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Restriction of Human Immunodeficiency Virus Type 1 Production in a Human Astrocytoma Cell Line Is Associated with a Cellular Block in Rev Function

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Chronically human immunodeficiency virus type 1 (HIV-1) strain IIIB-infected human TH4-7-5 astrocytoma cells show low-level virus production. Cocultivation of TH4-7-5 cells with myelomonocytic cells led to active virus production in these target cells after a lag period, indicating cell-determined restriction of virus replication in the glial cells. HIV-1 transcript patterns of TH4-7-5 cells contained only a small proportion of Rev-dependent mRNA species, mimicking a Rev-negative phenotype despite the presence of *rev* mRNAs and protein. Sequencing of the single provirus integrated in TH4-7-5 cells demonstrated that the *rev* gene and the Rev-responsive element are intact. These results suggested inhibited function of the Rev-regulatory unit in these astrocytoma cells. Transfection of TH4-7-5 cells with a Rev expression plasmid resulted in weak or no induction of proviral $p24^{gag}$ antigen levels compared with the dramatic increase observed in Rev-permissive HeLa cells. Immunofluorescence analysis of TH4-7-5 cells transfected with a *rev*-expressing plasmid revealed prominent cytoplasmic and nuclear-nucleolar localization of Rev, in contrast to the predominant nuclear-nucleolar localization pattern of Rev in HeLa cells. We conclude that restriction of virus production in TH4-7-5 cells is at least partially due to a block in Rev-dependent posttranscriptional regulation of HIV expression.

The human immunodeficiency virus (HIV) is capable of infecting a wide variety of cells (for a review, see reference 41). The extent of virus production is target cell dependent and can vary considerably, from a silent to a fully productive phenotype. Variability in virus expression is determined by the interplay of different regulatory circuits which together determine the final outcome of infection. These regulatory circuits involve the interaction of viral elements and cellular factors on the transcriptional and posttranscriptional levels. Viral elements include the long terminal repeat (LTR), the positive *trans*-acting regulatory proteins Tat and Rev, and their RNA-binding sites (for reviews, see references 12, 22, 29, 32, 54, 62, and 80). Whereas the HIV LTR and Tat influence expression of all viral transcripts, Rev selectively regulates posttranscriptional expression of transcripts encoding the viral structural proteins. Rev specifically stabilizes transcripts containing the Rev-responsive element (RRE; 18, 70), and Rev is thought to chaperone them through the processes of nuclear transport (17, 18, 31, 44) and translation (2, 14, 40).

Investigation of HIV regulation in nonpermissive human cells is important in understanding virus-related pathogenesis and has potential implications for design of antiviral strategies. Persistent HIV type 1 (HIV-1) infection of human glial cells is associated with diminished virus production, indicating that they are candidate cells for restricted HIV replication (38, 72, 78; for reviews, see references 3 and 27). We previously reported the establishment of this HIV-1-infected astrocytoma cell line as a model system for HIV-1 replication in astrocytes (7). These cells produce very low levels of infectious virus compared with HIV-1 producer fibroblasts and T cells. Analysis of HIV antigen expression in astrocytoma cell line TH4-7-5 indicated low quantities of viral structural proteins and increased levels of Nef, a protein expressed early after HIV infection from Rev-independent multiply spliced mRNAs.

In this study, we analyzed Rev-dependent posttranscriptional regulation in the chronically infected astrocytoma cell line TH4-7-5. This cell line contains a cell-determined block in Rev-RRE regulation. The block in Rev function was not due to lack of Rev expression from the provirus and could not be overcome by increasing intracellular levels of Rev by transfection with a *rev* expression plasmid. Analysis of subcellular localization of Rev in transfected TH4-7-5 cells indicated an unusually high level of cytoplasmic staining of Rev. We conclude that cell-determined suppression of Rev-RRE function contributes to restriction of virus replication in persistently HIV-infected astrocytoma cells.

MATERIALS AND METHODS

Northern blot analysis. Total cellular RNA was prepared by the guanidinium isothiocyanate-CsCl technique (11). Poly(A)$^+$ selection was performed with a Poly(A)-Quik mRNA purification kit (Stratagene) in accordance with the manufacturer's protocol. We subjected 10 μg of total RNA and 1 μg of poly(A)$^+$-selected RNA to Northern blot analysis as previously described (66). The blots were hybridized with a nick-translated probe spanning the 3′ end of HIV-1 which hybridizes to all HIV-1 transcripts (nucleotides [nt] 8443 to 9118 of the HXB2R sequence) (51). The blots were rehybridized with a PCR-generated human β-actin fragment (14). We noted that despite equal loading of RNA samples, the actin mRNA levels in HeLa cells were lower than those in TH4-7-5 and LC5-HIV cells. Quantitative evaluation of hybridization products was carried out by scanning the filter with an AMBIS radioanalytic system.

PCR amplification and sequence analysis. (i) DNA PCR. The provirus from TH4-7-5 cells was PCR amplified and sequenced. The virus isolate is referred to as HIV-$1_{IIIB\text{-}TH}$, and the sequence is available under GenBank accession no. L31963 (HIVTH475A). Essentially, the provirus was amplified in four overlapping subfragments, and molecular clones from at least three independent PCRs per fragment were obtained and subjected to DNA sequence analysis.

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The *rev*-RRE sequences of HIV-$1_{IIIB-TH}$ in RC-2A myelomonocytic target cells were analyzed 66 days after coculture with TH4-7-5 cells. PCR amplification of the proviral *rev*-RRE-containing segment was carried out with the primer pair rev2-S–rev1-A by using genomic DNA extracted from 3.5×10^6 cells by the standard procedure (4) or crude cell lysates. The sequences of the primers and the nucleotide positions (51) are as follows: rev1-A (nt 8240 to 8212), CAGC TACTGCTATAGCTGTGGCATTGAGC; rev2-S (nt 7261 to 7286), ACCCAC CAAGGCAAAGAGAAGAGTGG.

