Journal of NeuroVirology, 9: 372–389, 2003

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280390201263

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Identification of gene products suppressed by human immunodeficiency virus type 1 infection or gp120 exposure of primary human astrocytes by rapid subtraction hybridization

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Neurodegeneration and human immunodeficiency virus type 1 (HIV-1)-associated dementia (HAD) are the major disease manifestations of HIV-1 colonization of the central nervous system (CNS). In the brain, HIV-1 replicates in microglial cells and infiltrating macrophages and it persists in a low-productive, noncytolytic state in astrocytes. Astrocytes play critical roles in the maintenance of the brain microenvironment, responses to injury, and in neuronal signal transmission, and disruption of these functions by HIV-1 could contribute to HAD. To better understand the potential effects of HIV-1 on astrocyte biology, the authors investigated changes in gene expression using an efficient and sensitive rapid subtraction hybridization approach, RaSH. Primary human astrocytes were isolated from abortus brain tissue, low-passage cells were infected with HIV-1 or mock infected, and total cellular RNAs were isolated at multiple time points over a period of 1 week. This approach is designed to identify gene products modulated early and late after HIV-1 infection and limits the cloning of genes displaying normal cell-cycle fluctuations in astrocytes. By subtracting temporal cDNAs derived from HIV-1–infected astrocytes from temporal cDNAs made from uninfected cells, 10 genes displaying reduced expression in infected cells, termed astrocyte suppressed genes (*ASGs*), were identified and their suppression was confirmed by Northern blot hybridization. Both known and novel *ASGs*, not reported in current DNA databases, that are down-regulated by HIV-1 infection are described. Northern blotting confirms suppression of the same panel of *ASGs* by treatment of astrocytes with recombinant HIV-1 envelope glycoprotein, gp120. These results extend our previous analysis of astrocyte genes induced or enhanced by HIV-1 infection and together they suggest that HIV-1 and viral proteins have profound effects on astrocyte physiology, which may influence their function in the CNS. *Journal of NeuroVirology* (2003) **9,** 372–389.

Keywords: AIDS; gp120; neuropathogenesis; Northern hybridization; reverse Northern hybridization

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Recombinant gp120 was provided by ImmunoDiagnostics through the AIDS Research Reagent Repository, Rockville, MD. The present research was funded in part by a P01 grant NS31492 from the National Institute of Neurological Diseases and Stroke, PHS, an award from the Samuel Waxman Cancer Research Foundation, and the Chernow Endowment. PBF is the Michael and Stella Chernow Urological Cancer Research Scientist in the Departments of Pathology, Neurosurgery, and Urology.

Received 14 May 2002; revised 12 September 2002; accepted 24 October 2002.

Introduction

Human immunodeficiency virus type 1 (HIV-1) has evolved several modes of interaction with its host cells that may contribute to the diverse pathogenic effects caused by this virus (for review, see Levy, 1993). They range from productive infection of CD4-positive helper T lymphocytes, macrophages, and microglial cells, to low-productive, generally noncytopathic infections of CD4-negative astrocytes, brain microvascular endothelial cells, or retinal pigment epithelial cells, to proviral latency in resting T cells. Of these interactions, productive infection of T cells and macrophages with HIV-1 has been well characterized and it is believed to account for most of HIV-1–generated during disease and for the hallmark cytopathic effects of the virus, including depletion of helper T lymphocytes and presence of multinucleated giant cells in encephalitic brains.

The course and consequences of low-productive, noncytolytic HIV-1 infection of CD4-negative cells are less well understood but there is increasing evidence that one such interaction, that of virus with astrocytes, plays a role in the pathogenesis of HIV-associated dimentia (HAD) (Brack-Werner, 1999). Our laboratories have been interested in the progress and control of HIV-1 replication in human astrocytes and the effects of the virus on astrocyte physiology contributing to neuropathogenesis (Canki *et al*, 2001; Su *et al*, 2002). Astrocytes are attractive to consider in this context for three reasons. First, the glial tissue has emerged as a major target for HIV-1 infection in the brain in absolute terms (Nuovo *et al*, 1994; Saito *et al*, 1994; Tornatore *et al*, 1994; Ranki *et al*, 1995; Bagasra *et al*, 1996; Takahashi *et al*, 1996; An *et al*, 1999). Some of these studies indicate that the frequency of HIV-1–positive astrocytes can reach 1% or more in specific regions of the brain (Nuovo *et al*, 1994; Takahashi *et al*, 1996). Considering that about 50% (or 10^{11} to 10^{12}) of the cells in the brain are glia (Schubert, 1984; Rutka *et al*, 1997), the sheer number of potential virus target cells in this tissue is significant. Second, although HIV-1 infection of astrocytes is inefficient *in vitro* and *in vivo* (Dewhurst *et al*, 1987; Tornatore *et al*, 1991), there is a general agreement that HIV-1 can persist in these cells for prolonged times in a low-productive, noncytolytic state, from which it can be induced by physiological stimuli such as tumor necrosis factor-μ (TNF-μ) (Tornatore *et al*, 1991; Shahabuddin *et al*, 1992). Persistently infected astrocytes express HIV-1 regulatory genes, including Tat and Nef, that were shown to affect host cell gene expression (Roy *et al*, 1990; Conant *et al*, 1996). Third, astrocytes are responsible for a number of essential functions that impact the physiological activity and survival of neurons, the principal focus of damage in HAD. These include maintaining brain homeostasis, regulating the levels of extracellular glutamate, serving as a component of the blood-brain barrier (BBB), and responding to pathogens and brain injury (reviewed in Verkhratsky *et al*, 1998; Danbolt, 2001; Dong and Benveniste, 2001), as well as the recently discovered functional integration of astrocytes in neuronal signal transmission, which includes their role in enhancing synaptic activity and strength, increasing the number of synapses, and modulating neuronal activity (Iino *et al*, 2001; Oliet *et al*, 2001; Ullian *et al*, 2001; Beattie *et al*, 2002). Disruption of any of these functions by HIV-1 could have significant impact on the progression of HIV-1 disease in the brain.

