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Restricted HIV-1 infection of human astrocytes: potential role of *nef* in the regulation of virus replication

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A small percentage of astrocytes are consistently infected *in vivo* by HIV-1 and may contribute to neuropathogenesis despite a non-productive infection. Overexpression of the *nef* gene product has been associated with their infection both *in vivo* and *in vitro*. We examined the role of the *nef* gene during HIV replication in astrocytes (U251MG cells) following transfection with pNL4-3 proviral plasmid or isogenic strains containing a deletion or point mutation in the *nef* gene (pNL4-3ΔNef; pNL4-3-nef-stop). We were able to initiate virus replication which peaked at 5 days post-transfection and became non-productive after 21 days. Nef protein expression by wild type pNL4-3 was observed at low levels compared to control HeLa cells at peak virus replication. At later time points after development of a non-productive infection, viral antigen and Nef protein was not detectable however virus was readily recovered by co-culture with CD4+T-cells. Interestingly, virus production was significantly enhanced by a 222 base pair deletion in the *nef* reading frame. This was not observed with a frame shifting point mutation in *nef*, indicating a suppressive effect of *nef* on virus production in astrocytes. The enhanced virus production from *nef*-deleted pNL4-3 in U251MG cells was not reversed by co-expression of Nef from a second Nef-expressing plasmid, and in fact Nef expression in *trans* had a further positive effect on virus production. This suggested opposing effects of the Nef protein and elements contained within the *nef* sequence on virus production in astrocytes. Despite the low expression of Nef by U251MG astrocytes, relatively high amounts of multiply spliced 2 kb mRNA were present compared to HeLa cells. These data demonstrate that an acute low-level infection of astrocytes rapidly becomes a non-productive infection and this process is assisted by sequences in *nef*. The low level Nef protein expression, despite high levels of mRNA, suggests a block in translation of multiply spliced HIV mRNA in astrocytes, or a translational control mechanism not yet characterised.

Keywords: human immunodeficiency virus; astrocyte; Nef; non-productive; replication

Introduction

The neuropathogenesis of AIDS dementia complex (ADC), a frequent consequence of HIV infection, is unclear. However, macrophages and microglial cells constitute the major brain cells productively infected *in vivo* (Glass *et al*, 1995; Johnson *et al*, 1996; Bagasra *et al*, 1996; Brew *et al*, 1995; Takahashi *et al*, 1996). The role of a non-productive or low grade infection of other brain cell types including neurons, microvascular endothelial cells, oligodendrocytes (Bagasra *et al*, 1996), and astrocytes (Bagasra *et al*, 1996; Takahashi *et al*, 1996) in the development of ADC remains contentious. Studies by Bagasra *et al* (1996) would argue that productive infection of astrocytes, albeit low-level, is important for the development of ADC. The degree of astrocyte involvement roughly correlated with ADC, but a correlation could not be made with infected macrophage/microglial cells (Glass *et al*, 1995; Johnson *et al*, 1996). Normally, astrocytes perform key regulatory functions that are critical to

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brain function (Norenberg, 1994). Therefore, it is possible that astrocyte functions are impaired as a result of HIV infection.

In vitro studies have confirmed the susceptibility of astrocytes to HIV infection (Dewhurst *et al*, 1987; Erfle *et al*, 1991; Brack-Werner *et al*, 1992; Kleinschmidt *et al*, 1994; Neumann *et al*, 1995; Rytik *et al*, 1991; Tornatore *et al*, 1991, 1994a; Hatch *et al*, 1994). In contrast to infection of T-lymphocytes, infected astrocytes produce very low levels of virus in the acute phase (Erfle *et al*, 1991; Brack-Werner *et al*, 1992; Kleinschmidt *et al*, 1994). Concurrently the HIV regulatory protein, Nef, was expressed at high levels in a persistently infected astrocyte cell line (Brack-Werner *et al*, 1992). Studies of post-mortem brain tissue have demonstrated Nef in astrocytes without the detection of any other viral proteins (Tornatore *et al*, 1994b; Saito *et al*, 1994; Ranki *et al*, 1995), and a correlation between ADC and Nef expression (Ranki *et al*, 1995).

During persistent infection, astrocytes predominantly express multiply spliced 2 kb HIV-1 mRNA together with very little (Kleinschmidt *et al*, 1994) or no (Tornatore *et al*, 1994a) 4 kb and 9 kb mRNA encoding HIV-1 structural proteins. Since *nef* accounts for the majority of multiply spliced HIV-1 mRNA (Purcell and Martin, 1993), expression of Nef protein in chronically infected astrocytes may be attributed to the abundance of these transcripts. Stimulation of persistently infected astrocytes with the cytokines TNFα and IL-1β leads to a brief burst of virus production (Tornatore *et al*, 1991, 1994a). Similarly, co-cultivation with T-lymphocytes reactivates high levels of HIV-1 (Tornatore *et al*, 1991). This implicates astrocytes as a reservoir for HIV-1 in the brain which may disseminate virus upon appropriate triggering *in vivo*.

