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Human Chromosome 6- and 11-Encoded Factors Support Human Immunodeficiency Virus Type 1 Rev Function in A9 Cells

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The precise mechanism of Rev-mediated expression of human immunodeficiency virus (HIV-1) late genes is not well characterized. We recently proposed a requirement for HIV-1 Rev responsive element (RRE) RNA binding host nuclear proteins in Rev function. In this report, using a transient transfection assay of Rev function, we further demonstrate the role of host cell factors in HIV-1 Rev function. Murine A9 cells, which are inefficient in forming RRE-host protein ribonucleoprotein complexes, are also inefficient in supporting Rev function. We also show that host cell factor(s) encoded by human chromosomes 6 and 11 can support HIV-1 Rev-mediated expression of unspliced viral mRNAs in murine A9 cells.

Expression of the late genes of human immunodeficiency virus type 1 (HIV-1) is characterized by a predominant expression of unspliced or singly spliced viral mRNAs (23, 31). These mRNAs are required for the synthesis of viral enzymes and structural proteins (reviewed in references 1, 14, and 39). HIV-1 regulatory protein Rev facilitates the transport and translation of these viral mRNAs by interacting with an RNA element, the Rev-responsive element (RRE), present in the Env-encoding region (2, 8 [reviewed in references 7, 21, and 32]). The precise mechanism of Rev function is not well characterized (5); however, its binding to RRE RNA is essential (9, 10, 16, 28). Mutational analyses of Rev have identified two functional domains, an N-terminal basic domain which interacts with RRE RNA and contains the nuclear localization signal and a C-terminal leucine-rich activation domain which contains the nuclear export signal (7, 11, 21, 33). A requirement for cellular cofactors in Rev function has been proposed (5, 19, 32–34). Interactions of Rev with splicing factors and components of host cell nucleocytoplasmic RNA transport have been reported (6, 13, 27, 37). Such interactions may cause disruption of spliceosome formation and facilitate the transport and translation of RRE-containing viral mRNAs (5, 21, 25, 26, 32). Cellular factors which interact with activation (3, 13, 37) and basic (27) domains of Rev have been identified. Mutants of Rev carrying mutations in the activation domain bind to RRE RNA but do not support Rev function (reviewed in reference 21). These results have led to the hypothesis that host cell factors are essential for Rev function (reviewed in reference 21). The requirement for specific host factors in Rev function is further supported by the lack of Rev function in certain cell types (29).

Recently, we described the binding of nuclear proteins to RRE RNA in a cell-type-specific manner (34). Nuclear proteins from murine A9 cells do not form RRE-ribonucleoprotein (RNP) complexes; however, the presence of human chromosome 6 in these cells restores the binding of nuclear proteins to RRE RNA (34). In this report, we demonstrate that A9 cells, which do not form characteristic RNP complexes with RRE RNA, cannot support Rev function in vivo. Since A9 cells support human T-cell leukemia virus type 1 (HTLV-1) Rex function, we suggest that the lack of Rev function in these cells is specific and may provide a unique background for studying Rev regulatory factors. Using a Rev-green fluorescent protein (GFP) fusion protein expression, we show that the block in Rev function in A9 cells is not due to restricted nuclear localization of Rev. We also demonstrate that human murine somatic cell hybrids containing human chromosome 6 form RRE-RNP complexes and support Rev function in vivo.

