATTaattc
101 Q I L V E S P T V L E S G T K E N *

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Figure 1. A. Sequence of the synthetic *rev* gene in pUCsrev, and the deduced amino acid sequence of its translated product. 'Silent' mutations which introduced restriction sites are in upper case. B. Mutant *rev* proteins. MT-REV contains 8 amino acids of metallothionein in place of 26 amino acids at the N-terminus of *rev*. X11 has a deletion of the 14 C-terminal amino acids of *rev*. fB is a frameshift mutant after the 52nd *rev* codon. The diagram also shows the changes in mutants BL, SL and HS. Upward arrows mark the insertions in mutants BL and SL after residue 81. Downward arrows mark the substitutions in mutants SL and HS. The function of these mutants expressed either in bacteria or in human cells is shown at the right, as determined by complementation experiments. nd, not determined.

Transfections

Plasmid DNA was prepared using the Triton-X100 cleared lysate procedure and was purified twice by cesium chloride density-gradient centrifugation in the presence of ethidium bromide. Calcium phosphate mediated DNA transfer into cell line HLfB

was performed as previously described (17, 43,44). Cells were harvested after 24 hours in 0.4 ml of 0.5X RIPA buffer (15).

Immunoblots

Sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis and immunoblots were performed as previously described (15,

45). Intracellular HIV-1 proteins were detected using a mixture of patient antiserum and gp120 specific rabbit antiserum. Rabbit antibody directed against intact rev used in the immunoblot in Figure 2 was provided by Dr. P. Chanda, Wyeth, Inc.

RESULTS

Construction of rev mutants

Rev is a small protein of 116 amino acid residues. Functional analysis of a variety of deletion mutants after transfection in human cells has indicated that the amino and carboxy termini of the protein are not essential for function (Figure 1B). In initial experiments we replaced the 25 amino-terminal amino acids of rev with six amino acids of mouse metallothionein. This protein retained partial Rev activity (data not shown). In addition, functional analysis of the Tev hybrid protein produced by HIV-1 indicated that the 25 amino-terminal amino acid residues were not essential for rev function (46). Deletion of 14 amino acids at the carboxy terminus resulted in a functional rev protein, while deletion of 73 amino acids resulted in an inactive rev (15). Recently, it was reported that the 25 carboxy-terminal residues are dispensable (25). A loss of rev function was also observed in a frameshift mutant at the *Bam*HI site at codon 56 (14, 15, 17, 39). The phenotypes of these mutants suggested that a region including amino acid residues 56–91 was essential for the expression of a functional product. We therefore focused our initial efforts on this region. A series of highly conserved proline residues 70, 72, 76, and 77 separate a conserved basic domain (residues 35–50) from a conserved acidic domain (residues 84, 87, and 88). Three mutants in or near the acidic domain were analyzed (Figure 1B). To permit site-directed mutagenesis of double stranded plasmid DNA, a synthetic *rev* (*srev*) gene was constructed (Materials and Methods). Several 'silent' mutations were included which introduced a variety of convenient restriction sites. The deduced amino acid sequence of the synthetic *rev* gene (Figure 1A) was the same as in the infectious HIV-1 isolate HXB2. Mutant HS, which was constructed by oligonucleotide substitution between the *Hpa*I and *Spe*I sites (rev codons 81 and 91, respectively), contained lysine residues instead of the acidic residues at positions 84, 87 and 88. Mutant BL contained an insertion of four amino acid residues (Glu-Asp-Leu-Pro), after leucine residue 81. Mutant SL contained a substitution of phenylalanine for leucine (position 81), and an insertion of four amino acid residues (Pro-Arg-Gly-Glu) (Figure 1B).

Expression of rev in *E. coli*

The synthetic *rev* gene was constructed in the bacterial expression vector pUC12N to permit expression of the functional and mutant proteins in *E. coli*. In an effort to maximize the expression of rev in bacteria, the protease defective strain BU8049 (36, 47) was used. Plasmids encoding wild-type and mutant rev proteins were introduced into strain BU8049, and transformants were grown in minimal glycerol medium. Exponentially growing cultures were harvested and rev products were detected by immunoblot analysis (Figure 2). Mutants HS, BL and wild-type rev were expressed at approximately equivalent amounts (lanes 1, 3 and 2, respectively). The mutations in derivatives BL and HS markedly altered the electrophoretic mobilities of the mutant proteins.