Persistent HIV-1 infection of astrocytes resembles a Rev-defective phenotype, where only multiply spliced 2 kb mRNAs are exported to the cell cytoplasm (Neumann *et al*, 1995). A cell-determined block in the Rev/RRE regulatory axis in astrocytes that prevents the efficient synthesis and nuclear export of Rev-dependent transcripts has been proposed to account for this. The HIV-1 *nef*/LTR region of the genome may also regulate virus expression in astrocytes. The *cis*-acting negative regulatory element (NRE) of HIV-1 is partially contained within the *nef* open reading frame and has been shown to suppress HIV-1 LTR activity in persistently infected astrocytes, but not in other persistently infected cell types (Brack-Werner *et al*, 1992). Furthermore, Ludvigsen *et al* (1996) showed that this suppression of the LTR in infected astrocytes was due to a partial *nef* sequence flanking the NRE region and not the NRE itself. These results suggest that the *nef* gene of HIV-1 may play a role in restricting virus production to very low levels in astrocytes, but not necessarily in other cell types.

Using transient transfection assays with the molecular clone pNL4-3 and isogenic strains containing a deletion or point mutation in *nef*, we examined the influence of *nef* on the restricted virus production by astrocytes. A consistent finding in U251MG astroglial cells was that the *nef* sequence, but not the Nef protein, was responsible for a significant reduction in the kinetics of virus replication. These findings support Ludvigsen *et al* (1996) for a negative effect of *nef* upon transcription in astrocytes, and provide new evidence that *nef* suppresses HIV-1 replication in astrocytes at a post-transcriptional level to further impact upon virus production.

Results

High transfection efficiency

To assess the transfection efficiency of the astrocyte cell line U251MG, pGFP was used to transfect cells which were assayed daily for 4 days to determine when peak GFP (green fluorescent protein) expression occurred. At 48 h post-transfection, 58% of U251MG cells were expressing GFP as measured by flow cytometry, which was similar to the levels of expression in a control HeLa cell transfection (data not shown). In order to assess the relative transfection efficiencies of HIV-1 plasmids pNL4-3 and pNL4-3ΔNef, both plasmids were co-transfected with pGFP and fluorescence measured 48 h after by flow cytometry. Transfection efficiency for both co-transfections was similar to pGFP alone.

Negative effect of nef *on virus expression following transfection with HIV-1 proviral DNA*

Transient transfection assays using pNL4-3 and pNL4-3ΔNef were used to determine whether the HIV-1 *nef* gene has any effects on virus replication in astroglial cells. An acute infection occurred, peaking at day 5, which then decreased to background levels by day 21 (Figure 1a). The consistent observation for five transient transfection assays was that pNL4-3ΔNef produced significantly higher virus levels than wild type pNL4-3 (Figure 1b) ($P<0.009$, paired *t*-test for means). These results indicate that an intact *nef* gene is not only dispensable for virus production in U251MG cells, but may act to suppress replication in this cell type.

Expression of Nef protein is limited to HIV-1 expressing cells

At time points corresponding to peak virus production in U251MG cells (Figure 1a), the expression of Nef protein relative to other viral proteins was assessed by immunofluorescence and flow cytometry. Cell associated HIV-1 antigens were detected using a polyclonal HIV-1 immune sera in approximately 10% of the cell population transfected with both wild type and *nef*-deleted pNL4-3 (Figure 2a).

This level of expression is much lower than that observed in transfections of HeLa cells, which was equivalent to the level of GFP expression (data not shown). The high transfection efficiency with GFP indicates inefficient or suppressed viral antigen expression in U251MG cells compared with HeLa cells. Nef production was evident only in cells transfected with wild type pNL4-3 (Figure 2b), confirming that the 222 base pair deletion in *nef* rendered pNL4-3ΔNef incapable of synthesising Nef protein in U251MG astroglial cells. MT-2 cells acutely infected with the primary isolate HIV-1_{200} were included to demonstrate efficient antigen detection in these assays. Nef expression in a wild type astrocyte transfection was only detected at low levels also, a finding repeated using a polyclonal Nef antibody α-Nef_{15-27} (data not shown). The low levels of Nef expression were confirmed in transfected primary foetal astrocytes (unpublished data). Dual fluorescence labelling confirmed that expression was limited to HIV-1 positive cells, and not overexpressed relative to HIV-1 structural proteins (Figure 2c). At day 21 post-transfection Nef was not detected, nor were other cell associated HIV-1 proteins (data not shown). These results indicate that Nef protein is not overexpressed during the acute phase of virus production in transfected U251MG cells. Additionally, Nef protein expression was not evident after the acute replication phase, in contrast with other studies using a clonally derived, persistently infected astrocyte cell line (Brack-Werner *et al*, 1992).