HeLa and A9 cells were maintained in cell culture as previously described (34). Human mouse fibroblast (A9) somatic cell hybrids containing single human chromosomes were obtained from the laboratory of Eric J. Stanbridge (34). The Rev function reporter construct pDM128 was obtained from Tristram Parslow, University of California, San Francisco (18). The reporter construct pCMV128 is similar to pDM128, except that a cytomegalovirus (CMV) promoter drives the expression of cloned sequences. A reporter construct, pCMV110, in which the expression of the β-galactosidase gene is under the control of CMV promoter, was cotransfected to correct for transfection efficiency (17–20). Rev-expressing constructs pL3Crev and pRSVrev contain a Rev-encoding gene cloned under the HIV-1 long terminal repeat and in Rous sarcoma virus (RSV), respectively. pRSVrev, pCMV128, and pCMV110 were provided by Thomas J. Hope, Salk Institute, La Jolla, Calif.; pL3Crev was provided by George N. Pavlakis and Barbara Felber, National Cancer Institute, Frederick, Md. The reporter construct pCMVXRE, which was used to assay for HTLV-1 Rex function, and the HTLV-1 Rex expression construct pRSVrex were also provided by Thomas J. Hope (17). In transfection experiments, 10^6 cells (HeLa, A9, or somatic cell hybrids) were seeded in 100-mm-diameter plates containing 10 ml of culture medium 24 h prior to transfection (35). Cells were transfected with a total of 17 μg of DNA (reporter plasmids, Rev or Rex expression plasmid, and herring sperm DNA) per plate by a calcium phosphate precipitation protocol (35). Alternatively, cells were transfected with lipofectamine reagent (GIBCO BRL). Forty-eight hours after transfection, cells were harvested and cell lysates were prepared. Chloramphenicol acetyltransferase (CAT) enzyme activity in cell lysates

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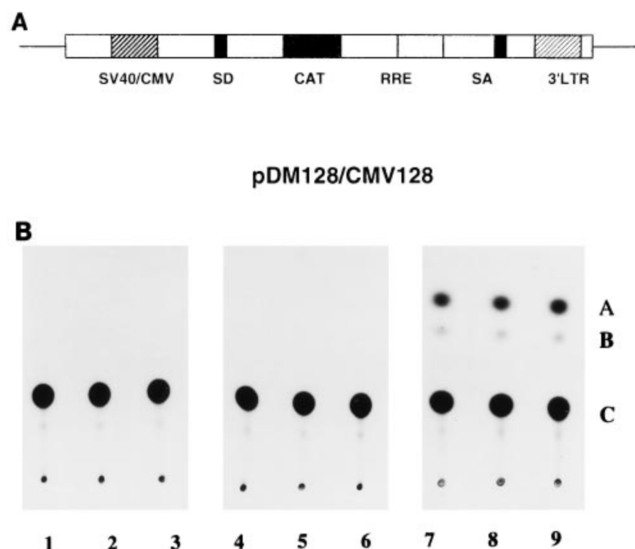

FIG. 1. (A) Reporter construct pDM128/CMV128. The design and construction strategy of this construct have been described previously (14). SD, splice donor site; SV40, simian virus 40; SA, splice acceptor site; LTR, long terminal repeat. (B) Rev Function in HeLa cells. HeLa cells were transfected with 10 μg of DM128 alone or cotransfected with 1 μg of Rev plasmid (pL3Crev) in a total of 17 μg of DNA by a calcium phosphate protocol. Control cells were transfected with 17 μg of herring sperm DNA (carrier DNA). Forty-eight hours after transfection, cells were harvested and lysed by freezing and thawing. CAT enzyme activity in the cell lysates was assayed by measuring the acetylation of chloramphenicol. Acetylated and unacetylated chloramphenicol species were separated by thin-layer chromatography. A and B, acetylated chloramphenicol; C, unacetylated chloramphenicol. Lanes: 1 to 3, cells transfected with carrier DNA; 4 to 6, cells transfected with reporter construct pDM128; 7 to 9, cells cotransfected with reporter construct pDM128 and Rev-expressing plasmid pL3Crev.

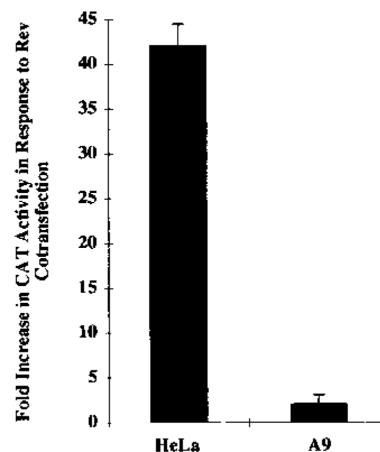

FIG. 2. Rev function in A9 cells. HeLa and A9 cells were transfected with reporter construct CMV128 alone or cotransfected with different amounts of pRSVrev. Each sample was also transfected with β-galactosidase reporter construct pCMV110. CAT enzyme activity was determined with cell lysates as described previously. β-Galactosidase activity was determined as described previously. Results are expressed as fold increase in CAT activity in Rev-transfected cells versus that in cells transfected with reporter construct alone after correction for the transfection efficiency. Each value represents an average of three replicates. Vertical bars represent standard deviation.