For preparation of crude cell lysates, aliquots corresponding to 1,000 cells were lysed at 100°C for 10 min in standard 1× PCR buffer (PEC; 10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% glycerol) containing 0.45% Tween 20 and 0.45% Nonidet P-40, and the lysates were used directly for PCR. PCRs were carried out as previously described (36) in a GeneAMP PCR System 9600 thermocycler (Perkin-Elmer-Cetus). A 5-μl volume of the reaction mixture of crude cell lysates was subjected to a second round of PCR with the primer pair MNSeq.31–rev1-A under the same reaction conditions. The sequence of primer MNSeq.31 (nt 7302 to 7320) is GAGCAGTGGGAATAGGAGC.

(ii) Reverse transcription (RT) PCR analysis. *rev* cDNA was amplified from RC-2A cells infected with HIV-$1_{IIIB-TH}$ by RT PCR with primer pairs SJ1.4A-S–rev1-A and SJ1.4B-S–rev1-A for 30 cycles as previously described (53). The sequences of the primers used are as follows: SJ1.4A-S (nt 276 to 289 and 5501 to 5506), AGGCGAGGGGAGGCGACTGCCTTAG; SJ1.4B-S (nt 276 to 289 and 5507 to 5511), AGGCGAGGGGAGGCGACTGGCATC. The resulting amplification products containing the complete *rev* open reading frame were purified by agarose gel electrophoresis and subjected to direct sequence analysis.

(iii) Sequence analysis of amplification products. DNA sequence analysis of plasmids and PCR amplification products was carried out by the dideoxy-mediated chain termination method (67). Plasmids were purified by CsCl-ethidium bromide centrifugation. Amplification products were purified by agarose gel electrophoresis and isolated by a modified freeze-squeeze technique (74) or, alternatively, by high-pressure liquid chromatography (10). Sequencing reactions were performed with 1/4 to 1/10 of the PCR product, as previously described (10), and samples were analyzed on an ABI 373A DNA Sequencer.

Cells, cell culture, and transfection techniques. Low-producer persistently HIV-1-infected human astrocytoma cell line TH4-7-5 was established by multiple rounds of cell cloning following exposure of parental 85HG-66 astrocytoma cells to HIV-1_{IIIB} (7). The U373MG and U251MG cell lines were derived from human malignant astrocytomas (6, 82). HIV producer cell line LC5-HIV is a persistently HIV-1_{IIIB}-infected human lung fibroblast cell line (46). HIV-1-susceptible T-lymphoma cell line KE37/1 (60) and syncytium-forming C8166 cells (65) were used for productive infection with HIV-1 and for assessment of infectious virus, respectively. Chronically HIV-1_{IIIB}-infected KE37/1 cells were used for production of HIV-1_{IIIB} virus stocks. RC-2A is a monocyte-macrophage type cell line isolated from peripheral blood of a patient with myelomonocytic leukemia (8) which supports productive infection with HIV-1 and HIV-2 (75). HeLa-derived cell line HLtat (69) is stably transfected with *tat* expression plasmid pL3tat (20). HeLa-derived cell line HLfB contains stably integrated copies of *rev*-defective mutant provirus fB (47). The Rev-expressing mouse fibroblast cell line 7.4/Rev is a bovine papillomavirus-transformed C127 cell line containing pBMcrev, which contains the mouse metallothionein promoter linked to HIV-1 *rev* cDNA and the human metallothionein (hMt1A) gene as a selection marker (19a). Cells were maintained in RPMI 1640 medium or in Dulbecco modified Eagle medium with 10% fetal calf serum under standard cell culture conditions as previously described (7). 7.4/Rev mouse fibroblast cultures contained 20 μM $CdCl_2$, which was added to induce Rev expression. Transfections were carried out by the calcium phosphate technique as previously described (20, 28). Assessment of intracellular Gag production was carried out 24 h posttransfection by $p24^{gag}$ antigen capture enzyme-linked immunosorbent assay as previously described (71).

Recombinant plasmids for cell transfections. Production of HIV-1 transcripts in HeLa cells was assessed by transfection with HIV-1 infectious proviral molecular clone HXB2. The following constructs for expression of Rev and Gag were used for transfection of human cells to study the effect of Rev: (i) Rev expression plasmid pBsrev, a derivative of pLsrev (47) in a Bluescript vector (13); (ii) Rev-independent Gag expression plasmid pCMV37M1-10, in which expression of the $p37^{gag}$ encoding region was mutated to inactivate inhibitory elements (67a, 68) is under control of the cytomegalovirus promoter; (iii) Rev-dependent Gag expression construct p37R (51a), which contains the HIV-1 5′ LTR promoter and the $p37^{gag}$ encoding region, followed by the RRE and the HIV-1 3′ LTR. p37R is similar to p37 (68) but is inserted into a Bluescript vector. pL3tat is a Tat expression plasmid (20). The *rev*, *gag*, and *tat* genes in plasmids pBsrev, p37R, and pL3tat are under control of the HIV-1 LTR.

Transfection efficiencies were assessed by immunofluorescence by using anti-Rev serum and found to be similar for the 85HG-66 and HeLa cell lines (~20 to 40% transfected cells). Cotransfection with luciferase expression plasmid pRS-Vluc (16) resulted in luciferase production that was similar among different plates of a cell line but differed between different cell types.

Infection of hematopoetic target cells by HIV-1. (i) Infection of RC-2A cells by cocultivation with TH4-7-5 cells. RC-2A cells (5×10^5) were added to subconfluent cultures of TH4-7-5 cells in 25-cm^2 flasks. After coculture for 6 days, RC-2A cells were removed from monolayers of TH4-7-5 cells. RC-2A cells were expanded through two cell passages to ensure removal of any contaminating glial cells before assessment for HIV-1 infection.

(ii) Infection of RC-2A and KE37/1 target cells by exposure to cell-free HIV-1_{IIIB} virus. Target cells (5×10^5) were resuspended in 1 ml of cell-free culture supernatant from HIV-1_{IIIB}-infected KE37/1 producer cells (≈10^6 50% tissue culture-infective doses per ml as assessed with C8166 indicator cells) and incubated for 2 h at 37°C. Cells were washed with phosphate-buffered saline and resuspended in culture medium for further propagation.

