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Use of a Human Immunodeficiency Virus Type 1 Rev Mutant without Nucleolar Dysfunction as a Candidate for Potential AIDS Therapy

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Applications of transdominant mutants of human immunodeficiency virus type 1 (HIV-1) regulatory proteins, especially Rev mutant, have been attempted for gene therapy against AIDS, because the Rev protein is essential for viral replication. We have previously reported that a mutant Rev protein (dRev) lacking its nucleolar targeting signal remained out of nuclei in expressed cells and strongly inhibited the function of Rev. To investigate the effects of dRev on HIV-1 replication, we established several dRev-expressing human cell lines with two different vector systems and examined virus production in these cells. An HIV-1-derived vector containing *drev* cDNA was constructed and introduced into CD4-positive HeLa cells and cells of the human T-cell line CCRF-CEM (CEM). In dRev-expressing HeLa cells, virus replication, syncytium formation, and cell death caused by HIV-1 infection were remarkably suppressed, and the same vector also conferred a resistant phenotype on CEM cells. The production was also suppressed in CEM cells containing the *drev* gene driven by a cytomegalovirus promoter. In addition, we found that dRev did not cause nucleolar dysfunction in a transient assay, in contrast to other transdominant mutants and wild-type Rev. Since dRev cannot migrate into the nuclei, it is expected not to interfere with nuclear/nucleolar functions of the host cell. We conclude that dRev is one promising candidate as an antiviral molecule for gene therapy against AIDS.

The genome of the pathogenic retrovirus human immunodeficiency virus type 1 (HIV-1) encodes some accessory proteins in addition to viral structural proteins (reviewed in reference 10). Among them, the nucleolar protein Rev has been characterized most extensively (5, 9, 11, 15). Rev binds to a region of RNA secondary structure known as Rev-responsive element (RRE) that is a part of the *env* region of unspliced or incompletely spliced HIV-1 mRNAs. These RNAs are accumulated in the cytoplasm, and viral structural proteins are synthesized (33, 40). Rev is essential for the production of infectious virions; hence, a strategy inhibiting Rev function has been mapped out to suppress HIV-1 production (3, 15, 26, 28, 29, 32). Rev is a 116-amino-acid protein composed of separate functional domains. The N-terminal domain at amino acid residues approximately 14 to 60 provides three different functions: (i) a specific binding domain for RRE (7, 34), (ii) a nucleolar targeting signal (NOS) identified as an arginine-rich stretch of amino acids extending from positions 35 to 50 (18, 24, 37, 47), and (iii) an overlapped oligomerization domain (40, 49). The C-terminal domain at residues approximately 78 to 90 is thought to be an activation domain of Rev (31, 33). Mutations of the activation domain confer a dominant negative phenotype, which inhibits Rev function. Several reports have demonstrated that the introduced *trans*-dominant *rev* mutant can suppress viral replication in human T-cell lines (3, 34). The other nonfunctional mutants with mutated N-terminal domains do not significantly inhibit wild-type Rev function in *trans* and are termed recessive negative mutants (31). However, we found that a deletion mutant of the N-terminal domain, named dRev, localized in the cytoplasm of transfected cells and effectively inhibited the function of wild-type Rev (23). In this report, we demonstrate the inhibitory effects of this Rev mutant against HIV-1 infection in stably transduced human cell lines and discuss the possible application of this mutant for gene therapy against AIDS, comparing it with other transdominant mutants.

MATERIALS AND METHODS

Plasmids. Plasmid pSE (14), which was produced by removing a 4.2-kb *Sph*I-*Eco*RI fragment from HIV-1 genomic clone pNL4-3 (1), was a gift from A. Adachi. After disruption of the unique *Eco*RI site of pSE, a 1.5-kb *Sal*I-*Nhe*I fragment was removed and an *Eco*RI linker was inserted. In the resultant plasmid, pSEEcoRI, the position of the *Eco*RI site was transferred behind a splicing acceptor, and the 5′ half of the *env* gene was removed. A selection marker plasmid, pSVbsr, purchased from Kaken (Kyoto, Japan) (19) was digested with *Bam*HI and *Pvu*II. Then the 1.8-kb fragment containing a simian virus 40 (SV40) early promoter, *bsr* gene, and poly(A) site was isolated and inserted into pSEEcoRI at the *Bam*HI site after removal of a 0.4-kb *Bam*HI-*Xho*I fragment. The resultant plasmid, pLbsr, contains (i) the HIV-1 long terminal repeat (LTR) at both the 5′ and 3′ ends, (ii) a packaging signal of HIV-1 (2, 27), (iii) a part of the *env* gene corresponding to RRE, and (iv) a selection marker gene in the opposite direction (Fig. 1B). pLbsr is an HIV-1 retroviral vector with an *Eco*RI cloning site requiring Rev expression for the packaging of the pseudogenome, and the expression of a introduced gene was upregulated by Tat protein. To construct the dRev protein expressor, pLdrevb, a 0.6-kb *Eco*RI fragment of pH2drev (23) was inserted at the *Eco*RI site of pLbsr. This plasmid is believed to express a Rev deletion mutant lacking 7 amino acid residues within its nucleolar targeting signal (Fig. 1A) (18, 23, 46). We constructed another dRev-expressing vector, pCdrev (Fig. 1C), to allow high and constitutive dRev expression. The parental vector pCMV-NEO-BAM (4), kindly given by B. Vogelstein, underwent digestion by *Bam*HI and blunting followed by *Xho*I linker insertion, into which a 0.6-kb *drev* fragment was ligated with *Xho*I linker. In pCdrev, the *drev* gene is expressed under the control of the cytomegalovirus (CMV) promoter. The following plasmids were used to observe the cytopathic effects of Rev. Plasmids pH2rev, pH2drev, and pH2revM10 express Rev, dRev, and RevM10, respectively, under the control of the SV40 early region promoter. One of them,