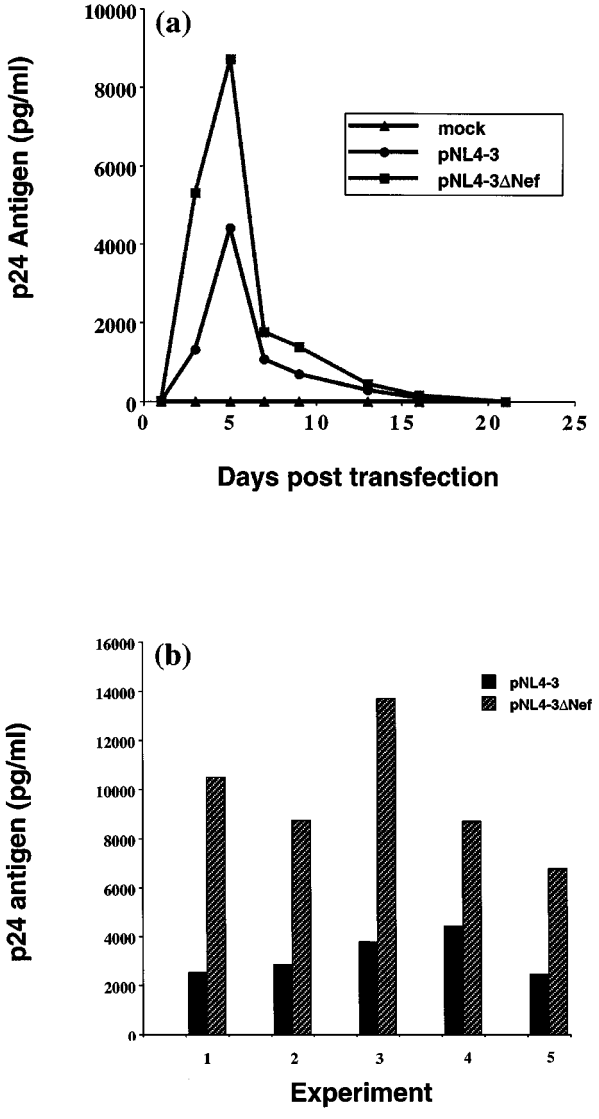

Figure 1 Replication kinetics after transfection with pNL4-3 or pNL4-3ΔNef. Virus replication kinetics following transfection with pNL4-3 or pNL4-3ΔNef (a). Supernatant samples were collected from transfected astroglial cells over a 21 day time course and analysed for the production of soluble p24 antigen. For five independent astrocyte transfections, supernatant samples collected at peak virus replication (day 5) were analysed for p24 antigen production (b).

Transfection leads to a non-productive infection

To determine whether a persistent infection was established on transfection of U251MG cells, virus reactivation experiments were performed. Cells were stimulated at time points corresponding to the low-level productive phase (day 13) and the non-productive phase (day 21) using T-cell co-culture according to Tornatore *et al* (1991). A3.01 cells were added to transfected U251MG cells at these time points, and culture supernatant samples collected over a 27 day time course and analysed for virus production. At day 13, HIV-1 could be recovered for both wild type and *nef*-deleted virus transfections (Figure 3a). Recovered HIV-1_{NL4-3} peaked at day 10 post co-culture and *nef*-deleted virus reached maximal levels 7 days later. At this time point, infecting virus may have been from reactivated HIV-1 provirus or the low levels still present in the transfection culture supernatant. However, by day 21 post-transfection no residual virus was detectable (Figure 1a) but upon co-culture, both HIV-1_{NL4-3} and HIV-$1_{NL4-3\Delta Nef}$ were recovered (Figure 3b). These results indicate that U251MG cells at day 21 post transfection contain a persistent, non-productive infection that can be reactivated to produce infectious virus particles that are capable of infecting and replicating in other cell types. At both days 13 and 21 post-transfection, wild type virus was more readily recovered in A3.01 cells than *nef*-deleted virus. This was despite restricted virus production upon transfection of astrocytes when compared to pNL4-3ΔNef. This suggests that while the *nef* gene or protein product of pNL4-3 acts to suppress virus production in U251MG cells, it maintains a positive role when virus is recovered in T-cells. Additionally, the enhanced virus recovery for wild type HIV-1_{NL4-3} indicates that the repressed virus production in