containing equal amounts of protein was determined by measuring the incorporation of acetyl groups in ^{14}C-labelled chloramphenicol (35). Acetylated chloramphenicol was extracted from unacetylated chloramphenicol either by xylene-TMPD elution or by thin-layer chromatography (24). β-Galactosidase activity was measured by a colorimetric assay with *o*-nitrophenyl-β-D-galactoside as a substrate (Promega Corporation). All of the transfections were carried out in triplicate and repeated three to five times. All results are expressed as fold increase in CAT activity in response to Rev or Rex cotransfection. Subcellular localization of Rev was studied with GFP constructs expressing GFP alone or as a Rev fusion protein under the CMV promoter. These constructs were kindly provided by Roland Stauber and George N. Pavlakis, National Cancer Institute (reference 36).

Use of the reporter construct pDM128 to study Rev function. Reporter plasmid pDM128 has been previously used to study Rev function in transient transfection assays in COS7 cells (18–20). Because of the presence of the CAT gene in an intron, transfection with pDM128 DNA alone leads to a nontranslated CAT sequence, since it is spliced out and presumably degraded within the nucleus (Fig. 1A). However, upon cotransfection with Rev-expressing plasmid, unspliced mRNA is transported to the cytoplasm and translated, and as a result, CAT enzyme activity is observed. To determine if transient expression of pDM128 could be used as an index of Rev function in HeLa cells, we transfected HeLa cells with pDM128 alone or along with Rev-expressing plasmid. Figure 1B shows that no CAT activity was observed in cells transfected with pDM128 alone (lanes 4 to 6) or with carrier DNA alone (lanes 1 to 3). Cotransfection with 1 μg of Rev-expressing plasmid, on the other hand, resulted in an approximately 33-fold increase in CAT activity (lanes 7 to 9). We reproduced the assays with a second reporter plasmid, pCMV128, a derivative of pDM128 in which the simian virus 40 promoter was replaced with a CMV promoter. As a source of Rev-expressing vector, we also used pRSVrev, in which the Rev-encoding sequences are under the control of the RSV promoter.

A9 cells do not support HIV-1 Rev function. We reported earlier that the host cell nuclear proteins form specific RNP complexes with RRE RNA and that certain cell types, such as murine fibroblast A9 cells, lack the ability to form these RNP complexes (34). To determine whether the formation of RRE RNA-cellular factor RNP complexes correlated with Rev function in vivo, we studied Rev function in A9 cells by using the reporter construct pCMV128. HeLa and A9 cells were transfected with pCMV128 plasmid alone or cotransfected with Rev-expressing plasmid pRSVrev, and the induction in CAT activity in the cell lysates was determined. In contrast to an over 40-fold increase in Rev-dependent CAT activity in HeLa cells, A9 cells showed negligible induction in CAT activity when cotransfected with the Rev expression plasmid (Fig. 2).

A9 cells support HTLV-1 Rex function. To determine whether the lack of Rev function in A9 cells is specific, we studied HTLV-1 Rex function with a similar CAT reporter plasmid, pCMVXRE, a pCMV128 equivalent which instead contains the Rex-responsive element sequences of HTLV-1 (17). Rex is the HTLV-1 regulatory protein equivalent to HIV-1 Rev and functions similarly to Rev in facilitating nucleocytoplasmic transport of unspliced RNA (3, 4, 17). Both HeLa and A9 cells were transfected with the pCMVXRE plasmid alone or cotransfected with a Rex expression plasmid, pRSVrex. As depicted in Fig. 3, both HeLa and A9 cells demonstrated a significant increase in CAT activity when cotransfected with pCMVXRE and pRSVrex plasmids compared with cells transfected with pCMVXRE alone. The increase in CAT activity, however, was more pronounced in HeLa cells than in A9 cells. These results demonstrate that the block in Rev function in A9 cells is specific for HIV-1 Rev. Since the activation domains of HIV-1 Rev and HTLV-1 Rex are interchangeable (17) and use the same pathway (11), it can be