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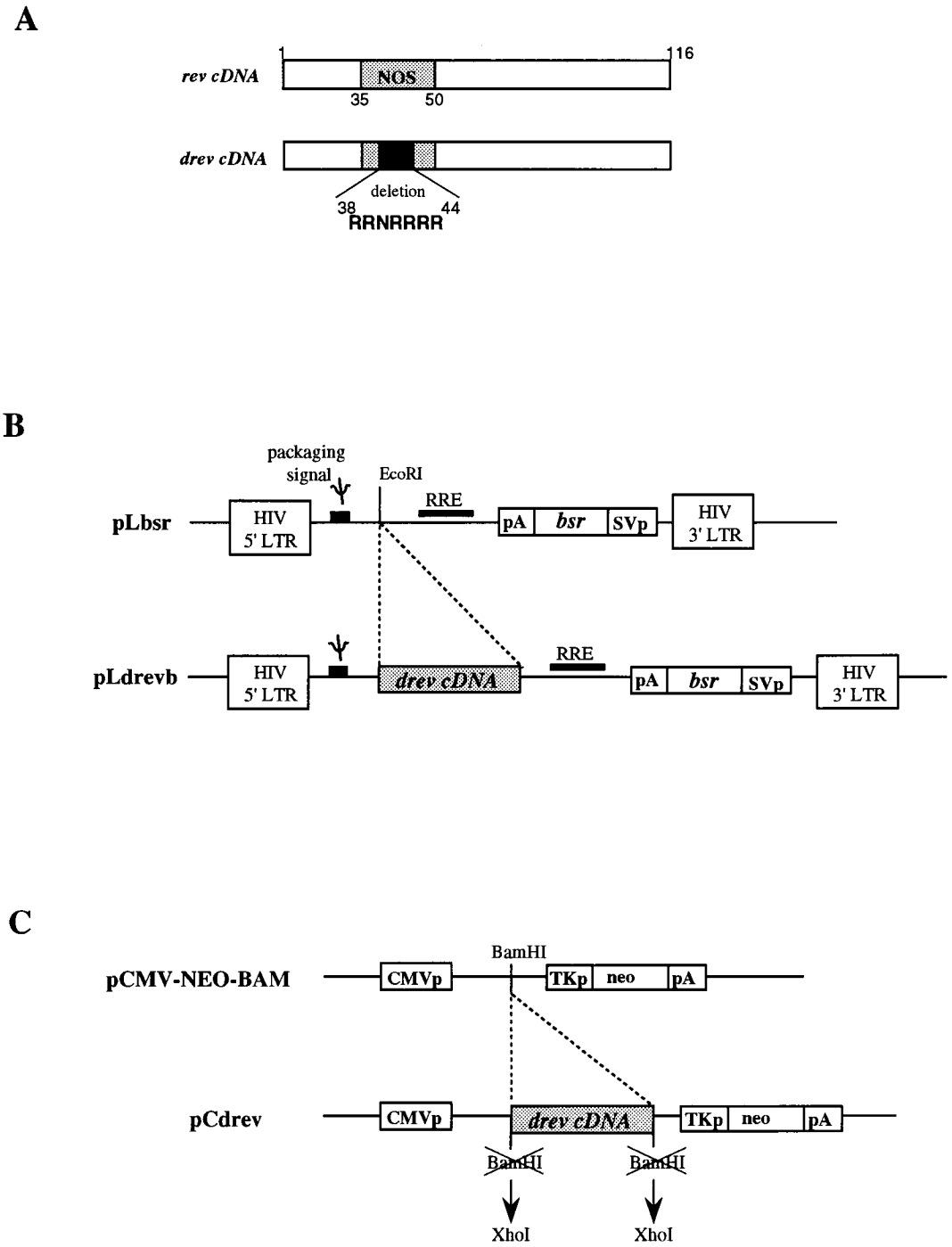

FIG. 1. (A) Structures of cDNAs encoding wild-type Rev and the mutant, dRev. The deduced amino acid sequence of the deletion is shown by single-letter abbreviations. Numbers below the hatched box of *rev* and above the deleted sequence represent residue numbers counted from the first methionine of Rev. (B) HIV-1 vector plasmids pLbsr and pLdrevb constructed from full-length proviral clone pNL4-3 (see Materials and Methods). The heavy lines represent RRE in the HIV-1 *env* sequence. (C) Structure of dRev expression vector under the control of the CMV promoter. The parental vector pCMV-NEO-BAM contains a eukaryotic expression unit with the CMV promoter, *Bam*HI cloning site, and selection maker gene. To make pCdrev, an *Eco*RI fragment of pLdrevb was inserted into the cloning site of pCMV-NEO-BAM with an *Xho*I linker. Abbreviations: *bsr*, blasticidin S resistance gene; NOS, nucleolar targeting signal; pA, SV40 late polyadenylation signal; SVp, SV40 early promoter; CMVp, CMV promoter; TKp, thymidine kinase promoter; neo, G418 resistance gene.

pH2revM10, which was constructed by substituting the cDNA portion of pH2rev for that of pcREVM10 (31) (provided by B. R. Cullen), expresses a *trans*-dominant mutant, RevM10. A *tat* expression plasmid, pH2Ftat, produces a full-length Tat protein of HIV-1 (13). Plasmid pKCRH2 (36) is a parental vector and was used as a control.

Cells and virus. CD4-positive HeLa cells (HeLa cl.1022 cells [6] provided by B. Chesebro) and COS7 cells were grown at 37°C under 5% CO_2 in air in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. To establish *drev*-transduced HeLa cells, 30 μg of linearized pLdrevb or pLbsr (as a control) were transfected into 10^6 HeLa cl.1022 cells by a standard calcium phosphate method, selected with 2.5 μg of blasticidin S per ml, and cloned by a cylinder cloning method. Cells of the human acute lymphoblastic leukemia de-

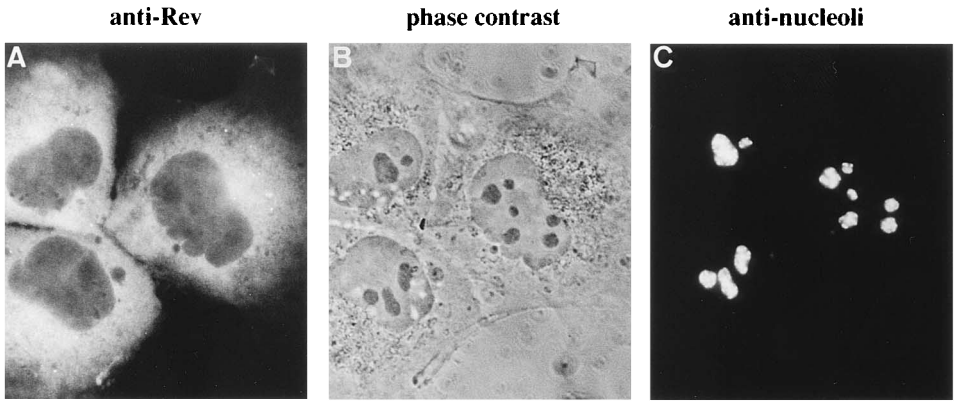

FIG. 2. Intracellular localization of dRev expressed by pLdrevb. COS7 cells were transfected with 6 μg of pLdrevb, fixed 48 h after transfection, and stained with both FITC for anti-Rev antiserum (A) and TRITC for serum from a patient with progressive systemic sclerosis (C). (B) Phase-contrast microscopic view of the same cells.