Assessment of virus production by HIV-infected cells. HIV-infected cells were detected by indirect immunoperoxidase staining with human anti-HIV-1 serum (46). Virus production in culture supernatant was assessed by $p24^{gag}$ antigen capture enzyme-linked immunosorbent assay (Du Pont) and evaluation of induction of HIV-positive syncytia in C8166 cultures as previously described (7). RT levels in culture supernatant were determined with a nonradioactive RT-Assay Kit (no. 1468120; Boehringer, Mannheim, Germany) in accordance with the manufacturer's instructions.

Immunoprecipitation and indirect immunoflourescence. Cells were metabolically labelled with [^{35}S]cysteine as previously described (20), with the following modifications. Cells were incubated in cysteine-free medium for 6 h and subsequently labelled with [^{35}S]cysteine overnight. Immunoprecipitation of Rev with rabbit antiserum raised against bacterially expressed and purified Rev protein (5) was followed by separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). Indirect immunofluorescence staining of Rev was carried out with cells fixed by treatment with formaldehyde–Nonidet P-40 as previously described (21). Staining of Gag was carried out with pooled human anti-HIV-1 serum (Scripps Laboratory, Inc., San Diego, Calif.).

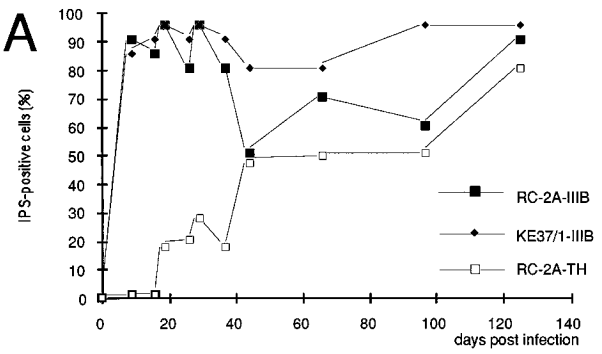
RESULTS

Persistently HIV-1-infected TH4-7-5 glial cells contain a functional virus. HIV-1-infected glial cell line TH4-7-5 produces very low levels of Gag (7). The single-copy proviral genome in this cell line was assessed by nucleotide sequence analysis of four overlapping segments amplified from genomic DNA of TH4-7-5 cells by the PCR technique. The TH4-7-5 proviral genome was found to be full length (9,759 bp) and displayed all of the features of a replication-competent provirus, including intact open reading frames for all essential gene products, as well as conservation of regulatory signal sequences (52a). Sequence comparison with other HIV-1 strains revealed that the TH4-7-5 provirus is clearly a member of the HIV-1_{IIIB} group and is most closely related to replication-competent proviral clone HXB2, with 99% amino acid sequence identity in the *gag* and *pol* genes (51). The virus isolate is referred to as HIV-$1_{IIIB-TH}$ in this report.

To show that replication-competent virus can be recovered from TH4-7-5 cells, we established persistently infected producer cells by cocultivation with myelomonocytic cell line RC-2A (Fig. 1), which is permissive for productive infection with HIV-1 (8, 75). HIV-1 infection and virus production were monitored by detection of HIV antigen-positive cells by indirect immunoperoxidase staining and assessment of culture supernatant for $p24^{gag}$ antigen levels, RT activity, and induction of HIV-positive syncytia with C8166 indicator cells. Production of progeny virus recovered from chronically infected glial cells was compared with virus production by persistently HIV-1_{IIIB}-infected RC-2A cells and KE37/1 T-lymphoma cells (7), which are routinely used for propagation of high-titer virus stocks.

Infection of RC-2A cells by coculture with TH4-7-5 cells was associated with an extended lag period, in agreement with the restricted-infection phenotype of chronically HIV-infected glial cells (Fig. 1A). However, during the persistent phase of infection, $p24^{gag}$ antigen levels and RT activity in culture supernatant of RC-2A cells infected by coculture with TH4-7-5 cells (RC-2A-TH) were in the same range as those detected in HIV_{IIIB} producer RC-2A (RC-2A-IIIB) and KE37/1 (KE37/1-IIIB) cells (Fig. 1B). HIV-1 produced by RC-2A-TH cells was infectious for C8166 cells and capable of syncytium induction, although virus titers were lower than those observed in the other HIV-1_{IIIB}-infected cells (Fig. 1B).

These results show that virus recovered from TH4-7-5 cells



B

	HIV-positive cells (%)	Virus production in culture supernatant: p24 antigen (ng/ml)	RT (ng/ml)	syncytia inducing capacity(log_{10} $TCID_{50}$/ml)
RC2A-TH	50	323	2.8	4
RC2A-IIIB	70	276	n.d.	6
KE37/1-IIIB	80	389	2.9	6.5

FIG. 1. Recovery of replication-competent virus from chronically HIV-1-infected astrocytoma cell line TH4-7-5 by cocultivation with myelomonocytic target cells (RC-2A). Virus production was assessed in parallel in RC-2A target cells infected with HIV-$1_{IIIB-TH}$ by coculture with TH4-7-5 cells (RC-2A-TH) and in RC-2A and KE37/1 T-lymphoma target cells (KE37/1-IIIB) inoculated with an HIV-1_{IIIB} isolate. (A) Kinetics of HIV infection as reflected by an increase in the percentage of HIV antigen-expressing cells with time. HIV-positive cells were detected by indirect immunoperoxidase staining with human anti-HIV serum. (B) HIV production in culture supernatant at 66 days postinfection. Culture supernatant was assessed for $p24^{gag}$ antigen levels, HIV RT activity, and induction of HIV-positive syncytia in C8166 indicator cells as described in Materials and Methods. A subset of these data is shown in reference 35. n.d., not done. $TCID_{50}$, 50% tissue culture-infective doses.

is replication competent and capable of active production in hematopoietic target cells. This suggests that low-level virus production in TH4-7-5 cells involves cell-directed restriction of a biologically functional provirus.