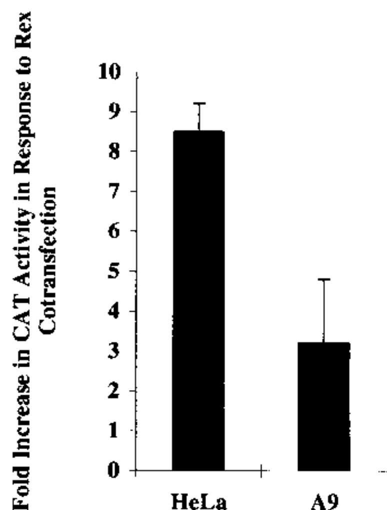

FIG. 3. HTLV-1 Rex function in A9 cells. HeLa and A9 cells were transfected with pCMVXRE plasmid alone or cotransfected with an HTLV-1 Rex expression plasmid (pRSVrex). Each sample was also transfected with β-galactosidase reporter construct pCMV110. CAT enzyme activity was determined in equal protein aliquots of cellular extracts. β-Galactosidase activity was determined as described previously. Results are expressed as the fold increase in CAT activity in Rex-transfected cells versus that in cells transfected with the reporter construct alone after correction for transfection efficiency, and each value represents an average of three replicates.

argued that A9 cells do not lack cellular factors which interact with the activation domain of Rev. Our previous observation that A9 nuclear proteins did not form RNP complexes with RRE RNA (34) would imply that impairment of Rev function in A9 cells may be due to lack of cellular factors (or the presence of inhibitors), which modulate the interaction of Rev with its target binding site in RRE RNA.

Rev is localized to the nucleolus in A9 cells. Understanding the mechanism of the block in Rev function in A9 cells may allow us identify cellular inhibitors of Rev. We therefore attempted to determine whether an impaired nuclear localization of Rev could be responsible for the block in Rev function in A9 cells. Subcellular localization of Rev in A9 and HeLa cells was studied with a Rev-GFP fusion protein.

Both A9 and HeLa cells were transfected with either GFP or Rev-GFP expression plasmids. Forty-eight hours after transfection, cells were observed under a fluorescent microscope. Fluorescence and bright-field images were collected with a Bio-Rad confocal imaging system. Figures 4 and 5 show the expression pattern of the GFP and Rev-GFP proteins in HeLa and A9 cells, respectively. Figures 4A and 5A show the fluorescence image while the Fig. 4B and 5B depict the bright-field image of the cells. As evident in Fig. 4 and 5, an overall diffused expression of GFP was observed in both A9 and HeLa cells. On the contrary, Rev-GFP was localized in the nucleoli in both HeLa cells and A9 cells. These results would indicate that the block in Rev function in A9 cells is not due to impaired nuclear localization of Rev protein in these cells.

Human chromosome 6- and 11-encoded factors rescue Rev function in A9 cells. We reported previously that human chromosome 6-encoded factors rescued the RRE RNA-host pro-