rived T-cell line CCRF-CEM (CEM) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C under 5% CO_2 in air. To obtain stable transfectants of CEM cells, 30 μg of linearized pLdrevb or pLbsr was transfected into 10^7 CEM cells by an electroporation method with a Gene Pulser (Bio-Rad, Richmond, Calif.) followed by selection with 2.5 μg of blasticidin S per ml and cloning by a limiting-dilution method. Integration of pLdrevb or pLbsr was confirmed by genomic PCR (data not shown). We also transfected 30 μg of pCdrev or pCMV-NEO-BAM into CEM cells by an electroporation method and selected cells with G418 at a concentration of 700 μg/ml (active dose) for 3 weeks. The expression of the *drev* gene of these cells was examined by Northern (RNA) blot analysis (data not shown). CD4 expression of cloned HeLa and CEM cells was determined by FACScan instrument analysis using fluorescein isothiocyanate (FITC)-conjugated Leu3a monoclonal antibody (Beckton Dickinson, Mountain View, Calif.). An HIV-1 HTLV-IIIB strain (provided by R. C. Gallo) was obtained from chronically infected H9 cells (human T-cell lymphoma cell line) grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

Viral infection. For efficient infection, we cultivated a HeLa cell series with H9 cells chronically infected with the HTLV-IIIB strain of HIV-1 (H9/IIIB). In order to produce a population of nondividing cells, H9/IIIB cells were exposed to 9,000 rads from a ^{137}Cs source. One day before infection, cloned HeLa cells were seeded in a 35-mm-diameter dish. After the culture medium was changed, 4×10^3 to 5×10^4 radiated H9/IIIB cells were added and cocultivated for 48 h. Then the medium including H9/IIIB cells and virions was removed, and HeLa cells were washed with phosphate-buffered saline (PBS) four times. Afterwards, the cells were grown in fresh growth medium for an additional 6 days. In the case of CEM cells, cell-free infection was applied. Filtered culture supernatant of H9/IIIB cells was added to each CEM transfectant at a multiplicity of infection (MOI) of 0.001 to 0.05. The infected cells were maintained at 2×10^5 to 10×10^5/ml in 25-cm^2 culture bottles, and virus production was monitored every other day.

Immunofluorescence. To confirm expression and localization of dRev, COS7 cells (5×10^4) were plated on glass coverslips in a 35-mm-diameter dish 1 day before transfection. DNAs were introduced into the cells by the DEAE-dextran method (8, 43). Forty-eight hours later, cells were fixed with 3.5% formaldehyde–PBS for 10 min at room temperature and permeabilized with 0.1% Nonidet P-40–PBS for 10 min at room temperature. Then the cells were incubated for 1 h at 37°C with both anti-Rev serum (provided by B. R. Cullen or purchased from ICN, Cleveland, Ohio) and the serum of a progressive systemic scleorisis patient as an antinucleolar antibody and then stained for 1 h at room temperature with FITC-conjugated anti-rabbit immunoglobulin G (IgG) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-human IgG antibody. To investigate nucleolar deforming effects, we incubated the cells with both anti-rRNA monoclonal antibody Y-10B (provided by J. A. Steiz) and anti-Rev serum. Then the cells were stained for 1 h at 37°C with both FITC-conjugated anti-rabbit IgG (for anti-Rev) and TRITC-conjugated anti-mouse IgG (for anti-rRNA). To confirm the Tat-dependent expression of the dRev protein in pLdrevb-transduced HeLa cells, pH2Ftat was transfected into the cells by a standard calcium phosphate method. After 48 h, the cells were fixed, permeabilized as described above, and incubated with both rabbit anti-Rev antiserum and mouse anti-Tat monoclonal antibody (purchased from American BioTechnologies Inc., Cambridge, Mass.) for 1 h at room temperature. Then the cells were stained for 1 h at 37°C with both FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG. To show subcellular localization of viral proteins of infected HeLa cells, *drev*-introduced or control CD4-positive HeLa cells were infected with filtered culture supernatant of H9/IIIB. Forty-eight hours postinfection, the cells were fixed and immunostained with both rabbit anti-Rev antiserum and mouse anti-$p24^{Gag}$ monoclonal antibody VAK4 (17) as described above.

Viral production assay. The multinuclear activation of a galactosidase indicator (MAGI) assay (20) was employed for titration of HIV-1 in culture supernatants of infected cells. The indicator cells were established by introducing the bacterial *lacZ* gene under the control of the HIV-1 LTR into HeLa cells expressing CD4. After infection with virus and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining, blue-stained cells were counted to estimate infectious units. In our assay, one stained cell represents one infectious unit in a chamber of a 24-well plate. Cell-free supernatants were also assayed for virus reverse transcriptase (RT) activity (25) and virus antigen $p24^{Gag}$ by a solid-phase enzyme immunoassay (Abbot Laboratories, North Chicago, Ill.). We also examined syncytium formation of the HeLa cell series in which a syncytium containing more than five nuclei was regarded as an HIV-induced syncytium. Since the ratio of small syncytia in parental cells was below 1% without HIV infection, we had confirmed that such large syncytia were HIV positive by immunofluorescence with VAK4 monoclonal antibody (data not shown).