Considering HIV-1 for perturbation of cellular physiology rather than cytolysis itself, studies of specific gene expression and gene-array analysis indicated that HIV-1 alters function of both infected T lymphocytes and macrophages (Nye and Pinching, 1990; Shahabuddin *et al*, 1994; Swingler *et al*, 1999; Geiss *et al*, 2000; Corbeil *et al*, 2001; Simm *et al*, 2001). We have also shown that both productive and nonproductive interactions of HIV-1 with macrophages induce expression of several cellular genes through activation of nuclear factor (NF)-μB (Choe *et al*, 2001, 2002). In addition, several viral proteins tested in isolation affect cellular gene expression. Tat was shown to modulate interferon-induced protein kinase (Roy *et al*, 1990) and interleukin-2 (Westendorp *et al*, 1994) in T cells and Nef can induce monocyte chemoattractant protein (MCP)-1 in macrophages (Swingler *et al*, 1999). In primary human astrocytes, HIV-1 infection *in vitro* was shown to activate the *c-kit* proto-oncogene (He *et al*, 1997), induce expression of complement factors (Speth *et al*, 2001), and as we have recently shown, induce transcriptional down-modulation of the glutamate transporter EAAT2 and inhibit glutamate transport by the cells (Wang *et al*, unpublished data). Using a sequential progressive genomic scanning (SPGS) cloning approach, we have cloned the human EAAT2 promoter and demonstrated that TNF-μ induces decreased EAAT2 transcriptional activity and consequently decreased mRNA and protein levels in primary human fetal astrocytes (Su *et al*, 2002, unpublished data). Transient expression of Tat in isolation from other viral products activates NF-μB and induces MCP-1 in fetal astrocytes (Conant *et al*, 1996; Conant and Major, 1998) and alters Ca^{2+} fluxes in response to glutamergic signals in a glial cell line (Köller *et al*, 2001). Exposure to envelope glycoprotein gp120 can induce tyrosine kinase activity (Schneider-Schaulies *et al*, 1992), influence astrocyte activation and expression of the glial fibrillary acidic protein, GFAP (Yeung *et al*, 1995), alter electrophysiological properties of human astrocytes (Patton *et al*, 2000), and, as recently reported, stimulate the signaling pathway effecting Ca^{2+}-dependent release of glutamate (Bezzi *et al*, 2001). Based on these considerations, it is evident that, independent of its level of replication, HIV-1 and viral proteins have profound effects on astrocyte gene expression and physiology. Moreover, these interactions are anticipated to alter the

function of these cells in the central nervous system (CNS).

The present study is part of our program to establish a comprehensive profile of human genes whose expression in astrocytes is altered by HIV-1 infection or exposure to gp120. Our long-term goal is to map the protein networks affected by HIV-1, gp120, and other neuropathogenic agents that ultimately impair astrocyte functions in maintenance of neuronal physiology, signal transmission, and response to injury. We employed our new rapid subtraction hybridization (RaSH) method (Jiang *et al*, 2000; Kang *et al*, 2002) to globally identify genes expressed aberrantly in astrocytes at discrete time points after HIV-1 infection. In a study presented elsewhere, we described 15 genes termed AEGs for astrocyte elevated genes, including 13 known and 2 novel genes, induced or up-regulated in early passage human fetal astrocytes by HIV-1 infection or treatment with gp120 (Su *et al*, 2002). Consistent with our cloning strategy, AEGs were identified that were induced early, 6 or 24 h after infection, and others were induced later, 3 and 7 days (Su *et al*, 2002). Here we describe extension of this research in the identification of 10 astrocyte genes, termed *ASGs* for astrocyte suppressed genes, that display reduced expression in human fetal astrocytes following HIV-1 infection or treatment with gp120. Eight *ASGs* are known genes and two have not yet been described in published databases. Our results confirm the utility and robustness of the RaSH approach for identifying genes differentially expressed in cells under particular conditions of interest, here HIV-1 infection or exposure to gp120 (Jiang *et al*, 2000; Kang *et al*, 2001, 2002; Simm *et al*, 2001; Su *et al*, 2002). The aberrant expression of specific ASGs described here may alter astrocyte physiology and function and could therefore contribute to HAD.

Results and discussion

Infection of human astrocytes with HIV-1 and cloning of ASGs *using the RaSH approach*

Human fetal astrocytes were cultured and infected with HIV-1 (NL4-3 clone) as previously described (Bencheikh *et al*, 1999; Canki *et al*, 2001; Su *et al*, 2002). Mock-infected cells were cultured and handled similarly in parallel as controls. HIV-1 infection was confirmed by following the levels of HIV-1 p24 core antigen in culture supernatants; generally, the supernatants contained 500 to 600 pg HIV-1 p24/ml 3 to 4 days after infection (data not shown). Samples were taken from infected and control cells at 6 h, 12 h, 24 h, 3 days and 7 days after infection, total cellular RNA was extracted and pooled for each kinetics series, and RNA from HIV-1–infected and control cells were then used in RaSH to identify cellular genes displaying suppressed expression as a function of HIV-1 infection. In general, the RaSH procedure followed that described for identification of cellular genes displaying elevated expression in astrocytes (Su *et al*, 2002), except that the tester and driver libraries were reversed, as shown schematically in Figure 1. The cDNA libraries were prepared by synthesizing double-stranded cDNAs, digesting the cDNAs with the restriction enzyme *Dpn*II, ligating the fragments to adapters and amplifying by polymerase chain reaction (PCR). The *Dpn*II-based approach was previously shown to generate fragments of at least 256 bp in average size (Jiang *et al*, 2000). Subtraction hybridization was then performed by incubating the tester (uninfected astrocyte library) and driver (HIV-1–infected astrocyte library) -denatured PCR fragments without further PCR amplification. Selection of subtracted cDNAs was achieved by ligation of the common *Xho*I restriction sites at the termini of the cDNA fragments and the plasmid vectors (Figure 1). Initial screening of a large number of clones representing differential expression of cellular genes was accomplished by reverse Northern hybridization (Jiang *et al*, 2000), followed by Northern blotting to confirm differential expression of genes in infected versus control cells, as we described (Jiang *et al,* 2000; Su *et al*, 2002). Previous studies document a high degree of conformity (~89%) between reverse Northern and Northern expression of RaSH-derived expressed sequence tags (ESTs) (Jiang *et al*, 2000; Simm *et al*, 2001). Using this approach, 10 distinct *ASGs* displaying reduced expression following HIV-1 infection were identified by reverse Northern blotting (Figure 2). Of these, *ASG-4, -6, -7,* and -9 were isolated in two independent RaSH analyses, whereas the remaining *ASGs* represented single cloning events (Table 1). Because only about 20% of the *Dpn*II RaSH–subtracted library was screened, it is estimated that this library may contain approximately 50 distinct differentially expressed *ASGs*. Sequence analysis of the RaSH-derived ESTs revealed eight previously identified *ASGs* and two unknown *ASGs*, designated *ASG-1* (novel) and *ASG-8* (novel), not reported in current databases (Table 1). Among the known *ASG* products are proteins involved in cell movement and cell differentiation, *ASG-3*, platelet-endothelial tetraspan antigen 3 (PETA-3) (Fitter *et al*, 1995; Yáñez-Mó *et al*, 1998); *ASG*-5, neuronatin (Dou and Joseph, 1996b); *ASG*-6, a neuroendocrine differentiation factor (Wilson *et al*, 2001), as well as intracellular regulators of signal transduction and gene expression; *ASG-4*, a guanine nucleotide–releasing protein C3G (Tanaka *et al*, 1994); *ASG-7*, cysteine/glycine-rich protein 1 (CSRP1) (Liebhaber *et al*, 1990); and *ASG-10*, signal-recognition particle 9 (SRP9) (Lütcke, 1995).