Functional assay for rev and rev mutants

We used the indicator cell line HLfB (Materials and Methods) to establish a functional assay for the bacterially expressed rev

Figure 2. Expression of wild-type and mutant rev proteins in *E. coli*. BU8049 transformants were grown to late logarithmic phase, harvested by centrifugation, lysed in gel loading buffer, and equal quantities of lysate were subjected to electrophoresis on a 15% SDS-polyacrylamide gel. Rev proteins were detected by immunoblot analysis with a rev-specific antibody. Lysates were from bacteria containing plasmids expressing mutant HS (lane 1), functional rev (lane 2), mutant BL (lane 3), and pUC12N control (lane 4). The positions of ^{14}C -Rainbow markers (Amersham, Inc.) are indicated.

Figure 3. Functional assay for bacterially expressed rev proteins. Protoplasts prepared from BU8049 transformants expressing the indicated proteins were fused with HLfB cells in 60 mm plates. Cellular lysates prepared 24 hours later were analyzed by immunoblotting. Each lane contained lysate from 1/10 of the plate. Proteins were detected with HIV-1 patient serum. (This serum did not recognize the env protein). Lane 1, rev; lane 2, pUC12N control; lane 3, BL; lane 4, HS; lane 5, lysate from chronically infected H9/HXB2 cells. The positions of p55^{gag} and p24^{gag} are indicated.

proteins. HLfB cells contain stably integrated *rev*⁻ mutant proviruses. Due to the absence of rev, these cells produce relatively low levels of viral structural gene products. Thus, immunoblots of lysates from HLfB cells reveal low levels of the gp120 and gp160 products of the *env* gene. Although HLfB cells contain readily detectable amounts of the gag precursor, p55^{gag}, no processed gag is detected on immunoblots and no virus is produced by these cells. Rev supplied by transfection results in increased expression of viral structural proteins. The most notable effects of providing rev to HLfB cells is the accumulation of large

Figure 4. Dose-response analysis of *trans*-dominant *rev* mutants. **A.** Complementation assay was performed as in Figure 3. Protoplasts were prepared from BU8049 transformants expressing the indicated proteins and mixed in the indicated ratios before fusion. 5×10^8 protoplasts containing functional *rev* protein were used throughout. Protoplasts derived from pUC12N/BU8049 were used to maintain 5×10^9 total protoplasts in each dish. Each lane contained concentrated lysate representing 1/2 of the 60 mm dish. Lanes are: lane 1, HeLa cell lysate; lane 2, pUC12N control; lane 3, BL + *rev*, 2:1; lane 4, 4:1; lane 5, 10:1; lane 6, HS + *rev*, 2:1; lane 7, 4:1; lane 8, 10:1; lane 9, *rev* (no competitor); lane 10, lysate from chronically infected H9/HXB2 cells. The proteins were detected using a mixture of HIV-1 patient serum and an anti-*env* rabbit antiserum. The positions of viral *env* and *gag* proteins are indicated. **B.** Radioactivity in the p55^{gag} and p24^{gag} bands of Figure 4A was measured by scanning the immunoblot with the AMBIS radioanalytic imaging system (Materials and Methods). The results are presented graphically as the normalized p24/p55 ratio. The ratio obtained in the presence of only functional *rev* (Fig. 4A, lane 9) was assigned the value of 100.

quantities of the processed gag product p24^{gag}, (a marker for mature virus particle production), and the production of virus particles. Since production of p24^{gag} in HLfB cells is detectable only in the presence of rev, the appearance of p24^{gag} serves as a convenient assay for functional rev protein.

We analyzed the ability of the bacterially expressed rev and the rev mutants to complement the *rev* defect in HLfB cells. Functional or mutant rev proteins were expressed in *E. coli* and were introduced into HLfB cells by protoplast fusion. The effect of rev proteins on the synthesis of viral structural proteins encoded by the mutant provirus in HLfB cells was assessed by immunoblotting (Figure 3). Control protoplast fusions (lane 2), gave immunoblot patterns which were indistinguishable from untreated HLfB lysates (not shown). The introduction of bacterially expressed rev protein resulted in the appearance of p24^{gag} (lane 1). In contrast, the mutant rev derivatives BL and HS caused no detectable accumulation of p24^{gag} (lanes 3 and 4), even after much longer autoradiographic exposure time.