DNA amplification. To analyze proviral DNA of infected cells, we extracted DNAs 48 h postinfection from *drev*-introduced or control CEM cells as follows: 2×10^6 cells were suspended in STE (100 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.5% sodium dodecyl sulfate), digested with proteinase K, phenol extracted, and ethanol precipitated. After RNase A digestion, DNAs were recovered by phenol extraction followed by ethanol precipitation. The DNAs were suspended with 1 ml of TE (10 mM Tris-Cl [pH 7.6], 1 mM EDTA). To amplify HIV-1 specific DNA, 1, 5, or 25 μl of the DNA solution was used for semiquantitative PCR with primer pair SRRE-3nef8978, which corresponds to part of the *env* gene (nucleotides 7759 to 8978 of pNL4-3). Primer pair 5βglo-3βglo was also used to amplify part of the human β-globin gene as an internal control. PCR was carried out using DNA thermal cycler PJ1000 (Perkin-Elmer Cetus Instrument) in 50 μl of the following reaction mixture: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM each deoxynucleoside triphosphate, and 0.25 μM each primer. The reaction mixture was incubated at 95°C for 5 min and chilled on ice for 5 min, and then 2 U of *Taq* polymerase (Perkin-Elmer Cetus Instrument) was added. PCR was repeated for 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 90 s. DNA fragments were analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining. Synthetic oligonucleotide sequences of the primers were as follows: SRRE, 5′-ATAGGAGCTCAGGAGCTTTGTTCCTTGGGT-3′; 3nef8978, 5′-CTCCT CTTGTGCTTCTAGCCA-3′; 5βglo, 5′-GGTTGGGCCAATCTACTCCCAG G-3′; and 3βglo, 5′-GCTCACTCACTGTGGCAAAG-3.

RESULTS

Efficient expression of dRev in COS7 cells by pLdrevb. We subcloned the cDNA of a Rev mutant with a 7-amino-acid deletion in its nucleolar targeting signal, named dRev (Fig. 1A) (23), into HIV-1 vector pLbsr (Fig. 1B). Expression and subcellular localization of dRev from pLdrevb were confirmed in COS7 cells in a transient transfection assay by an immunofluorescence technique (Fig. 2). In contrast to wild-type Rev and other transdominant mutants (31), the dRev protein efficiently expressed by pLdrevb remained in the cytoplasm of transfected cells like pH2drev with the SV40 promoter (23). We also examined expression and localization of the dRev protein under the control of a CMV promoter (Fig. 1C). pCdrev gave a

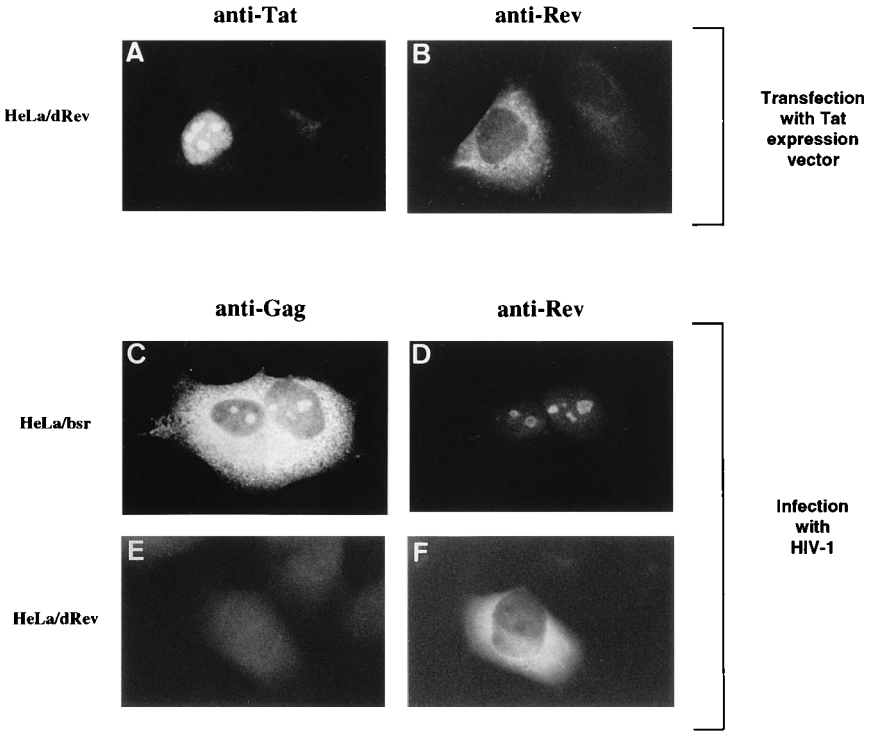

FIG. 3. (A and B) Tat-dependent expression of the dRev proteins in a dRev-introduced cell clone, HeLa/dRev; (C to F) subcellular localization of viral proteins in infected HeLa cells. HeLa/dRev cells were transfected with 4 μg of pH2Ftat, fixed 48 h after transfection, and stained with both TRITC for anti-Tat monoclonal antibody (A) and FITC for anti-Rev antiserum (B). HeLa/dRev and HeLa/bsr cells were infected with HTLV-IIIB, fixed 48 h postinfection, and stained with both TRITC for anti-$p24^{Gag}$ monoclonal antibody (C and E) and FITC for anti-Rev antiserum (D and F).

high level of expression of the dRev protein in the cytoplasm of transfected COS7 and HeLa cells (data not shown).

Establishment of dRev-introduced HeLa and CEM cell lines. First, we established a CD4-positive HeLa cell line stably transduced with the *drev* gene in order to show subcellular localization of introduced gene products and to examine the effects of dRev on virus infection. We chose HeLa cl.1022 constitutively expressing CD4 as a parental cell line because it was originally established for a focal immunoassay of HIV infectants with high sensitivity (6). After introduction of dRev expression vector pLdrevb or control plasmid pLbsr by transfection followed by selection with blasticidin S and cell cloning, integration of each vector was confirmed by PCR (data not shown). The dRev proteins were detected in established cells only when Tat was expressed by transfection with plasmid pH2Ftat (Fig. 3A and B), whereas control plasmid pKCRH2 did not give such an effect (data not shown). We also analyzed the level of CD4 expression in the cloned cell lines and parental HeLa cl.1022 cells. FACScan analysis using an anti-CD4 monoclonal antibody, Leu3a, revealed that there is no significant difference in the expression levels of CD4 molecules among the three cell clones (data not shown), assuming that the susceptibility to HIV of these cells could be the same. Doubling times of three cell lines were as follows: 19.6 h in HeLa cl.1022 cells, 21.9 h in the cell lines containing pLdrevb (HeLa/dRev), and 23.2 h in the cell line containing pLbsr (HeLa/bsr).