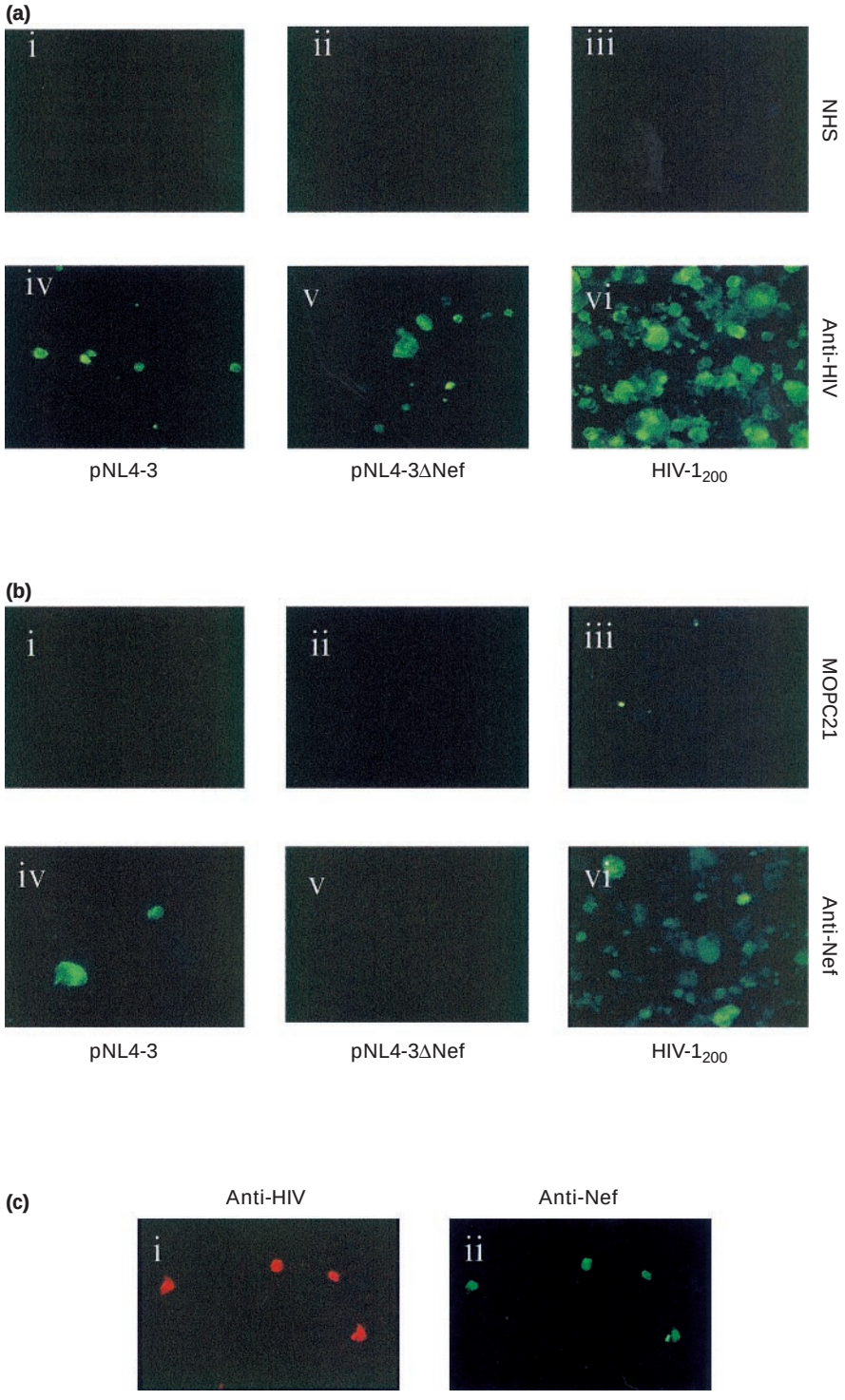

Figure 2 Immunofluorescent staining for HIV-1 antigen expression of transfected astrocytes and an infected T-cell line. Panels (**a**) and (**b**): Astroglial cells (i, ii, iv, v) transfected with pNL4-3 (i, iv) or pNL4-3ΔNef (ii, v) assessed for HIV-1 antigen (a) and Nef (b) expression. Transfected cells were collected just prior to peak soluble p24 antigen expression (day 4) and subjected to indirect immunofluorescence using primary antibodies BB10 (1 : 500) for HIV-1 antigen detection and NF2B2 (1 : 500) for Nef detection. Normal human serum (NHS) or an isotype control antibody (MOPC21) were included as antibody controls (i, ii, iii). MT-2 cells infected with the laboratory strain HIV-1_{200} (iii, vi) were also included as controls (Final magnification × 100). Panel (**c**): Astrocytes transfected with pNL4-3 and analysed for co-expression of HIV-1 antigens and Nef protein by dual immunofluorescence. Primary antibodies used were BB10 (1 : 500) and anti-$Nef_{15\text{-}27}$ (1 : 100). Shown is a representative field of cells expressing HIV-1 antigens (i) or Nef protein (ii).

U251MG cells may be due to a cell-specific effect of Nef or *nef* on HIV-1 replication.

Opposing effects of the nef *gene and protein product on virus replication*

The enhanced virus production by pNL4-3ΔNef in U251MG cells (Figure 1) indicates that the 222 base pair deletion in *nef* has a negative effect. However, it could not be determined whether this effect was provided by Nef protein or elements contained within the deletion. To investigate the possibility that Nef protein was responsible for restricted virus replication, Nef was provided in *trans* by means of a *nef*-expressing plasmid pLTR-Nef2 co-transfected with wild type and *nef*-deleted pNL4-3. To control for any effects due to the exogenous LTR and *nef* sequence delivered in this system, pLTR-Nef2-stop was included which contains a point mutation in *nef* and does not produce Nef protein. The LTR promoter used in these constructs requires Tat transactivation and so should produce Nef protein when co-expressed with pNL4-3ΔNef, but not when transfected alone. The efficiency of Nef production in *trans* was assessed by indirect immunofluorescence following co-transfection of U251MG cells. As predicted, pLTR-Nef2 (but not pLTR-Nef2-stop) successfully provided Nef in *trans* when co-transfected with pNL4-3ΔNef, and at levels similar to that observed in wild type pNL4-3 transfections (data not shown).

The effect of Nef compensation on virus replication kinetics in U251MG cells is shown in Figure 4. Nef protein delivered in *trans* to pNL4-3ΔNef did not reduce virus production to that of wild type; in fact virus production was further enhanced. This effect was also observed in HeLa cells (data not shown), and suggests that the expressed protein was not responsible for the negative effect observed in Figure 1. On the contrary, provision of the *nef* gene and/or Nef protein upregulated virus production when provided in *trans*, indicating that providing the gene in *cis* is quite different to that in *trans*.