Rev-independent transcripts are predominant in TH4-7-5 cells. To assess HIV-1 expression on the RNA level in TH4-7-5 cells, patterns of HIV-1-specific mRNAs were compared with those of chronically HIV-1-infected producer cells. Northern blot analysis (Fig. 2) revealed the predominance of multiply spliced transcripts over intermediate and unspliced transcripts in TH4-7-5 cells (lanes 1 and 2). HIV-1 RNA of LC5-HIV HIV-1 producer fibroblasts (lanes 3 and 4) and HeLa cells transfected with molecular clone HXB2 (lane 5) contained a higher proportion of intermediate and unspliced mRNA classes. In HeLa cells containing *rev*-defective mutant provirus HXB2/fB (30), HIV mRNAs consisted mainly of multiply spliced transcripts (lane 6). Similarity to the Rev-deficient phenotype suggests that Rev activity may be impaired in TH4-7-5 cells. Alternatively, the transcript pattern in TH4-7-5 cells could be the result of differences in the activities of cellular factors that regulate splicing.

Detailed analysis of HIV-1 expression in TH4-7-5 cells by the RT-initiated PCR method revealed largely the same RNA species in TH4-7-5 cells as in HIV-1 producer cells, including the presence of all major *rev*-, *tat*-, and *nef*-specific transcripts (35, 53). In agreement with the Northern blot data in Fig. 2, this method also revealed only low levels of RNA species relating to Rev-dependent intermediate and unspliced classes

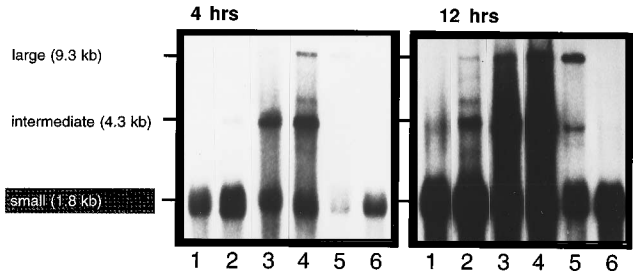


FIG. 2. Predominance of the small, Rev-independent class of transcripts over Rev-dependent intermediate and large transcript classes in low HIV-1 producer astrocytoma cells (TH4-7-5). Northern blot analysis (4- and 12-h exposures) was carried out with total RNA extracted from TH4-7-5 cells (lanes 1), HIV-1_{IIIB} producer fibroblasts (LC5-HIV; lanes 3), HeLa cells transfected with HIV-1 infectious molecular clone HXB2 (lanes 5), and HeLa cells transfected with Rev-defective molecular clone HXB2/fB (lanes 6). In addition, assessment of poly(A)$^+$-selected mRNA was performed for TH4-7-5 (lanes 2) and LC5-HIV (lanes 4) cells. Hybridizations were carried out with a *nef* LTR fragment containing sequences common to the 3′ ends of all HIV-1 transcripts.

of HIV-1 in TH4-7-5 cells. These results indicate that the same splice sites are used in both cell lines, leading to potentially functional *rev* transcripts. Thus, the major difference in HIV-1 mRNA expression in both cell types lies in the decreased steady-state levels of Rev-dependent mRNAs in this astrocytoma cell line.

HIV-1 Rev protein is produced in TH4-7-5 cells. We next demonstrated that the *rev* mRNAs identified above produce

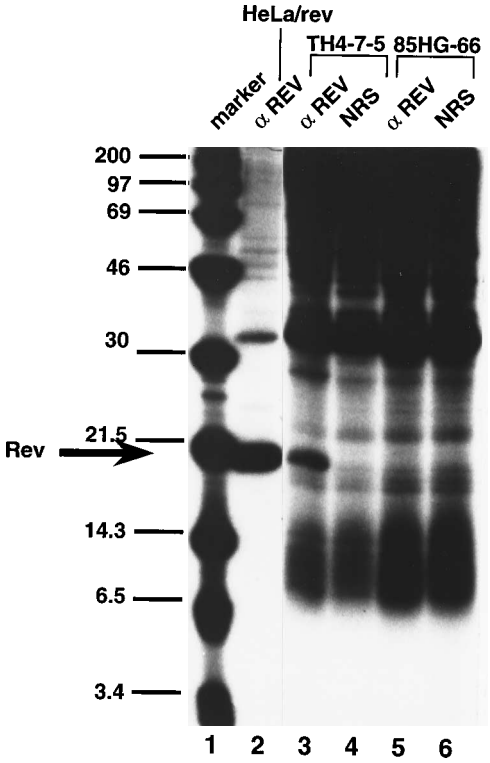
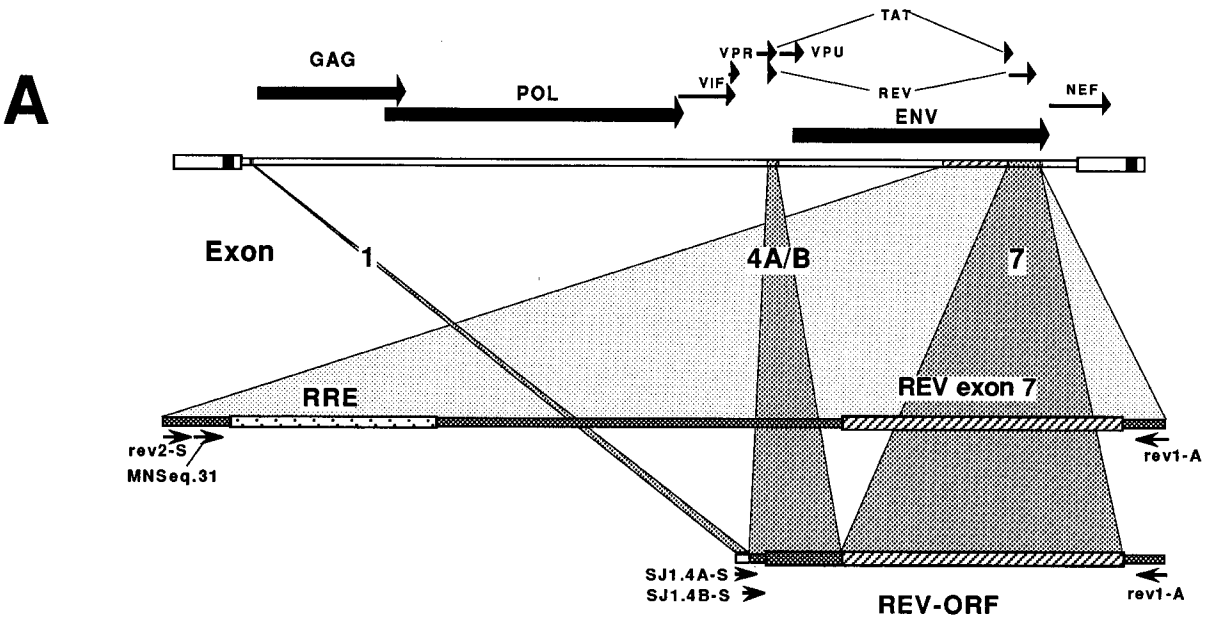