In order to examine the inhibitory effects of dRev on virus production in T cells in long-term infection, we next introduced pLbsr or pLdrevb into CEM cells and obtained stable transfectants after selection by blasticidin S and cell cloning. FACScan analysis also revealed that the mean fluorescence intensities given by an anti-CD4 monoclonal antibody were the same among the parental CEM cells, CEM *bsr* cells (CEM/bsr), CEM dRev mixed population (CEM/dRev mix.), and CEM dRev cloned cells (CEM/dRev cl.) (data not shown). The doubling times of these cell lines were also almost the same. In addition, we established CEM cell lines expressing constitutively the dRev protein under the control of a CMV promoter. CEM cells were transfected with the dRev-expressing plasmid, pCdrev, or parental vector pCMV-NEO-BAM (Fig. 1C) and selected in G418-containing medium for three weeks. The established cells (CEM/CdRev and CEM/neo) were subjected to virus infection assays.

HIV-resistant phenotype of a HeLa/dRev cell clone. We examined whether the stably transduced *drev* gene can inhibit HIV-1 replication in established cell lines. In the HeLa cell series, three clones, HeLa/dRev, HeLa/bsr, and HeLa cl.1022, were cocultivated with H9/IIIB, and then HIV-1 replication was monitored by MAGI assay. The representative data of four different experiments with similar results are shown (Fig. 4A). The replication of virus was suppressed in HeLa/dRev cells about 27% compared with that in HeLa cl.1022 cells and 35% compared with that in HeLa/bsr cells at 4 days postinfection without cell passage. Next, we compared the rate of syncytium formation among these cell lines (Fig. 4B). While we observed many syncytia 4 days after infection in control cells, the rate of syncytium formation was very low in HeLa/dRev cells. Since HIV-mediated syncytium formation depends upon the interaction of CD4 and gp120-gp41 complex (22, 30), it reflects the expression of the *env* gene induced by the Rev protein, suggesting that dRev efficiently blocked the Rev function of HIV-1 in stably transduced cells. In order to clarify that dRev conferred such an HIV-1-resistant phenotype on HeLa cells

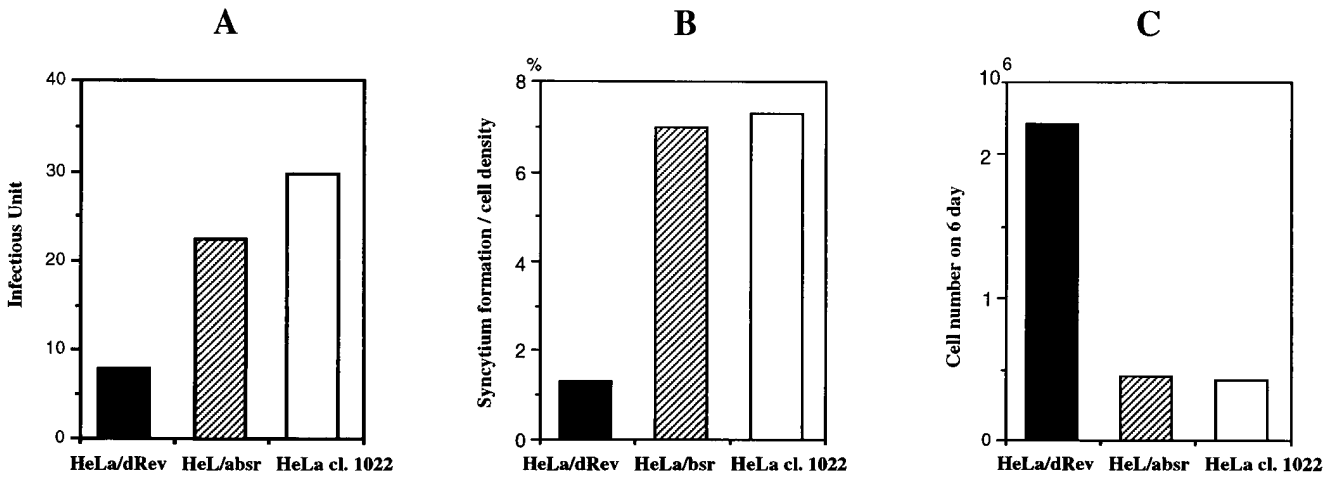

FIG. 4. Effects of the *drev* gene stably transduced in CD4-positive HeLa cells against infection by HIV-1. Cloned HeLa/dRev, HeLa/bsr, and parental HeLa cl.1022 cells were cocultivated with chronically infected H9 cells for 48 h. Four days after infection, HeLa cells were assayed for the virus replication that was monitored by MAGI assay (A) (see Materials and Methods). The rate of syncytium formation (B) and viable cell numbers counted by trypan blue exclusion (C) are also displayed.

through inhibition of the function of Rev, we examined subcellular localization of viral and antiviral proteins in the infected HeLa cell. Rev was localized in nuclei/nucleoli in infected HeLa/bsr, and viral structural protein $p24^{Gag}$ was strongly expressed in all of these cells (Fig. 3C and D). These results confirmed that Rev effectively functions in nuclei and induced the synthesis of viral structural proteins. On the other hand, in HeLa/dRev cells infected with HIV-1, Rev was detected only in the cytoplasm, and $p24^{Gag}$ was not detected in the same cells (Fig. 3E and F). We believe that the cells positively stained in the cytoplasm by anti-Rev antibody were infected, because the antibody used here recognizes both wild-type and mutant Rev and also because the gene expression of both *rev* and *drev* depended on Tat, as shown in Fig. 3A and B. These data indicate that dRev interfered with the localization of Rev and that the viral replication was suppressed in these cells.

In addition, many HeLa/dRev cells were alive, unlike other control cells on day 6 postinfection (Fig. 4C). Since the doubling times and the levels of CD4 expression of these three cell lines have been almost equal, the viable cell numbers represent actual populations that escaped cell death by HIV-1 infection. Syncytium formation and cell viability were almost the same in HeLa/bsr and HeLa cl.1022 cells, suggesting that the titration effects of Tat and Rev by the *trans*-activation-responsive region (TAR) and RRE of pLbsr vector were not observed in this assay system. Considering the above findings together, we concluded that the dRev-harboring HeLa cells escaped cell death by HIV-1 infection through inhibition of the function of Rev.