To determine whether the *nef* region of the genome was providing the negative effect on virus production, pNL4-3-nef-stop was used to transfect U251MG cells. This plasmid contained a point mutation in the *nef* gene and did not express any detectable Nef protein in U251MG cells (data not shown). Upon transfection with pNL4-3-nef-stop, the kinetics of virus production were similar to that

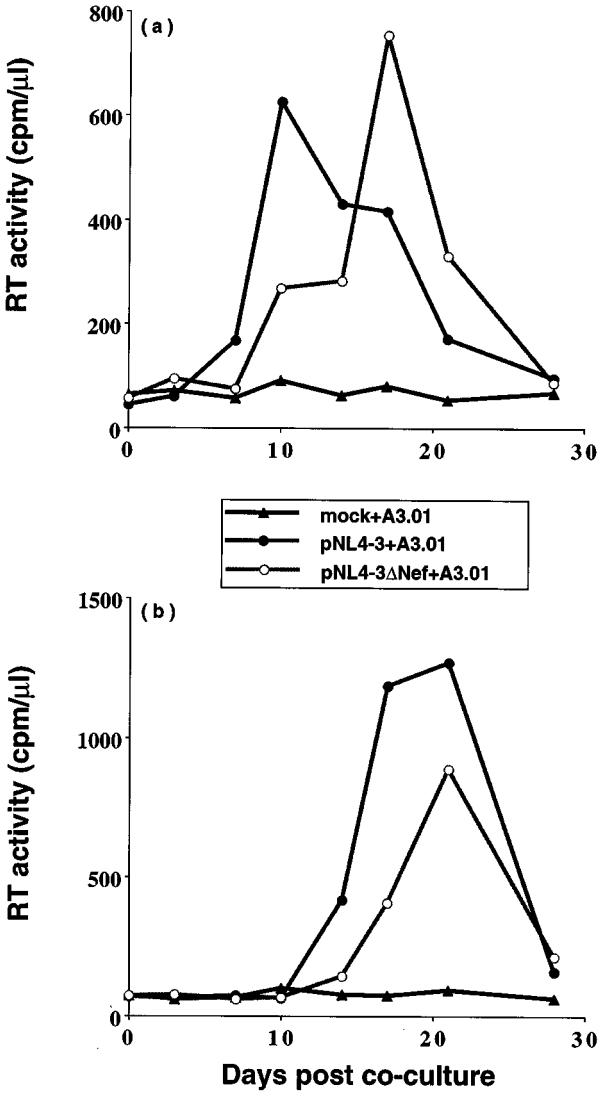

Figure 3 Co-culture of the A3.01 T-cell line with latently infected astrocytes. Recovery of virus from astroglial cells transfected with pNL4-3, pNL4-3ΔNef or mock-transfected cells at day 13 (a) or day 21 (b) post-transfection. Transfected astrocytes were co-cultured with A3.01 cells and supernatant samples assessed for viral RT production over 27 days.

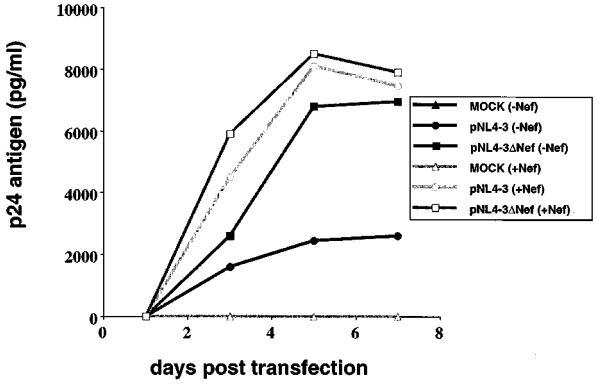

Figure 4 Replication kinetics of *trans* complemented virus in astroglial cells. Cells were transfected with pNL4-3 or pNL4-3ΔNef together with pLTR-Nef2 (+Nef) or pLTR-Nef2-stop (−Nef). Mock transfections received pNef2 or PNef2-stop alone. p24 antigen was measured in supernatant samples over a 7 day period.

of wild type pNL4-3 (Figure 5). This suggests that the *nef* sequence of pNL4-3, and not Nef protein, was responsible for the reduced virus production observed in U251MG cells. Furthermore, the positive effect of Nef protein observed when provided in *trans* suggests opposing effects of Nef protein and the *nef* gene on virus replication in U251MG cells.

High levels of HIV-1 mRNA expression

Due to the low level of Nef protein expression together with previous reports of overexpression in astrocytes (Brack-Werner *et al*, 1992), we evaluated the production of HIV-1 mRNA transcripts.

Total cellular RNA was prepared from U251MG and HeLa cells transfected with pNL4-3, pNL4-3ΔNef and pNL4-3-nef-stop. This was done just prior to peak virus production: 48 and 72 h post-transfection for HeLa cells and U251MG cells, respectively. Equivalent amounts were electrophoresed, subjected to Northern transfer and hybridised with a specific DNA probe designed to detect all HIV-1 mRNA species. The three major classes of HIV-1 mRNA could be readily identified in both HeLa and U251MG cells transfected with each of the three plasmids (Figure 6).

Interestingly, the amount of HIV-1 specific mRNA produced by U251MG cells was equivalent, if not greater, than in HeLa cells. This was despite severely restricted virus production in the U251MG cells at the same time points (Table 1). Since total cellular RNA was analysed, this suggested that a large proportion of HIV-1 mRNA produced in U251MG cells may be retained in the nucleus, possibly by a Rev-defective mechanism similar to that proposed by Neumann *et al* (1995).