FIG. 3. Immunoprecipitation of Rev from persistently HIV-1-infected astrocytoma cells (TH4-7-5). Cells were metabolically labelled with [^{35}S]cysteine, and the cell lysates were immunoprecipitated with normal rabbit serum (NRS) and rabbit antiserum against Rev (αREV). Lanes: 1, protein molecular size markers (sizes [in kilodaltons] are listed on the left); 2, HeLa cells transfected with *rev* expression plasmid pBsrev; 3 and 4, chronically HIV-1-infected astrocytoma cells (TH4-7-5); 5 and 6, uninfected parental astrocytoma cell line 85HG-66.



B

IIIB-consensus	MAGRSGDSDE eLlkaVRLIK fLYQSNPPPn PEGTRQARRN RRRRWRERQR QIHSISERIL	60
REV-RC2A-TH	....IRT..... L.....	60
REV-TH	....IRT..... L.....	60
REV-HXB2	....IRT..... L.....	60
REV-LAI	..... D.....	60
IIIB-consensus	sTYLGRSAEP VPLQLPPLER LTLDCNEDCG TSGTQGVGSP QILVESPTvL ESGaKE	116
REV-RC2A-TH	....I.	116
REV-TH	....I.	116
REV-HXB2	G..... ....T..	116
REV-LAI	....T..	116

FIG. 4. Analysis of *rev* and RRE sequences in myelomonocytic producer cells for HIV-$1_{IIIB\text{-}TH}$. *rev*-RRE sequences were determined in RC-2A target cells 66 days after transfer of virus by coculture with TH4-7-5 cells (Fig. 1). The *rev* and RRE sequences were identical to the corresponding sections of the HIV-$1_{IIIB\text{-}TH}$ provirus in TH4-7-5 cells. (A) A proviral fragment containing the RRE and *rev* exon 7 was amplified from RC-2A-TH cells by one-step (high-molecular-weight genomic DNA) and two-step (crude cell lysates) PCRs, respectively, with the primers indicated. *rev* cDNA was obtained by RT PCR with primers spanning the region between exons 1 and 4A (SJ1.4A-S) or 1 and 4B (SJ1.4B-S) and the rev1-A primer. Purified amplification products from at least two independent PCRs were subjected to direct sequence analysis without additional cloning steps. The locations of the amplified regions in the proviral genome are indicated by light grey shading for sequence analysis of proviral *rev*-RRE-containing DNA and by dark grey shading for analysis of *rev* cDNA. (B) Comparison of the Rev amino acid sequences in RC-2A-TH and TH4-7-5 cells (***REV-RC2A-TH*** and ***REV-TH***) with the HIV-1_{IIIB} consensus sequence (51) and HIV-1_{IIIB} representatives HXB2 (REV-HXB2) and LAI (REV-LAI). The nucleotide sequence of the RRE in TH4-7-5 and RC-2A-TH cells was identical to the HIV-1_{IIIB} consensus sequence and HXB2 and LAI (data not shown). ORF, open reading frame.

Rev protein in TH4-7-5 cells. TH4-7-5 cells were metabolically labelled with [^{35}S]cysteine, and Rev was immunoprecipitated from the cell extract with anti-Rev serum (Fig. 3). Parallel immunoprecipitations were carried out with HLtat cells transfected with *rev* expression plasmid pBsrev as a positive control. Extracts from parental uninfected astrocytoma cell line 85HG-66 (7) were assayed as negative controls.

The anti-Rev serum precipitated a 19-kDa protein from extracts of TH4-7-5 cells (lane 3) not detected with normal rabbit serum (lane 4) or in cell extracts from parental uninfected astrocytoma cell line 85HG-66 (lanes 5 and 6). This band comigrated with the Rev protein immunoprecipitated from Rev-expressing HeLa cells by anti-Rev serum (lane 2). These results demonstrate that Rev protein is produced in TH4-7-5 cells.

Proviral components of the Rev-RRE regulatory axis of HIV-$1_{IIIB\text{-}TH}$ are functional. It has previously been shown that virus cannot be recovered from cells transfected with *rev*-defective provirus clone fB by coculture with sensitive indicator cells (30). In contrast, coculture of TH4-7-5 cells with myelomonocytic cells (Fig. 1) and with T-lymphoma cells (7) results, after a lag period of over 2 weeks, in active production of infectious virus in the hematopoetic target cells. This suggests that TH4-7-5 cells contain a provirus capable of Rev regulation in a nonglial intracellular environment.

Nucleotide sequence analysis of proviral DNA and the *rev*-encoding mRNA was carried out to confirm that the *rev*-RRE viral components in RC-2A-TH producer cells are identical to the corresponding sections of the HIV-$_{IIIB\text{-}TH}$ provirus. A 980-bp region encompassing the RRE and *rev*-encoding sequences in exon 7 was amplified by PCR from RC-2A-TH producer cells 66 days after infection. This region was amplified from high-molecular-weight genomic DNA by one-step PCR with appropriate primers, as well as two-step PCR from crude cell lysates (Fig. 4A). *rev*-encoding mRNAs 1.4A.7 and 1.4B.7 (69) were amplified specifically from RNA of RC-2A-TH cells by combination of primers spanning exons 1 and 4A (SJ1.4A-S) and 1 and 4B (SJ1.4B-S), respectively, with downstream primer rev1-A for RT PCR (Fig. 4A). All amplification products were sequenced directly without molecular cloning to ensure that the derived sequence information would reflect the predominant species in the sample.