The inhibitory effects of dRev on virus production in CEM cells. We established *drev*-introduced CEM cells to examine whether dRev does work as an antiviral molecule in human T-cell lines as well as in CD4-positive HeLa cells. First, we examined the effects of dRev under the control of the HIV-1 LTR. Parental CEM cells and stably transduced CEM series (CEM/bsr, CEM/dRev mix., and CEM/dRev cl.) cells were infected with HTLV-IIIB via cell-free infection at a MOI of 0.001 to 0.05. Virus production of each cell type was quantified by MAGI assay. Figure 5A shows one of the typical results among five experiments independently performed with various MOIs. The production of virus in control CEM cells increased from day 18 postinfection. An increase in the number of infectious units of CEM/bsr cells was observed on day 20. Virus production in both the dRev-introduced mixed population and cloned cell line remained very low, though a little increase was observed on days 20 and 24. This increase could be explained by the insufficient amount of dRev, because dRev was expressed under the control of the HIV-1 LTR in these cells. Therefore, we next performed another infection assay using cells expressing dRev by the CMV promoter. The RT activities of control CEM/neo cells peaked on day 12 (Fig. 5B). In CEM/CdRev cells, however, no significant virus production was seen through the experimental period.

In order to confirm that these cells were equally susceptible to HIV-1, we analyzed the viral DNA in the infected cells by a PCR method (Fig. 5C). Forty-eight hours postinfection, genomic DNAs were prepared from the CMV/CdRev and CMV/neo cells and subjected to semiquantitative PCR. Almost the same levels of HIV-1 specific fragments were amplified from the DNA of both CEM/neo and CEM/CdRev cells. PCR products of the human β-globin gene of CEM/neo and CEM/CdRev cells 48 h postinfection were observed as the same, assuming that the DNA of these cells had been amplified equally under these PCR conditions; therefore, there was no great difference in the amounts of the viral DNA between these cells. These data clearly demonstrated that the HIV-1 infection in these cells occurred equally. We therefore concluded that dRev inhibited the virus production in T cells as well as in HeLa cells.

dRev is free from nucleolar side effects observed in another transdominant Rev mutant. We have previously found that overexpressed Rev proteins have cytotoxic effects in some cell lines, and these effects were also observed in the cells introduced with Rev mutants, including a transdominant mutant (38). It has been reported that transdominant mutant RevM10 was able to block HIV-1 replication in stably transduced cells, and its potential use for gene therapy has been suggested (3, 32). Cytotoxic effects by dRev, Rev, and RevM10 were examined. We transfected wild-type or mutant Rev expression vectors into COS7 cells and doubly immunostained the cells with anti-Rev and anti-rRNA antibodies. The nucleoli of COS7 cells were deformed and ballooned 48 h after transfection with wild-type Rev or RevM10 expressor, and rRNAs mostly disappeared in those cells (Fig. 6A to F). These findings are not dependent on cell cycle (36a). In contrast, dRev has shown neither nucleolar destructive effects nor an aberrant distribu-