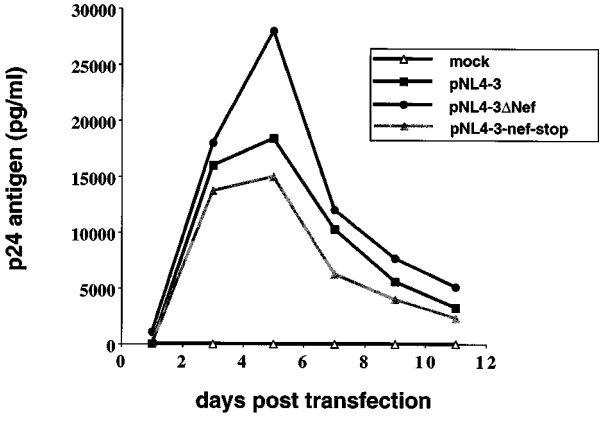

Figure 5 Virus replication kinetics in astroglial cells following transfection with pNL4-3, pNL4-3ΔNef or pNL4-3-nef-stop. Mock transfections were treated identically but did not receive any plasmid. p24 antigen was measured in supernatant samples over an 11 day time course.

The proportion of the multiply spliced 2 kb mRNA species was assessed by comparison to the unspliced, 9 kb transcripts. Densitometry revealed that HIV-1 mRNA produced in U251MG cells contained a higher proportion (2–8-fold) of 2 kb mRNA when compared to HeLa cells. When expressed as a percentage of total HIV-1 mRNA, the 2 kb species was 2–3-fold more abundant in U251MG cells (data not shown). This finding is in accordance with other reports that demonstrate overexpression of multiply spliced HIV-1 mRNA *in vitro* (Kleinschmidt *et al*, 1994; Tornatore *et al*, 1994a; Neumann *et al*, 1995), however it does not correlate with the Nef protein expression observed.

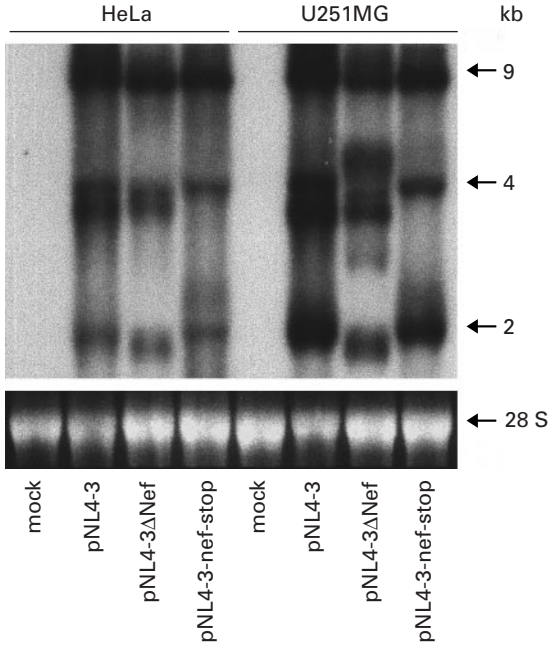

Figure 6 HIV-1 mRNA production in HeLa and astroglial cells transfected with pNL4-3, pNL4-3ΔNef or pNL4-3-nef-stop. Mock-transfected cells were treated identically but did not receive any plasmid. Total cell RNA was purified from transfected cells prior to peak virus production (at day 2 for HeLa cells and day 3 for astrocytes) and HIV-1 transcripts detected by Northern hybridisation.

Table 1 Soluble p24 antigen production upon transfection. Cell culture supernatant samples were taken from transfections harvested for mRNA analysis (Figure 6) and analysed for p24 antigen.

Transfection	*p24 antigen (ng/ml)*
HeLa pNL4-3	540
HeLa pNL4-3ΔNef	270
HeLa pNL4-3-nef-stop	370
U251MG pNL4-3	12
U251MG pNL4-3ΔNef	16
U251MG pNL4-3-nef-stop	11

Discussion

Replication kinetics of astrocytes transfected with wild type pNL4-3 or *nef*-altered constructs (see Figures 1, 2, 4 and 5) demonstrated a suppressive effect of the *nef* gene but not the protein. Enhanced virus production rather than the kinetics of replication indicated that the amount of virus antigen produced per cell was altered as opposed to the number of cells infected/transfected. This confirmed early studies which initially characterised *nef* as a negative factor, and implicated *nef* in maintaining viral latency (Ahmad and Venkatesan, 1988; Terwilliger *et al*, 1986; Luciw *et al*, 1987). More recently the *nef* gene has been shown to suppress HIV-1 LTR activity in a persistently infected astrocyte cell line, an effect not observed in other cell types (Ludvigsen *et al*, 1996). Our results, and those of Ludvigsen *et al* (1996), suggest that the *nef* gene may act as a negative factor during infection of astrocytes. Hence, the characteristic low grade virus infection of astrocytes may be, at least in part, due to effects of HIV-1 *nef*. No obvious impairment of transcription was found from proviral plasmids containing an intact *nef* reading frame (Figure 6). This indicates that the effect of *nef* on viral replication is probably at a post-transcriptional level, and not at the level of LTR activity as proposed by Ludvigsen *et al* (1996).

Providing Nef in *trans* failed to duplicate the suppressed HIV replication. The *nef* gene and protein product may have opposing effects in these cells since exogenous Nef had an enhancing effect on replication. However, levels of Nef protein were not noticeably higher in these co-transfection experiments and the overall expression of Nef was very low. To determine whether the *nef* gene was providing the negative effect on virus replication, U251MG cells were transfected with a plasmid that contains the *nef* gene but does not produce Nef protein (pNL4-3-nef-stop). Here, replication kinetics were similar to that of wild type pNL4-3. This confirmed that the Nef protein of pNL4-3 did not provide the negative effect observed, and that suppression of viral replication was associated with the *nef* gene.