Expression analysis and characterization of ASGs

The differential expression of *ASGs* was confirmed by Northern blot analyses of RNA isolated at various times from control or HIV-1–infected fetal astrocytes (Figures 3 to 5). For comparison, we also tested *ASG* expression in astrocytes treated with HIV-1

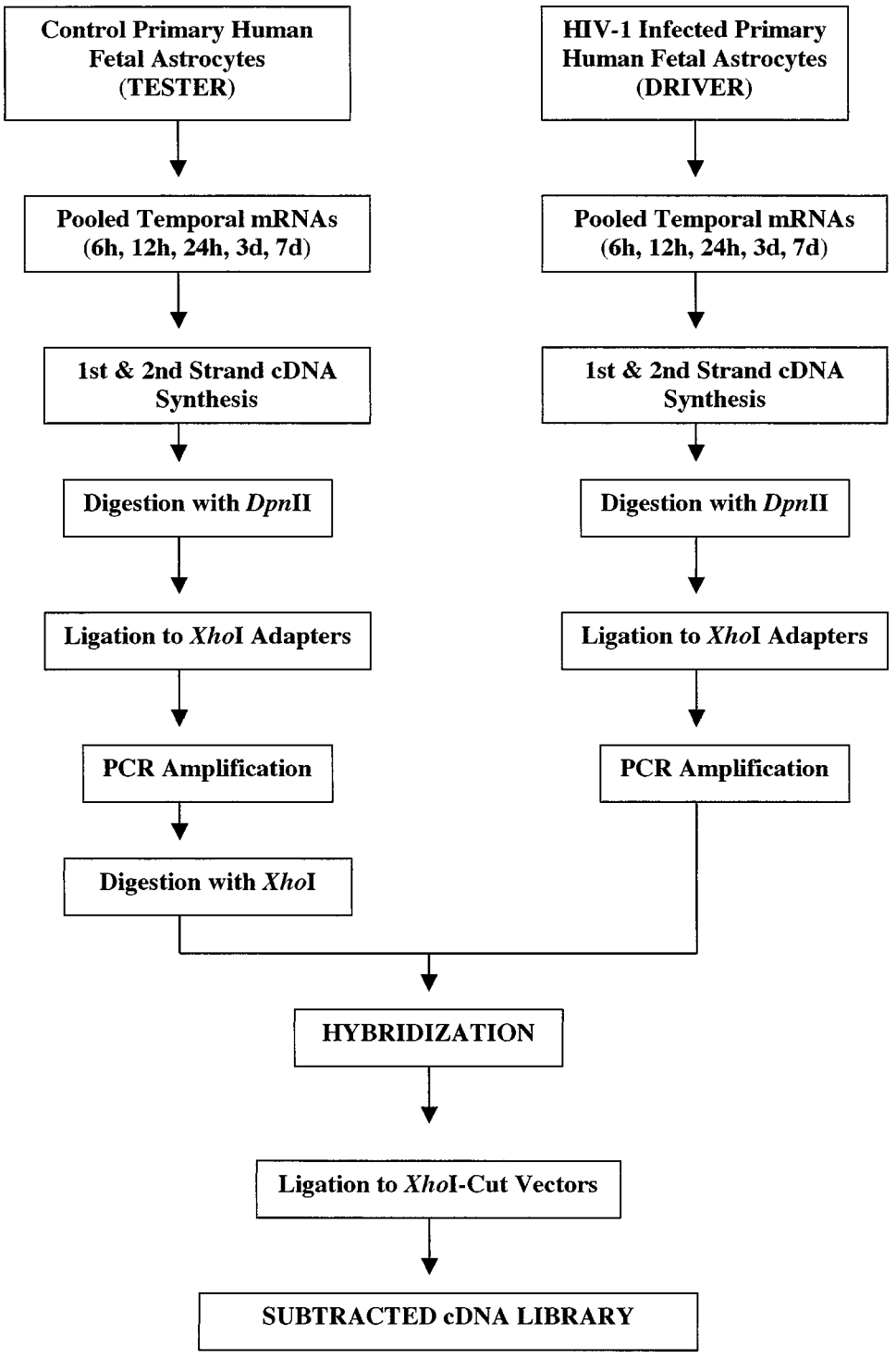

Figure 1 Schematic representation of the RaSH approach as applied to HIV-1–infected early passage human fetal astrocytes. For this scheme tester (control [mock infected], 6 h, 12 h, 24 h, 3 days, and 7 days) and driver (HIV-1 infected, 6 h, 12 h, 24 h, 3 days, and 7 days) early passage human fetal astrocytes libraries are constructed followed by digestion of only the tester library with *Xho*I. After hybridization, differentially expressed sequences are cloned into *Xho*I-digested vectors, resulting in a subtracted cDNA library enriched for *ASGs* displaying suppressed expression in human fetal astrocytes.