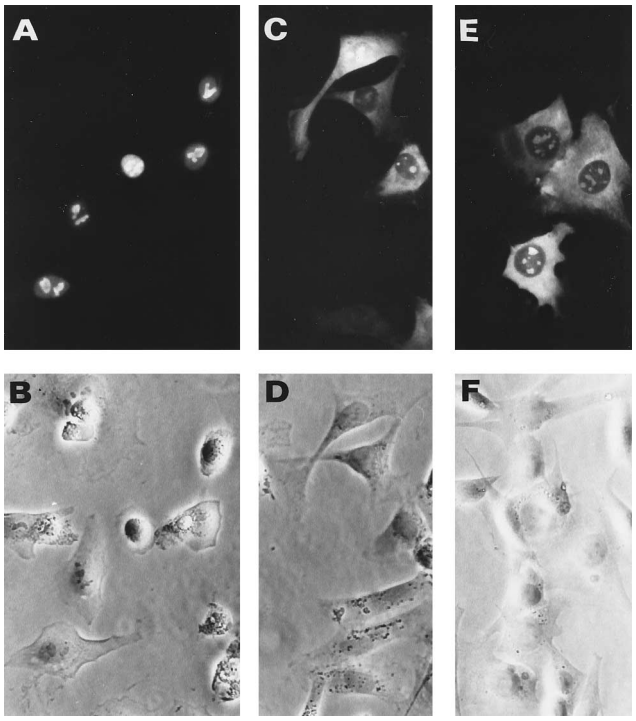


FIG. 5. Indirect immunofluorescence staining of Rev in Rev-expressing cell lines. HLtat and TH4-7-5 cells were transfected with *rev* expression plasmid pBsrev and immunofluorescence staining was carried out with polyclonal rabbit antiserum against Rev. The staining pattern of Rev in TH4-7-5 cells is distinctly different from the exclusive nuclear-nucleolar staining in Rev-permissive HLtat cells. Panels: A and B, HeLa-derived cell line (HLtat); C and D, HIV-1-infected astrocytoma cell line (TH4-7-5); E and F, murine fibroblast cell line (7.4/Rev) persistently producing high levels of Rev protein. Panels A, C, and E show immunofluorescence staining; panels B, D, and F show phase-contrast microscopy.

There was complete nucleotide agreement between the RRE sequences of the HIV-$1_{IIIB-TH}$ provirus and the other members of the IIIB family (data not shown). Analysis of the *rev* coding region revealed no changes between the RC-2A-TH-derived virus and the HIV-$1_{IIIB-TH}$ provirus (Fig. 4B). REV-TH and REV-HXB2 differ from the REV-IIIB consensus by one amino acid (V to I at position 109), which is a conservative exchange. Isoleucine is also found at this position in several other *rev* genes (51). REV-TH and REV-HXB2 differ from the IIIB consensus by a few amino acids in the amino-terminal portion. Taken together with the finding that the TH4-7-5 virus infects RC-2A cells (Fig. 1), these data indicate that the HIV-$1_{IIIB-TH}$-derived Rev-RRE regulatory unit is functional and capable of supporting virus replication in a nonglial intracellular environment.

Distinct subcellular localization of Rev in TH4-7-5 cells. Subcellular localization of Rev was assessed in transfected TH4-7-5 cells by indirect immunofluorescence with anti-Rev serum (Fig. 5). While no staining was observed in untransfected or mock-transfected cells (data not shown), transfection with pBsrev resulted in intense staining in 20 to 40% of TH4-7-5 cells (Fig. 5C). Besides confirming that transfection of TH4-7-5 cells had actually occurred, these results indicate that provirus-encoded Tat is active in TH4-7-5 cells, since pBsrev is a *tat*-dependent plasmid. Staining of Rev was detected in the cytoplasm, nuclei, and nucleoli of TH4-7-5 cells. In contrast, *rev*-transfected HeLa-derived cells (HLtat) showed staining of the nuclei with predominant staining of nucleoli (Fig. 5A). The subcellular localization pattern of Rev in TH4-7-5 cells resembled that of Rev-expressing murine 7.4/rev fibroblasts, which also show strong cytoplasmic and nucleolar subcellular localization of Rev (Fig. 5E).

Expression of additional Rev protein does not overcome the block in Rev function in TH4-7-5 cells. To examine whether additional Rev would increase Gag production in TH4-7-5 cells, these cells were transfected with increasing amounts of *rev* expression plasmid pBsrev. Despite the presence of Rev (Fig. 5), no significant increase of $p24^{gag}$ antigen levels in TH4-7-5 cell compared with mock-transfected cells was detected (Table 1). Varying the amount of plasmid pBsrev from 0.1 to 5 μg in the transfection reaction had no effect on $p24^{gag}$ production. The average $p24^{gag}$ values of three individually transfected plates in a representative transfection experiment are shown in Table 1. As a control, transfection of HeLa cells containing a *rev*-defective molecular clone (HLfB [47]) with the same DNA mixture resulted in a dramatic (>100-fold) increase of $p24^{gag}$ levels. To control for transfection efficiency within one cell line, luciferase expression plasmid pRSVluc was cotransfected. Production of luciferase was higher in HLfB cells than in TH4-7-5 cells (Table 1). Since the indirect immunofluorescence assay revealed similar transfection efficiencies in both cell lines, the different luciferase levels most likely reflect distinct promoter activities in these cell lines. In the presence of increasing amounts of pBsrev, expression of cotransfected pRSVluc decreased three- to fivefold (Table 1), in agreement with previous observations (73). Note that maximal Rev response was achieved by addition of 0.5 to 1 μg of this Rev-producing plasmid (our own observation). Addition of 5 μg of this plasmid is a large excess and results in a slight decrease in expression of non-RRE-containing mRNAs, such as the luciferase mRNA. In summary, these results indicate