Nef was detected at low levels in U251MG cells, and co-localised to the HIV-1 positive population, as assessed by double labelling fluorescence experiments. Several studies characterising a restricted HIV-1 infection of astrocytes have used a persistently infected astrocyte cell line that produces high levels of Nef protein relative to HIV-1 structural proteins (Brack-Werner *et al*, 1992; Kleinschmidt *et al*, 1994; Neumann *et al*, 1995). However, this cloned cell line was selected for Nef expression, and probably represents a very minor proportion of the infected astrocyte population. The small amounts of Nef protein expressed agrees with the observations of Fiala *et al* (1996) in primary human fetal astrocytes infected with HIV-$1_{JR\text{-}FL}$. However, the astrocyte population expressed high levels of Nef when co-cultivated with macrophages. Since the presence of monocyte/microglial cell populations is a characteristic of ADC (Glass *et al*, 1995), the interaction of these cells (or secreted factors) with infected astrocytes *in vivo* may trigger the production of Nef in astrocytes.

Virus replication in U251MG cells following transfection with various HIV-1 plasmids peaked at day 5 followed by reduction to background levels by day 21. At this later time point no cell-associated HIV-1 antigens, Nef protein expression or HIV-1 mRNA could be detected, however viral DNA was consistently present (data not shown). Due to the frequent passage of transfected cells, trypsin treatment ensured removal of any residual undetectable cell associated HIV-1. Recovery of virus from these astrocytes was achieved upon co-cultivation with the T-cell line, A3.01. This demonstrated ready recovery of virus from a non-productively infected cell which from all virus replication markers was ostensibly latently infected.

Despite overall efficient mRNA expression in transfected U251MG cells, the synthesis of p24 antigen was severely restricted compared to HeLa cells. This indicates that low-level virus production characteristic of HIV-1 infected astrocytes is the result of a block at a stage in the replication cycle between mRNA expression and viral protein synthesis. Neumann *et al* (1995) have proposed that the restricted viral expression from persistently infected astrocytes is due to a cell-determined block in the Rev/RRE regulatory axis. While our results do not address this directly, the restricted virus production in U251MG cells is more likely to result from a block in the translation of HIV-1 mRNA. This interpretation may be predicted by the low levels of Nef protein produced from high levels of 2 kb mRNA. In fact 2 kb mRNA production was approximately 3 fold greater in U251MG cells compared to HeLa cells. In contrast, Nef expression in astrocytes was at least 3–4-fold lower than that in HeLa cells at a similar transfection efficiency (data not shown).

In conclusion, restricted virus production in U251MG cells is clearly influenced by a post-transcriptionally regulated mechanism. The poor synthesis of Nef protein, despite high levels of available 2 kb mRNA, suggests a block in translation as a possible mechanism. Furthermore, even in this restricted state, the *nef* gene may play a role in suppressing virus replication to very low levels in astrocytes. Finally, HIV-1 enters a potentially latent state in U251MG cells which can be reactivated to produce virus that is capable of replicating in other cell types. Taken together, these findings may have several implications for understanding HIV-1 neuropathogenesis and antiviral therapies. Firstly, although *nef* may be required for progression to AIDS in SIV-infected macaques (Kestler *et al*, 1991)

and rare cohorts of HIV-infected individuals (Deacon *et al*, 1995; Kirchhoff *et al*, 1995), these attenuated strains of HIV-1 may have an increased capacity to replicate in the brain astrocyte population. Secondly, current antiviral strategies target actively replicating virus. A latent infection of brain astrocytes would not be susceptible to antiviral drugs, and even if astrocytes were targeted their loss *per se* would highly compromise normal brain function.

Materials and methods

Cell culture

The astrocytoma cell line U251MG (Bigner *et al*, 1981) was obtained from Dr J Kort, Department of Medicine, Albany Medical College, Albany, New York, USA. U251MG cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% v/v foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (25 μg/ml) (DMEM10). MT-2 cells (Harada *et al*, 1985) was provided by Dr Y Hinuma, Institute for Virus Research, Kyoto University, Kyoto, Japan. A3.01 cells (Folks *et al*, 1985) were provided by Drs T Folks and G Poli, National Institute for Allergy and Infectious Diseases, Bethesda, MD, USA. These cell lines were maintained in RPMI 1640 medium supplemented with 10% v/v FCS.

Plasmids used for transfection

The proviral plasmids pNL4-3, pNL4-3ΔNef and pNL4-3-nef-stop were constructed from plasmids obtained from Dr M Martin (Department of Molecular Microbiology, NIAID, NIH, Bethesda, MD) and Dr R Desrosiers (Department of Microbiology and Molecular Biology, Harvard Medical School, Boston, MA) (pNL4-3, ARRRP no. 114; HIV-1_{NL43}5′ clone, ARRRP no. 2479; HIV-1_{NL43}3′ clone, ARRRP no. 2480; HIV-1_{NL43} *nef* deletion mutant, ARRRP no. 2485). pNL4-3ΔNef is identical to pNL4-3 except for a deletion of 222 base pairs surrounding an *Xho*I restriction site in the *nef* gene. pNL4-3-nef-stop is identical to pNL4-3 except for a premature termination codon resulting from end filling and religation of a *Xho*I restriction site in the *nef* gene. pGFP (Clontech, Palo Alto, CA, USA) encodes jellyfish green fluorescent protein, and its expression is under the control of the cytomegalovirus (CMV) immediate-early promoter. pLTR-Nef2 produces Nef protein upon Tat activation of its LTR promoter (unpublished data). pLTR-Nef2-stop contains a premature termination codon resulting from end filling and religation of a *Xho*I restriction site and is incapable of synthesising Nef protein upon Tat activation.