gp120 and cultured, sampled, and analyzed in parallel (Figures 3 to 5). This experimental system was of interest because astrocytes exposed to gp120 in the absence of other viral proteins were found to undergo many of the functional and gene-expression changes observed in cells infected by intact virus (Schneider-Schaulies *et al*, 1992; Yeung *et al*, 1995; Patton *et al*, 2000; Su *et al*, 2002), suggesting the

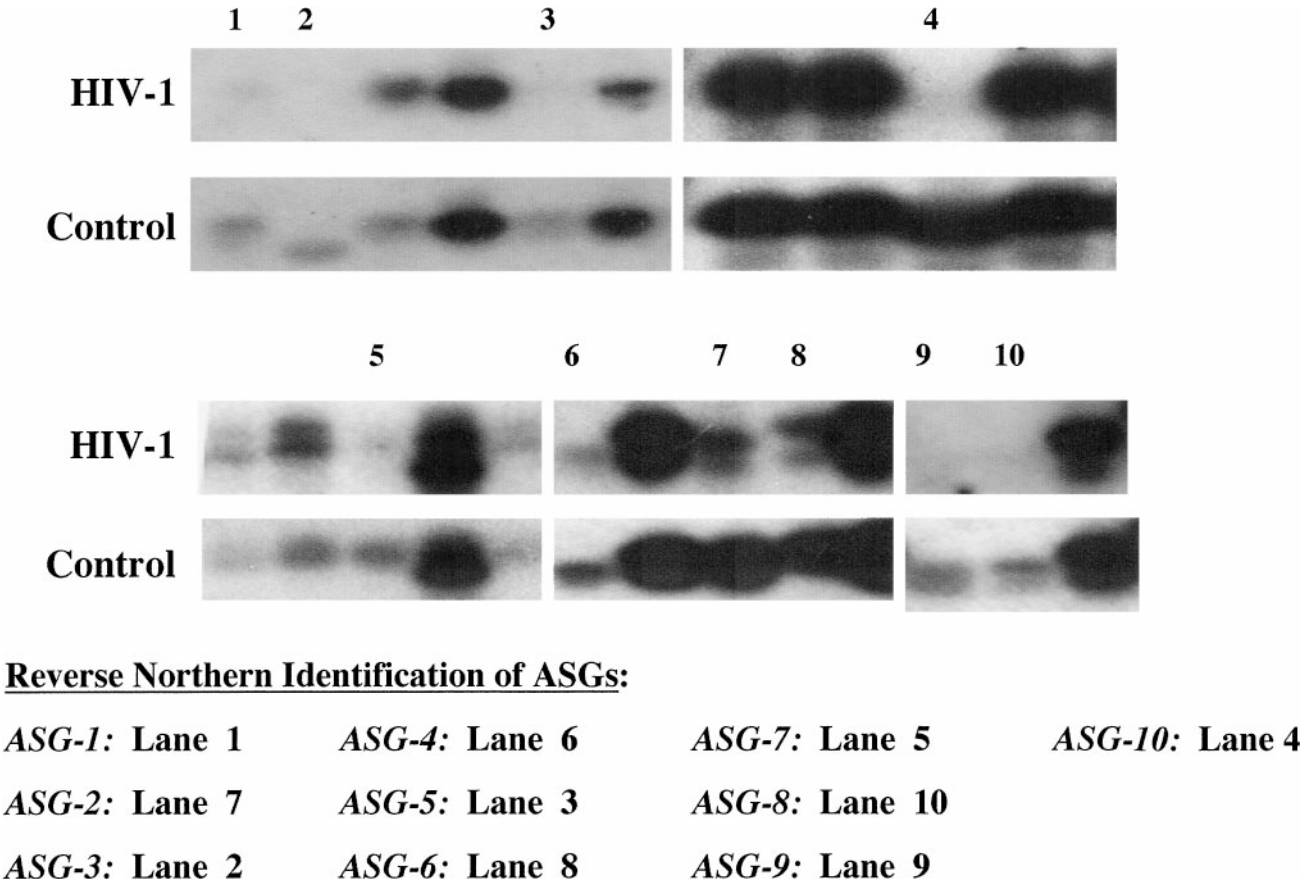

Figure 2 Reverse Northern blot analysis of differentially expressed sequence tags identified by RaSH. Equal quantities of PCR-amplified products from random bacterial clones of RaSH-derived libraries were loaded onto 1.2% agarose gels. Samples were electrophoresed for 1 h under 100 V and transferred to nylon membranes. The blots were hybridized with ^{32}P-labeled putative *ASGs* cDNAs reverse-transcribed RNA samples. Blots were exposed for autoradiography. The lane numbers (1 to 10) indicate the various suppressed ESTs, which were designated *ASG-1* to *-10*.

potential importance of virus- and gp120-cell surface interactions in the responses under consideration here. RNAs were isolated after 6 h, 24 h, 3 days, and 7 days from uninfected, HIV-1–infected, and gp120-treated human fetal astrocytes. *ASG* RNA levels were determined by Northern blotting, first by probing with a random-primed [^{32}P]-labeled *ASG* cDNA, followed by stripping of the blot and reprobing with a probe for the transcript of the human housekeeping enzyme, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). After autoradiography, relative change in the expression of individual *ASGs* was determined by densitometry, by first normalizing all *ASG* signals to *gapdh*, then comparing *ASG* signal intensities from uninfected samples to samples from HIV-1–infected or gp120-treated cells at the same time point. The results of Northern blot hybridization analysis for all 10 *ASGs* from a typical experiment are shown in Figures 3 to 5. The experiments and analyses were repeated two to three times using different preparations of astrocytes and virus or gp120 and qualitatively similar results were observed. The results of densitometry analysis from two separate experiments with standard deviations and statistical significance ($P < .05$) are shown in Figures 6 and 7. These data are summarized in Table 1. Several points can be made about these differentially expressed genes.