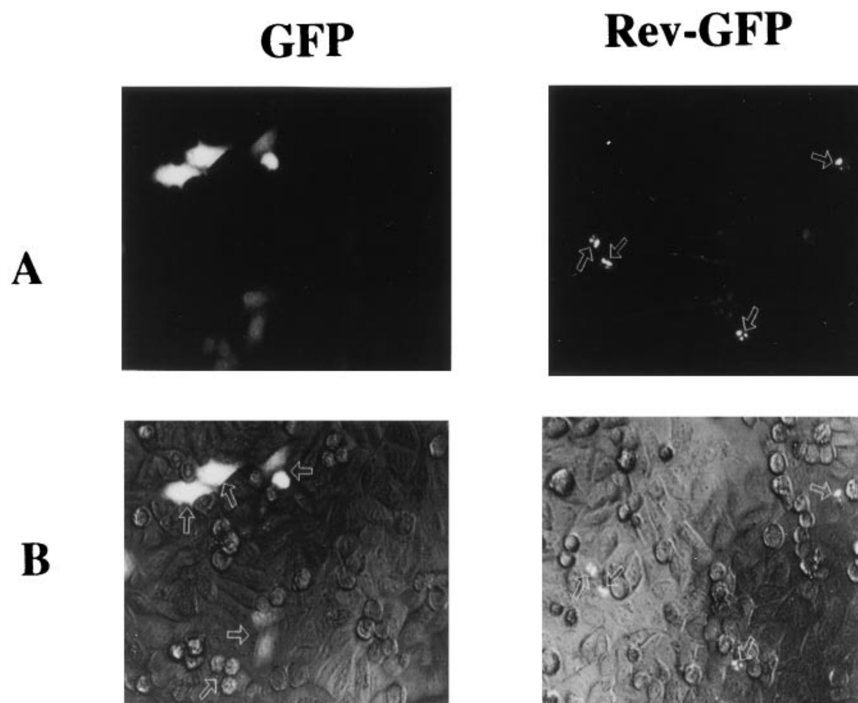

FIG. 4. Subcellular localization of HIV-1 Rev-GFP fusion protein in HeLa cells. HeLa cells were transfected with a GFP expression plasmid, pCMVGFP, and a Rev-GFP fusion protein expression plasmid, pCsRevsg25GFP. Forty hours after transfection, cells were examined under a fluorescence microscope with a ×40 objective. Images were examined with a Bio-Rad confocal imaging system. (A) Fluorescence image. (B) Bright-field image merged with the fluorescence image. Arrows indicate the cells expressing GFP.

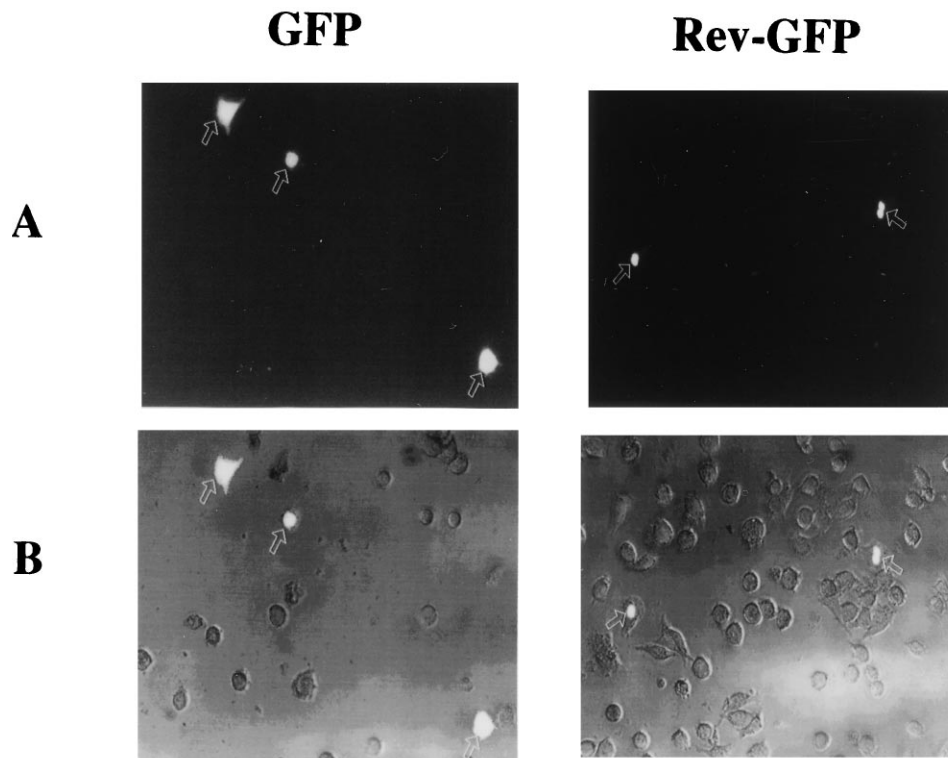

FIG. 5. Subcellular localization of HIV-1 Rev-GFP fusion protein in A9 cells. A9 cells were transfected with a GFP expression plasmid, pCMVGFP, and a Rev-GFP fusion protein expression plasmid, pCsRevsg25GFP, and examined as described in the legend to Fig. 4. (A) Fluorescence image. (B) Bright-field image merged with the fluorescence image. Arrows indicate the cells expressing GFP.