Antibodies

BB10 (a gift from Dr E Dax, National HIV Reference Laboratory, Fairfield) is pooled human HIV-1-immune sera that recognises the major HIV-1 structural proteins p24, p55 and gp120. NF2B2 is a monoclonal antibody directed to the amino terminus of HIV-1 Nef, and was obtained from the AIDS Research and Reference Reagent Program (catalogue number 456). Sheep anti-Nef_{15-27} (provided by Dr A Greenway) is an affinity purified polyclonal antibody raised against a peptide corresponding to amino acids 15-27 of HIV-1 Nef (Greenway *et al*, 1994).

Measurement of virus production in culture supernatants

HIV-1 p24 antigen production was measured using a commercially available kit (Organon Teknika, Durham, NC, USA) and following the manufacturers' instructions. Cells were assessed for viral replication by measuring cell-free reverse transcriptase (RT) activity as described by Willey *et al* (1988) with the following modifications: 6 μl of supernatant was added to 30 μl of RT mix and 6 μl was spotted onto glass fibre filtermats with DEAE active groups (Wallac, Turku, Finland); 10 μCi ^{33}P (200 Ci/mM) was used per ml rather than 0.5 μCi ^{32}P (400 Ci/mM).

Indirect immunofluorescence

Transfected cells were washed twice in PBS and resuspended at a density of 5×10^6/ml in PBS. 20 μl was spotted onto microscope slides and left to dry. Cells were fixed in acetone at -20°C for 20 min, then washed twice in PBS. Cells were incubated in PBS containing 1% v/v FCS for 10 min at room temperature, and then subjected to the primary antibody diluted as indicated in PBS containing 1% v/v FCS for 1 h at 37°C. After washing three times in PBS, a fluorescein isothiocyanate (FITC)-labelled secondary antibody (Silenus, Melbourne, Australia) was applied to the cells, diluted 1 : 50 in PBS, and incubated for 1 h at 37°C. Cells were washed three times in PBS and examined using an Axiovert 100 fluorescence microscope (Zeiss, Germany). For double labelling experiments, immunofluorescence was performed as above with the following additional steps: cells were incubated with a combination of two primary antibodies. Nef protein was detected using an FITC-labelled secondary antibody, HIV-1 antigens were detected using an anti-human-biotin conjugate followed by streptavidin-Texas Red (1 : 200).

Oligonucleotide primers

For the generation of a DNA probe for use in Northern hybridisation experiments, primers were used to amplify a sequence at the 3′ end of pNL4-3 that is common to all HIV-1 mRNA species: forward primer (+) Odp.141: 5′-ACTGGAAGGGCTAATTCACTCCCAA-3′ NL4-3 nucleotides 9071-9096; reverse primer (−) Odp.106: 5′-TTTATTGAGGCTTAAGCAGTGGGTTC-3′ NL4-3 nucleotides 9582-9608.

Calcium phosphate transfection

Transfection media was prepared by reconstituting DMEM powder (Gibco BRL, Gaithersburg, MD, USA) in 1 litre of water containing 44.04 mM $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (25 μg/ml). The pH was adjusted to 7.5, and the media filter sterilised. The medium was supplemented with 10% v/v FCS.

Twenty μg of plasmid DNA was combined with 62 μl of 2 M $CaCl_2$, and adjusted to a final volume of 500 μl with 10 mM Tris-HCl (pH 7.6). The DNA/$CaCl_2$ solution was added dropwise to 500 μl HEPES buffered saline (50 mM HEPES, 1.5 mM Na_2HPO_4, 180 mM NaCl, pH adjusted to 7.13) while being vortexed at low speed. The mixture was allowed to sit at room temperature for 30 min to allow the formation of the precipitate. During this time, the existing media was removed from 75% confluent cell monolayers in 25 ml cell culture flasks (Nunc), and replaced with 5 ml transfection media. The DNA precipitate was added dropwise to the cells and then incubated at 37°C, 5% CO_2 for 12 h. The media was then removed and replaced with 5 ml fresh transfection media.

Reactivation of HIV-1 from persistence

Monolayers of persistently infected U251MG cells, seeded after trypsin treatment (approximately 75% confluent), were washed twice with PBS. The T-cells (2.5×10^6 A3.01 cells), resuspended in 6 ml of DMEM10, were added to the monolayers and allowed to co-culture for 28 days. Supernatant samples were taken at days 0, 3, 7, 10, 13, 17, 21, and 28 and assessed for virus production.

Analysis of HIV-1 mRNA

Extraction of cellular RNA was carried out using TRIzol reagent (Gibco BRL). 12 μg of total cellular RNA obtained from transfected cells was subjected to Northern blot analysis according to Sambrook *et al* (1989), and probed with a specific ^{32}P-labelled DNA probe corresponding to amino acids 9071 to 9582 of pNL4-3 which is common to all HIV-1 mRNAs. Radiolabelling of the DNA probe was carried out using 4 cycles of PCR with oligonucleotide primers Odp 141 and Odp 106, and 10 μCi of [^{32}P]dCTP. HIV-1 mRNA was detected by autoradiography and analysed by densitometry.

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