In general, specific *ASGs* were reduced in their expression at defined time points up to ~5.3-fold by HIV-1 infection and up to ~4.8-fold following gp120 treatment (Table 1, Figures 6, 7). Similar to differentially expressed gene products described in our previous applications of the RaSH technique (Jiang *et al*, 2000; Su *et al*, 2002), the cellular genes identified here display distinct patterns of expression over time in response to HIV-1 infection of astrocytes, stratifying into early and sustained versus late responders (Table 1, Figures 3 to 7). Six out of the 10 *ASGs* (*ASG-1, -2, -3, -5, -6,* and *-9*) can be considered late-response genes with maximum changes apparent in their expression 3 and 7 days after HIV-1 exposure (Figures 3 to 7). A temporal decrease in expression of *ASG-1, -3, -5, -9*, and *-10* was apparent over the 7 days of observation. *ASG-4, -7*, and *-8* were early, sustained-response genes whose expression was suppressed within 6 to 24 h and remained suppressed

Table 1 General characteristics of genes suppressed in HIV-1–infected and gp120-treated astrocytes (ASGs)

Gene designation	*Maximum down-regulation*[a]		*Approximate size of mRNA (kb)*	*No. of clonal isolates*~~	*Sequence homology*
	HIV-1 infected	*gp120-treated*			
ASG-1	4.2 ~ 0.5 (7d)*	4.8 ~ 0.9 (3d)*	~4.0 & ~2.0	1	Novel sequence (maps to position 11q23)
ASG-2	3.2 ~ 0.5 (3d)*	2.1 ~ 0.1 (3d)*	3.0 & 1.5	1	Human cDNA FLJ10705 (unpublished) (mRNA from NT2 neuronal precursor cell after 2-week RA induction)
ASG-3	3.4 ~ 0.8 (7d)*	3.3 ~ 0.4 (7d)*	1.5	1	Platelet-endothelial cell tetra-span antigen 3 mRNA (CD151/PETA-3)
ASG-4	3.5 ~ 0.1 (7d)*	2.9 ~ 0.5 (3d)*	4.1	2	Guanine nucleotide-releasing factor, C3G
ASG-5	4.1 ~ 0.1 (7d)*	2.9 ~ 0.5 (7d)*	1.3	1	Neuronatin
ASG-6	3.2 ~ 0.5 (3d)*	3.2 ~ 0.4 (7d)*	3.0, 1.0 & 0.7	2	Neuroendocrine differentiation factor; CGI149 mRNA
ASG-7	3.5 ~ 0.1 (3d)*	3.0 ~ 0.7 (3d)*	1.8	2	Cysteine/glycine-rich protein 1 mRNA (CSRP1)
ASG-8	3.1 ~ 0.6 (24h)*	3.0 ~ 0.1 (24h)*	~3.0 & ~1.5	1	Novel sequence (human Bac clone CTB-152G17 from 7q22-q31.1)
ASG-9	5.3 ~ 0.4 (3d)*	3.0 ~ 0.7 (7d)	16.5	2	Human mitochondrion (encoding rRNA)
ASG-10	3.7 ~ 0.3 (7d)*	1.6 ~ 0.2 (7d)	2.5 & 1.5	1	Signal recognition particle 9 KD (SRP9KD), human clone 45620

[a]Maximum down-regulation of *ASG* mRNA determined in HIV-1–infected or gp120-treated human fetal astrocytes relative to the same time point in control (untreated) cells. Fold HIV-1 down-regulation = C-*ASG*/C-*gapdh* divided by H-*ASG*/H-*gapdh*. Fold gp120 down-regulation = C-*ASG*/C-*gapdh* divided by G-*ASG*/G-*gapdh*. Time of maximum fold down-regulation indicated in parenthesis. Average fold change from two independent experiments using different primary human fetal astrocyte cultures ~ SD. Qualitatively similar results were obtained in an additional experiment (data not shown).

*Statistical analysis of the gene expression changes between 6-h control and the time point showing maximum change were analyzed using the Student's *t* test. *Indicates that the change in expression is significant ($P < .05$). All maximum changes indicated in this table were significant ($P < .05$), with the exception of *ASG-10* in gp120-treated PHFA at day 7.

**Reverse Northern blotting identified potential down-regulated *ASGs*. Sequencing indicated that *ASG-4, ASG-6, ASG-7*, and *ASG-9* had been cloned two times, whereas the rest were identified one time in the ~20% of the subtracted library screened by reverse Northern blotting.

for the rest of the period of observation (Figures 4, 5). The observation that the extent and time course of perturbation of cellular gene expression by a given agent is specific to the particular transcript examined reproduces the cellular response pattern seen in our previous study in astrocytes (Su *et al*, 2002) and in terminally differentiated human melanoma cells (Huang *et al*, 1999a, 1999b; Jiang *et al*, 2000), and in work by others (Corbeil *et al*, 2001), underscoring the generality of this phenomenon and the importance of assessing RNA levels at multiple times after a single stimulus or multiple stimuli.