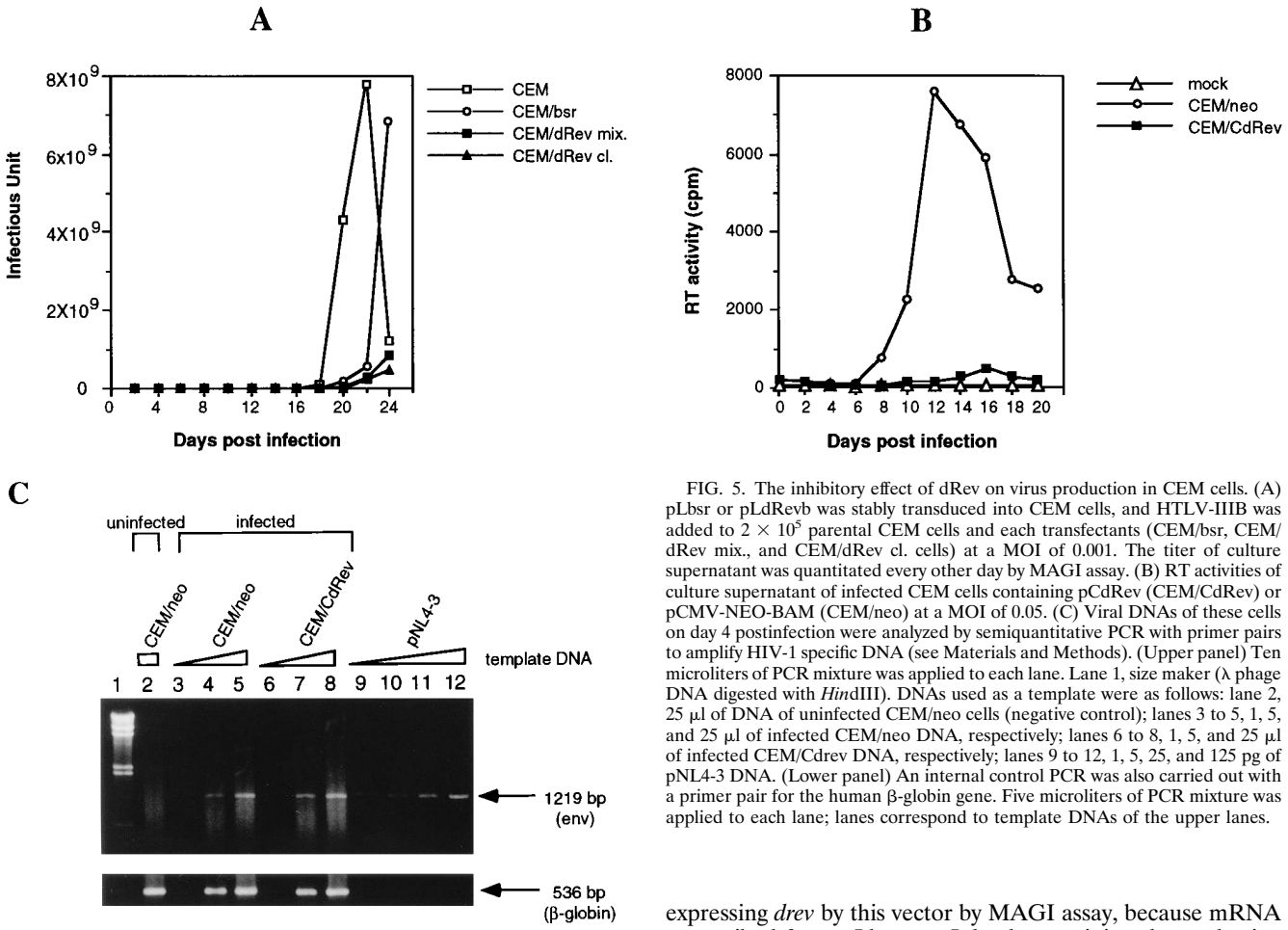

FIG. 5. The inhibitory effect of dRev on virus production in CEM cells. (A) pLbsr or pLdRevb was stably transduced into CEM cells, and HTLV-IIIB was added to 2×10^5 parental CEM cells and each transfectants (CEM/bsr, CEM/dRev mix., and CEM/dRev cl. cells) at a MOI of 0.001. The titer of culture supernatant was quantitated every other day by MAGI assay. (B) RT activities of culture supernatant of infected CEM cells containing pCdRev (CEM/CdRev) or pCMV-NEO-BAM (CEM/neo) at a MOI of 0.05. (C) Viral DNAs of these cells on day 4 postinfection were analyzed by semiquantitative PCR with primer pairs to amplify HIV-1 specific DNA (see Materials and Methods). (Upper panel) Ten microliters of PCR mixture was applied to each lane. Lane 1, size maker (λ phage DNA digested with *Hin*dIII). DNAs used as a template were as follows: lane 2, 25 μl of DNA of uninfected CEM/neo cells (negative control); lanes 3 to 5, 1, 5, and 25 μl of infected CEM/neo DNA, respectively; lanes 6 to 8, 1, 5, and 25 μl of infected CEM/Cdrev DNA, respectively; lanes 9 to 12, 1, 5, 25, and 125 pg of pNL4-3 DNA. (Lower panel) An internal control PCR was also carried out with a primer pair for the human β-globin gene. Five microliters of PCR mixture was applied to each lane; lanes correspond to template DNAs of the upper lanes.

tion of rRNA in transduced cells, although the excess amounts of the dRev proteins were expressed (Fig. 6G, H, and I) under the same conditions in experiments using Rev and RevM10.

DISCUSSION

In this study, we demonstrated that dRev suppressed HIV-1 production in the several stable transformants with the *drev* gene and clearly show that this suppression was due to interference with Rev localization by dRev in infected HeLa cells, which we have previously reported in a cotransfection assay (23).

We introduced the *drev* gene into two human cell lines, CD4-positive HeLa cells and CEM cells, by two different vectors and found that virus production by the dRev-harboring cells was apparently lower than that by control cells in the all cells tested here. The vector, pLbsr, used in this study was designed as a retroviral vector so that the introduced gene is expressed under the control of the HIV-1 LTR and that the genomic RNA of the vector is expected to be packaged into a virion in the presence of the Rev and viral structural proteins. Furthermore, mRNA of pLbsr contains two important secondary structural regions, TAR and RRE, which are expected to titrate Tat and Rev of HIV. We, however, transduced it into the cells by transfection in this study because packaging devices for HIV vectors are still under development (42, 44). We measured virus production in culture supernatants from cells expressing *drev* by this vector by MAGI assay, because mRNA transcribed from pLbsr or pLdrevb containing the packaging signal of HIV-1 might be packaged into virions and so that the amount of p24 and RT activity in culture supernatants of these cells may reflect production of such pseudovirions in addition to wild-type ones.

The dRev protein was efficiently expressed in the cytoplasm of the transfected COS7 cells from this vector (Fig. 2), and the expression of dRev was regulated in stably transduced HeLa cells in a Tat-dependent manner (Fig. 3A and B). Moreover, dRev was highly expressed and worked as an antiviral molecule when the *drev*-introduced HeLa cells were infected with HIV-1 (Fig. 3C to F), in which case we found different localization of the functional or nonfunctional Rev by immunofluorescence. In the infected HeLa/dRev cells, syncytium formation, which is one of the typical cytopathic effects observed in HIV-infected cultured cells, was suppressed (Fig. 4B) and virus production was also suppressed compared with that in parental HeLa cl.1022 cells (Fig. 4A). A slight inhibition was also seen in HeLa/bsr cells, probably reflecting the titration effects of TAR and RRE against Tat and Rev as discussed above. Furthermore, the number of viable HeLa/dRev cells on day 6 postinfection far exceeded than that of the control cells (Fig. 4C). Therefore, we concluded that dRev conferred an HIV-resistant phenotype on the introduced HeLa cells.

We also performed a cell-free infection study using CEM cells and found that virus production was also suppressed in CEM/dRev cells. The delay in virus production seen in CEM/bsr cells was probably due to the same effects observed in HeLa/bsr cells. Virus production was not completely suppressed by dRev in the HIV-1 vector system, probably