TABLE 1. Transfection of chronically HIV-1-infected astrocytoma cell line TH4-7-5 and HeLa cells containing Rev-defective provirus HXB2/fB with Rev expression plasmid pBsrev

Plasmid (amt [μg])	TH4-7-5 cells			HXB2/fB-infected HeLa cells		
	Luciferase activity (RLU)[a]	$p24^{gag}$ (pg/ml)	Fold induction	Luciferase (FFU)	$p24^{gag}$ (pg/ml)	Fold induction
pBluescript (mock transfection)	10[b]	1,071[c]		11[b]	814[c]	
pBsrev (0.1)[d]	84	1,622	1.5	1,641	46,939	58
pBsrev (1)[d]	57	1,328	1.2	763	103,552	127
pBsrev (5)[d]	27	1,262	1.2	289	86,049	106

[a] RLU, relative light units.
[b] Negative control value for test; average of two plates.
[c] Average of four plates.
[d] Average of three plates.

TABLE 2. Rev-mediated Gag induction in two different cell lines

Cell line and source of data	$p24^{gag}$ (pg/ml)		Fold induction	$p24^{gag}$ (pg/ml); luciferase activity (RLU)[a]		Fold induction
	Tat^- Rev^-	Tat^- Rev^+		Tat^+ Rev^-	Tat^+ Rev^+	
85HG-66						
Multiple expts	31[b]	178[b]	6[c]	1,391[d]	7,901[d]	8[c]
Expt 8[e]	ND[f]	ND		2,945; 33	11,912; 29	4
HeLa						
Multiple expts	<7[b]	10[b]	ND	200[g]	52,821[g]	279[c]
Expt 8[e]	ND	ND		195; 130	22,705; 102	116

[a] RLU, relative light units.
[b] Average of two experiments.
[c] Average of inductions calculated for each experiment.
[d] Average of eight experiments.
[e] The same transfection mixture was added to three individual plates of each cell line; the p24 value represents the average of these three plates.
[f] ND, not done.
[g] Average of seven experiments.

that the block in the Rev-RRE regulatory axis in TH4-7-5 cells cannot be overcome by increasing intracellular levels of Rev after transfection.

Rev functions inefficiently in uninfected parental cell line 85HG-66. To evaluate Rev-dependent expression of Gag in uninfected parental 85HG-66 cells, we studied *gag* expression from plasmid p37R in the absence and presence of Tat-producing plasmid pL3tat. Plasmid p37R contains the $p37^{gag}$-encoding region ($p17^{gag}$ and $p24^{gag}$) and the RRE and thus depends on Rev for efficient expression. Transcription of p37R, like that of pL3tat and pBsrev, is controlled by the HIV LTR promoter.

In the absence of Tat, very low levels of Gag were produced in 85HG-66 cells, while no Gag production was detected in HeLa cells (Table 2). Cotransfection with Rev-expressing plasmid pBsrev in 85HG-66 cells resulted in a sixfold increase in Gag production, demonstrating that Rev-mediated Gag production is independent of Tat. Cotransfection of these cells with p37R and Tat-producing plasmid pL3tat led to an over-40-fold increase in Gag levels (Table 2), confirming previous observations that Tat is functional in transient transfection assays (7). Similarly, Tat elevated basal-level Gag expression in HeLa cells as expected.

Rev-dependent induction of Gag expression in the presence of Tat was assessed further. 85HG-66 and HeLa cells were cotransfected with p37R and pL3tat in the absence or presence of pBsrev, and Gag production was measured 1 day later. Table 2 shows the average of seven or eight experiments, as well as the results of a typical experiment (no. 8). In experiment 8, the same transfection mixture was added to three individual plates of 85HG-66 and HeLa cells. The presence of Rev resulted in an average eightfold increase of Gag production in 85HG-66 cells. This agrees with the Rev-dependent induction of Gag expression observed in the absence of Tat. In contrast, in HeLa cells, the Rev response was greatly increased (>200-fold average induction). Luciferase values indicate that similar transfection efficiencies were achieved in the presence and absence of Rev for each cell line (see above; Table 1).

The experiments whose results are summarized in Table 2 were designed to simulate the situation in HIV-infected cells, in which expression of all viral components is directed by HIV LTR-Tat activity and production of Gag is dependent on the Rev-RRE regulatory axis. Under these conditions, Rev-dependent induction of Gag expression in parental 85HG-66 glial cells was less than 10% compared with induction in Rev-permissive HeLa cells. These results indicate that decreased Rev-RRE-dependent posttranscriptional regulation is a property of chronically HIV-infected TH4-7-5 cells, as well as parental 85HG-66 cells.

To evaluate whether uninfected astrocytoma cells are capable of producing higher levels of Gag, we transfected parental astrocytoma cell line 85HG-66 with *tat*- and *rev*-independent Gag expression construct pCMV37M1-10 (67a, 68). This plasmid contains an HIV-1 *gag* gene with all of the identified instability-inhibitory elements eliminated and allows Rev-independent expression of Gag in HeLa cells. Transfection of pCMV37M1-10 into 85HG-66 cells resulted in Gag production that was >10-fold higher than in persistently HIV-infected TH4-7-5 cells and was comparable to that in transfected HeLa cells (data not shown). In summary, these data indicate that Gag can be produced in the astrocytoma cell line at high levels, but only if expressed in a Rev-independent manner.

DISCUSSION

Comparison of restricted-infection phenotype in TH4-7-5 cells with virus suppression in model cell lines for HIV-1 proviral latency. Several persistently HIV-1-infected low-producer cell lines have been proposed as model cell lines for HIV-1 latency (i.e., T-lymphocytic cell line ACH2 [23], monocytic cell line U1 [24], and monocytic THP1-derived cells [50]). In U1 and ACH-2 cells, the primary cause of viral latency seems to be transcriptional, although different mechanisms are responsible for transcriptional repression of HIV-1 in each cell line (8, 9, 39, 57, 83). Whereas virus production in U1 and ACH-2 cells is dramatically raised by cytokines and phorbol esters (25, 49, 56, 59), only modest stimulation is observed in TH4-7-5 cells (35) and in other persistently HIV-1-infected astrocytes (72, 77). This suggests that suppression of virus production in astrocytoma cells differs from that in latently infected hematopoetic cells.