Although our *ASG* library was constructed on the basis of transcripts from HIV-1–infected astrocytes (Figure 1), all of the *ASGs* thus far identified were also suppressed in expression in astrocytes treated with gp120. Neither can the extent of suppression distinguish specific genes modulated by HIV-1 versus gp120 treatment. The maximum suppression observed was induced by HIV-1 in four of the cases, *ASG-2, -5, -9*, and *-10*, whereas statistically similar maximal changes were observed with the other *ASGs* following HIV-1 infection or gp120 treatment (Table 1, Figures 6, 7). It is perhaps most interesting that the suppression of gene expression was as sustained in cells exposed to gp120 as in HIV-1–infected cells, for example *ASG-8* was suppressed by both agents for 7 days of observation. In our previous study, we found that cellular genes displaying up-regulated expression in astrocytes after HIV-1–infection (*AEGs*) are also up-regulated in gp120-treated cells (Su *et al*, 2002). The similar patterns of altered cellular gene expression in infected and gp120-treated astrocytes indicate that intact HIV-1 and gp120 activate similar cellular pathways, leading to transcriptional modulation of these genes. It should be noted, however, that because our RaSH library was based on HIV-1–infected cells, our data couldn't identify cellular genes whose expression might be affected by gp120 but not by HIV-1. Another implication of these results is that some modulatory effects of HIV-1 on cellular gene expression in human astrocytes, such as the ones represented by changes in *ASG* (this work) and *AEG* (Su *et al*, 2002) expression, are independent of virus replication because they can be reproduced to a significant extent by treatment of cells with recombinant gp120 in the absence of other viral products (Figures 3 to 7, Table 1). A similar conclusion was reached in our recent study on the regulation of glutamate transporters and glutamate transport in human astrocytes by HIV-1 (Wang *et al*, unpublished data). This conclusion is less surprising if one considers that productive infection of astrocytes by HIV-1 is inefficient and only a small proportion of infected cells, about 1%, express virus products that could possibly affect host cell physiology (Tornatore *et al*, 1991; Takahashi *et al*, 1996; Bencheikh *et al*, 1999). However, the viral effects observed here are "global," that is, they must occur in a majority of cells in order to be detectable. For example, the ca.

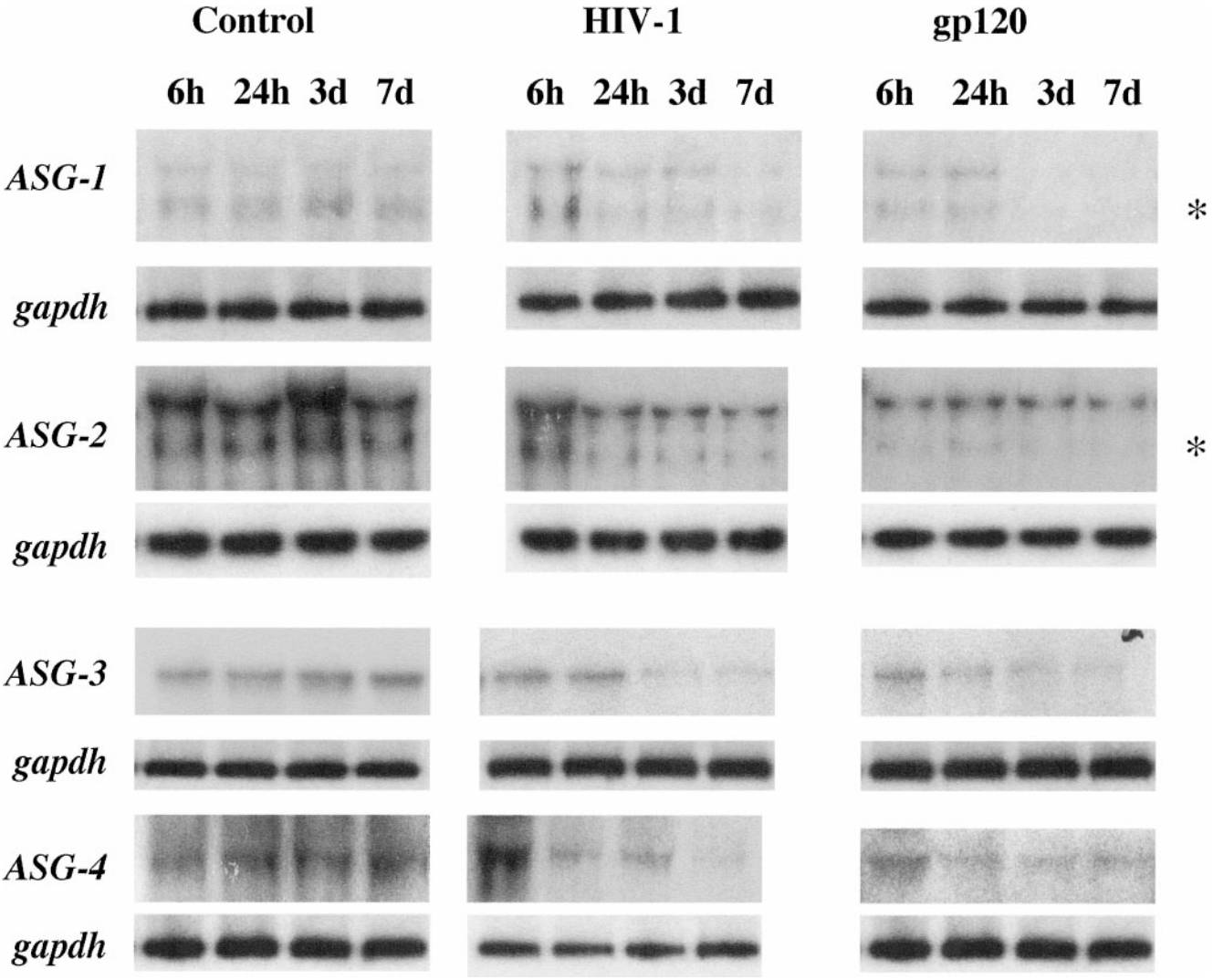

Figure 3 Confirmation of differential expression using Northern blot analysis of *ASG-1* to *ASG-4* displaying temporally suppressed expression in astrocytes following HIV-1 infection or treatment with gp120. Early passage human fetal astrocytes were mock infected (control), infected with HIV-1 at an M.O.I. of 1, or treated with 1 nM gp120 for 6 h, 24 h, 3 days, or 7 days, total RNA was isolated and analyzed by Northern blotting. Membranes were probed with the indicated radiolabeled [^{32}P]-*ASG* EST, identified by RaSH, the blots were stripped and probed with a radiolabeled [^{32}P]-*gapdh* cDNA probe. Expression was quantitated by densitometric analysis. *ASG-1* and *ASG-2*, ~ indicates RNA transcript monitored in control, HIV-1–infected, and gp120-treated human fetal astrocytes.