tein complex formation in A9 cells. Our next goal was to determine whether the inclusion of human chromosome 6 in A9 cells could rescue Rev function in vivo. A9 cells and somatic cell hybrids containing single human chromosomes 6, 8, 11, or 12 were transfected with the reporter plasmid pCMV128 alone or cotransfected with Rev expression plasmid pRSVrev. CAT activity in cellular lysates was determined as described previously (24); the results are shown in Fig. 6. Chromosome 6- and 11-containing microcell hybrids supported Rev function, while chromosome 8- or 12-containing cells showed only background-level activity. However, compared with a 33-fold increase in HeLa cells, human chromosome 6-containing A9 cells showed only a 3.5-fold increase in CAT activity, while chromosome 11-containing cells showed a 6-fold increase in CAT activity. These results support the hypothesis that HIV-1 Rev function requires host cell cofactors and that chromosomes 6 and 11 encode such factors.

The role of host proteins in Rev function has been suggested by several direct and indirect pieces of evidence, such as a requirement for non-Rev binding domains of RRE for optimal Rev function and inhibition of Rev function by TD Rev mutants (19). The fact that RNA splicing and nucleocytoplasmic transport of RNA require a complex set of RNA-protein interactions further substantiates the argument that HIV-1 Rev function would require a complex interaction with host cell factors. Our results clearly demonstrate the requirement for host factors in HIV-1 Rev function and that A9 cells lack such cofactors. Alternatively, A9 cells may contain a specific inhibitor of Rev function. A cell-specific block in Rev function has been reported in other cell types, such as glioma cells (29). It

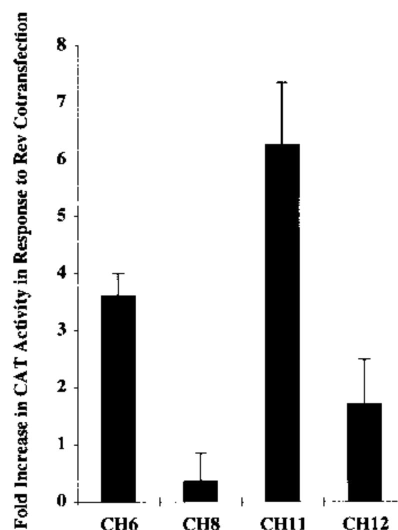

FIG. 6. Rev function in A9 somatic cell hybrids containing single human chromosomes. Parent A9 cells and their somatic cell hybrids containing single human chromosomes were transfected with reporter construct CMV128 alone or cotransfected with Rev-expressing plasmid pRSVrev. Each sample was also transfected with β-galactosidase reporter construct pCMV110. CAT enzyme activity in the cell lysates was determined as described previously. β-Galactosidase activity was determined as described previously. Results are expressed as the fold increase in CAT activity in Rev-transfected cells versus that in cells transfected with the reporter construct alone after correction for transfection efficiency, and each value represents an average of three replicates. Vertical bars represent standard deviation.

can be argued that the block in Rev function of A9 cells is cell type specific rather than species specific, since Rev function has been documented in cells from a variety of species (avian, murine, *Drosophila melanogaster*, and *Xenopus laevis*) (11, 12, 22, 38). A fairly conserved mechanism of RNA splicing and transport in animals will suggest that the factor(s) lacking in A9 cells is not a common splicing or transport factor. Restoration of the Rev function in somatic cell hybrids containing human chromosomes 6 and 11 suggests that these chromosomes encode such cofactors, which may play a crucial role in HIV-1 Rev function. However, the only 4- to 6-fold increase in CAT activity in these cells upon Rev cotransfection compared with the 40-fold increase in HeLa cells suggests that complementary cellular Rev cofactors are encoded by different chromosomes.

The absence of Rev function in chromosome 12-containing cells, which were reported earlier to support Tat-mediated transactivation, further suggests that host factors required for Tat-mediated transactivation and Rev-mediated RNA transport are different and are encoded by different chromosomes (15, 30). Our results are in agreement with the observation of Winslow and Trono, who earlier demonstrated the lack of Rev function in human chromosome 12-containing somatic cell hybrids (40). Further studies are needed in order to define the defective step of Rev-mediated gene expression in A9 cells. Defining the mechanism of Rev function in A9 cells may prove useful in a search for natural inhibitors of Rev function.

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