This difference is further supported by the HIV-1 transcript pattern of TH4-7-5 cells. Whereas latently infected U1 and ACH2 cells lack mainly unspliced genomic mRNAs (59), in TH4-7-5 cells, both singly spliced and unspliced classes of HIV-1 transcripts are present but underrepresented. The similarity of the transcript pattern of TH4-7-5 cells to that of replication-defective, *rev*-defective mutant provirus (19, 30; Fig. 2) suggests that Rev activity may be impaired in TH4-7-5 cells. Alternatively, the transcript pattern in TH4-7-5 cells

could be the result of differences in activity between cellular factors that regulate splicing of HIV-1. Such a possibility needs to be addressed experimentally. Note, however, that *gag* expression plasmid p37R (Table 2) contains no functional splice sites and still cannot be regulated by Rev in 85HG-66 cells as in Rev-permissive HeLa cells.

Block of Rev-RRE regulatory axis in human glial cells. Our data suggest that restriction of virus expression in chronically HIV-1-infected TH4-7-5 glial cells involves a cellular block in Rev-RRE-dependent regulation. This block is not due to expression of Rev at levels below a critical threshold postulated for Rev function (58), since it is not overcome by increasing intracellular levels of Rev by transfection with a *rev* expression plasmid. Preliminary experiments with additional astrocytoma cell lines U373MG and U251MG showed only a low Rev response of p37R, like that which we have observed in 85HG-66 and TH4-7-5 cells. Experiments intended to analyze these glial cell lines in more detail are in progress. These results indicate that decreased Rev-RRE-dependent posttranscriptional regulation is not a unique property of chronically HIV-infected TH4-7-5 cells or parental 85HG-66 cells and suggest that it may extend to other, unrelated astrocytoma cell lines.

The block in Rev-RRE function in astrocytoma cells could be due to either lack of necessary positive cellular cofactors (1, 5, 18, 79, 84) or the presence of negative cellular factors capable of repressing Rev-RRE regulation. Expression and activity of Rev in TH4-7-5 cells share several features with those in the murine 7.4/Rev cell line, which is nonpermissive for Rev function (22a) and shows unusually high levels of cytoplasmic Rev. Analysis of the subcellular localization of the unrelated simian virus 40 T antigen and of HIV-1 Tat clearly revealed their exclusive nuclear and nucleolar accumulation, respectively (data not shown). This further supports our finding that the unusual localization of Rev in TH4-7-5 and 85HG-66 cells is specific to Rev. Experiments are in progress to further examine the underlying mechanism resulting in the cytoplasmic accumulation of Rev. The block in Rev function in TH4-7-5 cells was not relieved by fusion with Rev-permissive cells, in contrast to that in murine 7.4 cells (data not shown). In addition, initial experiments revealed that Rex of human T-cell leukemia virus type I, the functional homolog of HIV-1 Rev, also does not function in 85HG-66 cells (data not shown). Although these results are preliminary, they suggest that human *trans*-dominant cellular factors are involved in inactivating the Rev and Rex functions in TH4-7-5 cells.

Both the Rev protein and the Rev-dependent mRNAs could serve as targets for such inhibitory factors. Inactivation of Rev protein could occur by obstruction of domains crucial for its activity, such as the RNA-binding and effector domains (33, 34, 43, 45, 47, 55), or by inhibition of Rev oligomerization (85) or nuclear localization (37). Furthermore, shuttling of Rev between the nucleus and cytoplasm (48, 61) may be imbalanced in TH4-7-5 cells. Possible RNA targets for inhibitory cellular factors are represented by the RRE (15) and by inhibitory elements responsible for poor expression of Rev-dependent HIV-1 RNAs without Rev (52, 68, 70). Inactivation of Rev would be achieved either by interference with direct binding of Rev to the RRE or by preventing Rev from indirectly counteracting the effect of such inhibitory elements.

Consequences of restricted infection of human astrocytes in HIV pathogenesis of the brain. Study of HIV-1 expression during virus persistence in astrocytes indicates accumulation of viral regulatory RNAs and proteins over structural proteins (this report and reference 77). Use of probes targeted to exons in multiply spliced transcripts and antibodies against Nef led to identification of HIV-1-positive astrocytes in brains of pediatric patients with AIDS encephalopathy (64, 76). Thus, astrocytes may be target cells for HIV-1 in the brains of AIDS patients.

In the brain, HIV-1-infected astrocytes may constitute a virus reservoir for productive infection of brain macrophages, as suggested by recovery of infectious virus from TH4-7-5 cells by coculture with monocytic target cells. In addition, HIV-1-infected astrocytes may be a source of potentially neuropathogenic viral proteins. Neurotoxic properties have been attributed to viral regulatory proteins Tat (26, 63), Rev (42), and Nef (81). We have shown that these proteins are expressed in TH4-7-5 cells (7), and expression of Nef protein has been detected in HIV-1-infected primary fetal astrocytes (77). Therefore HIV-1-infected astrocytes may contribute to the pathogenesis of AIDS-related neurological disease by production of viral regulatory proteins in the absence of virus production.

In conclusion, TH4-7-5 cells show a restricted HIV-1 infection phenotype also observed upon exposure of primary human fetal brain astrocytes to HIV-1 (77) and in some brains from pediatric AIDS cases (64, 76). Therefore, TH4-7-5 cells are a suitable model system for unraveling the molecular mechanisms underlying restriction of virus production in astrocytes. Since HIV-1 repression in these cells is associated with a cell-determined constitutive block in Rev function, study of HIV-1 regulation in these cells should allow insight into cellular elements involved in the Rev-RRE regulatory axis. Finally, human glial cells may be a source of potential Rev-inhibitory factors which may provide the basis for design of antiviral strategies aimed at repression of virus production.

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