Identification of Trans-Dominant rev mutations

Protoplast fusion was also used to determine if the rev mutants HS and BL might interfere with normal rev function. Protoplasts containing each of the mutants were mixed at various ratios with a constant quantity of protoplasts carrying wild-type rev protein, and the protoplasts were fused with HLfB cells. The total number of protoplasts in each fusion was kept constant by the addition of protoplasts carrying the pUC12N expression vector alone. Figure 4A shows an immunoblot analysis of this dose-response experiment. Fusion using rev-containing protoplasts resulted in the production of p24^{gag} (lane 9). The addition of increasing amounts of BL-containing protoplasts to a constant amount of protoplasts carrying functional rev progressively inhibited the production of p24^{gag} (lanes 3–5). A similar pattern of inhibition was observed by the addition of increasing amounts of mutant HS protoplasts (lanes 6–8). Thus, both mutants displayed a dose-dependent dominant phenotype in this assay, as evidenced by the decreasing levels of p24^{gag}. Quantitation of the radioactivity on the immunoblot (Materials and Methods) revealed that at a ratio of 10:1 (HS:rev), p24^{gag} production was inhibited by 80% (Figure 4B). In addition, the levels of gp160^{env} and gp120^{env} were also decreased in the presence of the mutants. The levels of p55^{gag} were affected to a lesser extent.

Transfection analysis of trans -dominant mutants

To determine whether the *trans* -dominant phenotype was a result of the synthesis of the proteins in bacteria, expression of the mutant rev proteins in eukaryotic cells was used to confirm the phenotypes identified by the protoplast fusion experiments. In addition to the insertion mutant BL, another insertion mutant, SL (Figure 1B), was selected for this analysis. The phenotype of mutant HS was not addressed by transfection experiments. The mutant synthetic *rev* genes were transferred to a vector suitable for expression in eukaryotic cells (LdK3pA, see Materials and Methods). Plasmid pL3crev (17) which expresses the HXB2-derived *rev* gene in a similar vector, was used for the expression of functional *rev* in these experiments. The immunoblot analysis of viral proteins produced after transfection is presented in Figure 5. As expected, transfection of HLfB cells with pL3crev caused an increase in gag and env expression and the appearance of p24^{gag} (lane 1). Transfection with mutants BL (lane 2) or SL (lane 3) had no effect on viral expression. When the mutant expression plasmids were cotransfected with plasmid

Figure 5. Transfection analysis of *trans*-dominant rev mutants. *Rev* genes encoding the mutants BL and SL were transferred to eukaryotic expression vector pLdK3pA (Materials and Methods), generating plasmids pL3BL and pL3SL, respectively, for transfection of HLfB cells. Plasmid pL3crev, which expresses the HXB2 *rev* gene was used to complement the mutation in HLfB cells. Two days later, the cells were harvested and the proteins were separated on a 12.5% SDS polyacrylamide gel. HIV-1 proteins were detected with a mixture of patient serum and gp120 antibody. Cells were transfected with the following plasmids: 1 μ g pL3crev (lane 1), 16 μ g pL3BL (lane 2), 16 μ g pL3SL (lane 3), 1 μ g pL3crev + 16 μ g pL3BL (lane 4), 1 μ g pL3crev + 16 μ g pL3SL (lane 5), 17 μ g pLdK3pA expression vector. All DNA mixtures were adjusted to 17 μ g with salmon sperm DNA. The positions of HIV-1 gag and env proteins and of the mutant rev products (mrev) are indicated.

pL3crev, the mutant phenotype was dominant (lanes 4 and 5). At a ratio of 16:1, the activation of p24^{gag} production by rev was completely abolished. Subsequent experiments demonstrated that 60% inhibition of Rev function was achieved by cotransfection of an equal amount of SL mutant (46). As indicated in Figure 5, the mutant rev proteins were detectable on the immunoblot, verifying their expression in HLfB cells. The BL and SL mutant proteins had distinguishable mobilities on these gels.