fourfold decline in *ASG-5* expression at 7 days after HIV-1 exposure (Figure 7) can be explained only by a total loss of *ASG-7* expression in 75% of affected cells or 75%loss incurred by all the cells in the population. The replication-independent modulation of cellular gene expression by HIV-1 is likely mediated by efficient viral interaction with surface receptors on astrocytes. Although these cells lack surface CD4, the canonical HIV-1 receptor on T cells and macrophages (Klatzmann *et al*, 1984; Cheng-Mayer *et al*, 1987), they do express a high-molecular-weight protein of about 260 kDa, which was shown to bind gp120 with high affinity (Ma *et al*, 1994) and which may be responsible for binding intact HIV-1 as well. Astrocytes also express the chemokine receptors CXCR4 and CCR5 (Andjelkovic *et al*, 1999; Rezaie *et al*, 2002) as well as galactocerebroside (Harouse *et al*, 1989), all of which can bind the HIV-1 envelope. Further studies are needed to determine the identity of the membrane receptors on astrocytes that may mediate the HIV-1 effects on cellular gene expression observed here and in our other studies (Su *et al*, 2002).

Although RNA transcripts of a single size are apparent in astrocytes after probing with *ASG-3, -4, -5, -7*, and *-9*, multiple hybridizing RNA species are apparent in Northern blots after probing astrocyte RNAs with *ASG-1, -2, -6, -8*, and *-10* (Figures 3 to 5). The presence of multiple RNAs may indicate alternative processing of the respective gene, resulting in multiple-sized transcripts, or it could reflect a cloning artifact resulting from two sequences being cloned together in a single *RaSH*-derived clone. In cases where a decrease is apparent in the multiple RNA species as a consequence of infection with HIV-1 or treatment

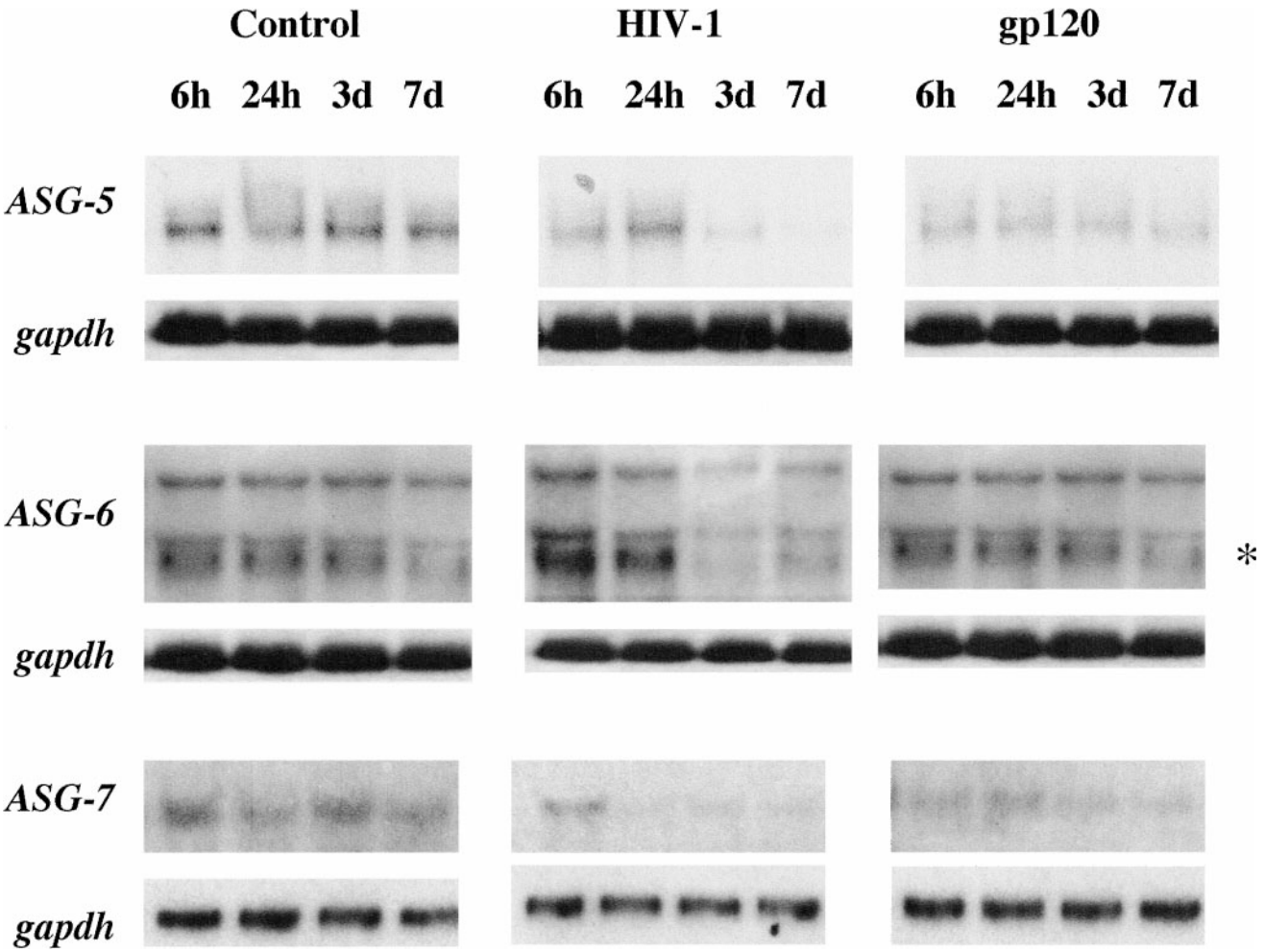

Figure 4 Confirmation of differential expression using Northern blotting analysis of *ASG-5* to *ASG-7* displaying temporally suppressed expression following HIV-1 infection or treatment with gp120. *ASG-6*, ~ indicates RNA transcript monitored in control, HIV-1–infected, and gp120-treated human fetal astrocytes. See Materials and methods and Figure 3 for experimental details.