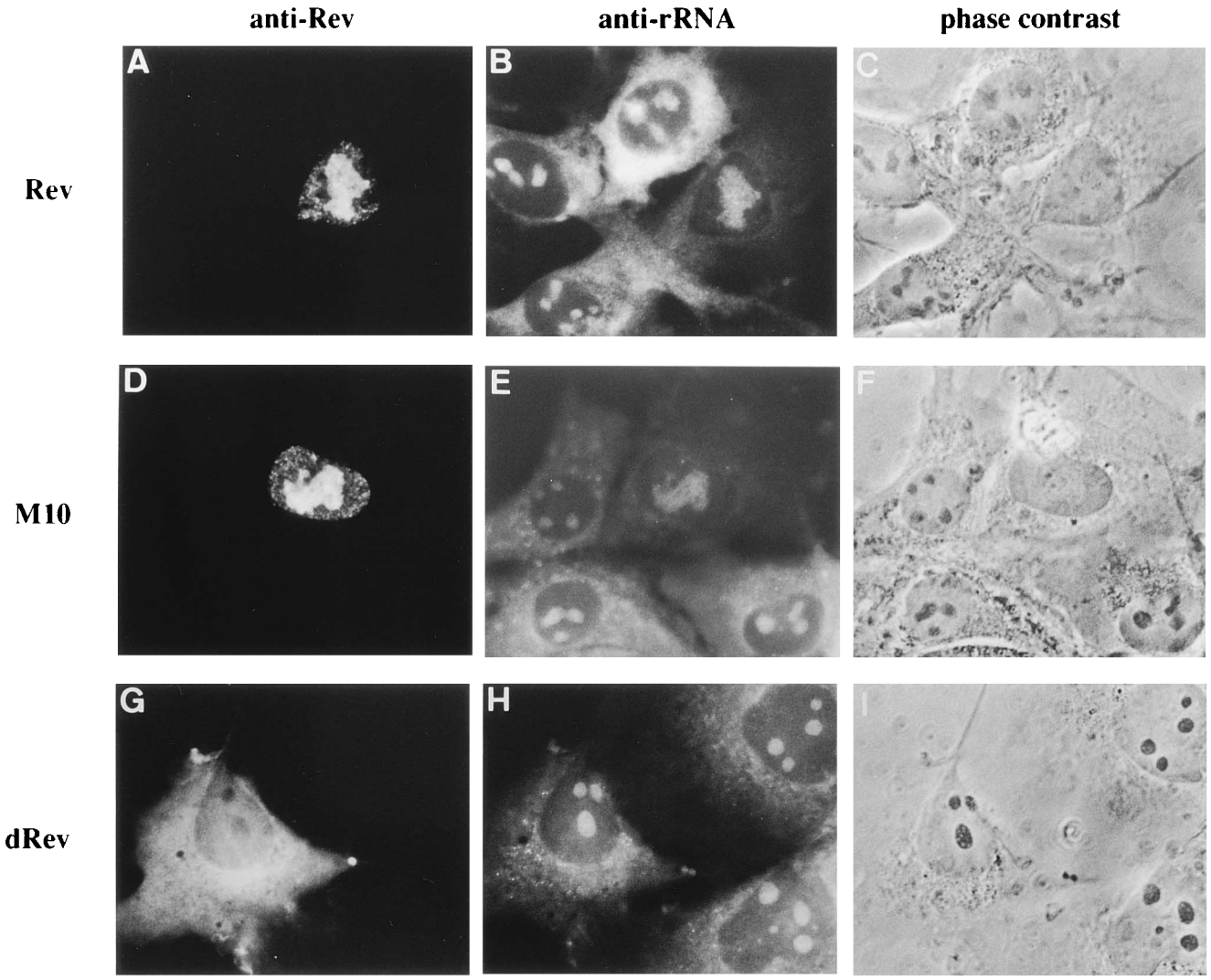

FIG. 6. Distribution of rRNA in COS7 cells transfected with Rev or mutant Rev expression vector. Cells transfected with 5 μg of pH2rev (A to C), pH2revM10 (D to F), or pH2drev (G to I) were doubly stained 48 h after transfection. Panels A, D, and H were stained with rabbit anti-Rev serum and FITC-conjugated anti-rabbit IgG antibody. Panels B, E, and I were stained with mouse anti-rRNA monoclonal antibody Y-10B and TRITC-conjugated anti-mouse IgG antibody. Panels C, F, and G were viewed by phase-contrast microscopy.

because the expression level of dRev was not sufficient for complete inhibition in this experimental condition. In contrast, almost complete inhibition was observed in CEM/CdRev cells in which *drev* was driven by the CMV promoter. Bahner et al. (3) also reported that the retroviral vectors containing a transdominant mutant Rev gene driven by the CMV promoter worked more effectively than that driven by the HIV-1 LTR. We believe that Tat-dependent expression of anti-HIV molecules may be safe in gene therapy against AIDS because transduced gene products are not expected to be expressed at a high level without HIV infection and the HIV vector could transfer genes into the HIV target cells in vivo, yet the suppressive effect in this system was not complete. Further investigation for a more efficacious transmission and expression system of anti-HIV molecules would be necessary.

Although the precise mechanism of the inhibitory effect of dRev has not been proved yet, it is evident that Rev cannot migrate into the cell nucleus/nucleolus and does not function in the presence of dRev. Very recently, Duan et al. reported an intracellularly expressed anti-Rev single-chain antibody. They claimed that the antibody changed the localization of Rev and decreased HIV-1 replication in human cells, results very similar to ours (12). The mechanism of how dRev can retain Rev in the cytoplasm has not been clarified. It is possible that dRev competes with Rev in the cytoplasm for some cellular factors which carry Rev into the nucleus/nucleolus. We presume, however, that dRev may not interact with such molecules, since dRev lacks its nucleolar targeting signal. Alternatively, dRev may form a hetero-oligomer with Rev which cannot migrate into the nucleus/nucleolus as hypothesized previously (23). It was reported that an excess amount of Rev makes an oligomer without RRE and some Rev mutants with substitutions in the nuclear targeting signal form a hetero-oligomer with wild-type Rev (40). Multimer formation of Rev with or without RRE, however, remains unsettled.

It is important to mention that dRev works only in the cytoplasm of the introduced cell. Until now, a variety of ret-

roviral vectors have been created to inhibit HIV replication, but most of them were designed to work on the events in the nuclei of infected cells, for example, antisense RNA for viral mRNA (28, 35), ribozymes for viral RNAs (39, 45), so-called TAR decoys as competitors of TAR RNA (47), and transdominant mutants of HIV regulatory proteins (3, 29, 32). Since interactions with cellular components may be necessary for the viral regulatory proteins to function in the nucleus of the infected cell (48), these anti-HIV viral analogs can interact with such cellular factors. Thus, if large amounts of shams of viral products are introduced into cell nuclei as anti-HIV reagents, they may affect not only viral replication but also cellular function. We recently reported that the Rev protein has cytotoxic activity with nucleolar dysfunction caused by the failure of ribosomal biosynthesis, and the effect was observed in transfected cells and in T cells acutely infected with HIV-1 but not in chronically infected cells (38). These observations indicated that the high expression of Rev may be lethal to cells. Here, we also demonstrated that highly expressed transdominant mutant RevM10 induced nucleolar ballooning and deformity with aberrant accumulation of rRNAs like wild-type Rev in transfection experiments (Fig. 6). Although these phenomena could be explained by the excess amount of expression, dRev did not show such effects at the same level of expression. To sum up, dRev remains in the cytoplasm, inhibits viral replication effectively, and does not affect nuclear events directly. Malim et al. showed that the transduced RevM10 gene produced an effective blockade against HIV-1 in CEM cells without remarkable side effects on some T-cell functions using selected clones of transduced cells (32). Also similar results have been shown by Bahner et al., and they noted that it is important to check the toxicity of these genes on normal cellular function (3). We think that much should be done to examine the toxicity of transduced gene products in various conditions.

Our data reported previously (23, 38) and here suggest that it is possible that the dRev protein blocks cell death caused by HIV-1 through two pathways. One is an inhibitory effect on viral protein production, including the *env* gene product, which induced cell death (reviewed in references 16 and 21). The other is the fact that dRev is able to retain Rev in the cytoplasm to prevent Rev from nucleolar accumulation which may cause nucleolar dysfunction, occasionally leading to cell death. Considering the above aspects together, we may reasonably conclude that dRev is a good candidate for gene therapy against AIDS.

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