DISCUSSION

A large number of transcriptional activators have recently been described which function by the concerted action of distinct binding and activation domains within the protein (29). This

structural feature may render regulatory proteins susceptible to mutations producing *trans*-dominant variants. Mutations which selectively interfere with activation would thus be predicted to inhibit the function of the wild-type product in a *trans*-dominant manner. We have shown that insertions (mutants BL and SL) and substitutions (mutant HS) in the area of residues 81–88 in rev, a protein which acts at the post-transcriptional level, result in a *trans*-dominant mutant phenotype. Although the understanding of the structural basis for this phenomenon requires further investigation, these mutations may have disrupted one site involved in rev function without affecting the binding to a critical substrate. While it is possible that the mutations in BL, SL, and HS directly perturbed an active site, it is equally likely that the effect was due to conformational changes. Another *trans*-dominant mutant with substitutions at residues 79 and 80 was recently reported (35). Taken together with the results reported here, it can be concluded that a variety of mutations between residues 79–88 render the product *trans*-dominant. This supports the contention that this phenotype results from the loss of some property rather than the acquisition of one (35).

An alternative mechanism could account for a *trans*-dominant phenotype. Oligomeric proteins may be rendered nonfunctional by the presence of one or more mutant monomers in the complex (27). It is not currently known if rev functions as an oligomer. Since the proteins analyzed in the mixed protoplast fusion assay (Figure 4) were synthesized in separate bacterial strains, we think it unlikely that they were dominant by virtue of this mechanism, assuming that putative oligomers formed in bacteria would not reassort following fusion. The precise nature of the rev target is unknown and may include cellular and viral components. The rev responsive element (RRE), a unique sequence element in the *env* region of viral RNA, is required for rev function (15, 17–22). *Trans*-dominant mutants which saturated viral target sites like RRE would be particularly useful for the specific inhibition of viral expression. Such mutants would be preferable to mutants which exerted an inhibitory effect by saturating putative cellular targets since this could prove toxic to the cell.

Agents which inhibit the function of essential viral factors may protect cells from viral infection. In the case of HSV, for example, a *trans*-dominant mutant viral protein has been shown to have this protective property (28). The *trans*-dominant rev mutants reported here would thus be candidates for further analysis as inhibitors of HIV-1 replication in susceptible cells. In light of the complexity of the HIV-1 replication cycle, it is likely that a variety of factors will be relevant to the outcome of an infection. For example, the dose-response properties of these mutants suggest that the levels of expression of rev after infection will be a critical parameter. As indicated in Figures 4 and 5, high levels of inhibition required high ratios of mutant to wild-type proteins. Efficient screening systems may facilitate the discovery of other *trans*-dominant mutants which may be even more effective inhibitors.

We have used a bacterial expression system for the functional analysis of the HIV-1 rev gene. The complementation of HLFb cells by bacterially expressed rev protein indicated that the protein expressed in these heterologous cells was biologically active. After completion of this work, another group reported the purification of functional rev protein from *E. coli* (48). We have also used protoplast fusion to demonstrate that the bacterially expressed BL and HS mutants were biologically active as *trans*-dominant mutants. Bacteria can therefore serve as a useful source of these proteins for comparative biochemical studies.

The protoplast fusion approach for screening and analyzing mutants is an alternative to methods involving transfection with purified eukaryotic expression plasmids. This method eliminates the requirement for purified plasmid DNA for transfection. As indicated in Figure 4, protoplast fusion can be used to identify *trans*-dominant mutants when the mutant and wild-type protein are expressed in separate bacterial cells. This suggests that a productive cell-protoplast fusion event involves multiple protoplasts, confirming previous reports describing protoplast-mediated DNA transfer (49). The *trans*-dominant inhibition observed in the protoplast fusion assay was not due to non-specific effects of overexpression of rev-related proteins, since the complementation of HLFb cells by functional bacterial rev increased linearly with increasing amounts of rev-expressing protoplasts throughout the range used in the competition experiment (data not shown). This method might in principle have some limitations: Mutants which are dominant by virtue of incorporation into oligomers may not be readily detected by this method, unless expressed in the same bacterial cell. Also, mutations which render proteins more susceptible to intracellular degradation in bacteria will likely lead to a recessive phenotype, whereas the same gene expressed in human cells could produce a stable product. Additional differences between bacteria and human cells such as post-translational modifications may result in differences in the phenotypes of the produced proteins.

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