with gp120, such as *ASG-1* and *-6*, differential processing of a single gene is a more plausible explanation for the different sized mRNAs. However, in the case of *ASG-2*, *-8*, and -10, further analysis was performed to determine if chimeric clones have been produced during the RaSH procedure. Use of RaSH in its presently described form highlighted two potential problems that can readily be addressed by minor modifications in the protocol as addressed by Kang *et al* (2002). As indicated above, although the majority of *ASG* RaSH clones contained single inserts, some clones contained more than one insert that were ligated in tandem (Jiang *et al*, 2000). Multiple inserts can obscure differential expression in screening procedures, such as reverse Northern hybridization. Moreover, if the gene has not been reported previously, the hybrid molecule can inappropriately serve as a basis for attempting to clone a spurious molecule. Careful consideration, especially with respect to the presence internally of the restriction site used in library construction (*Dpn*II) in the RaSH clone could be used to circumvent this problem. This inspection has been done for the *ASGs* described in the present study, indicating that those genes hybridizing to multiple transcripts are not chimera genes, i.e., produced as a result of cloning artifacts during the RaSH procedure. Additionally, digestion of a cDNA with a frequent cutter could increase additional redundancy due to cloning different parts of the same gene. Immobilization of the 3′ end by using biotinylated reverse transcriptase (RT) primer with a cloning site (e.g., *Xho*I) and ligation of adapter with another cloning site (*Bam*HI) may prove useful in ameliorating this problem of redundant clone isolation and the isolation of clones containing multiple inserts. This modification in the original protocol will also enhance the cloning efficiency of the differentially expressed insert into the vector (Kang *et al*, 2002).

HIV-1–mediated dysregulation of cellular gene expression in human astrocytes and neuropathogenesis

The major implication of the findings presented here and in our previous work (Su *et al*, 2002) is that

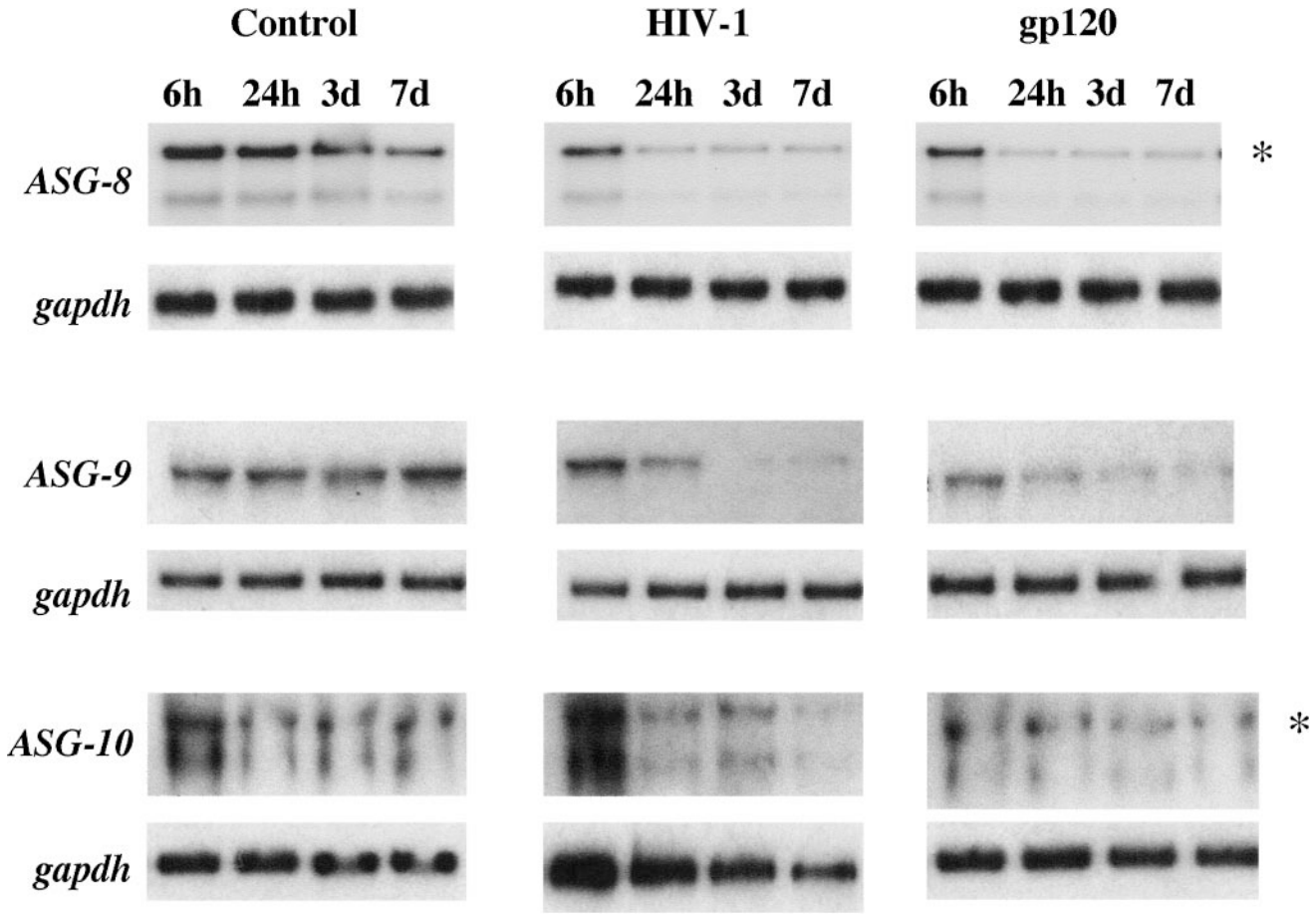

Figure 5 Confirmation of differential expression using Northern blotting analysis of *ASG-8* to *ASG-10* displaying temporally suppressed expression following HIV-1 infection or treatment with gp120. *ASG-8* and *ASG-10*, ~ indicates RNA transcript monitored in control, HIV-1–infected, and gp120-treated human fetal astrocytes. See Materials and methods and Figure 3 for experimental details.

HIV-1 has profound, global effects on expression of a broad array of cellular genes in astrocytes, suggesting that this may be one route through which HIV-1–infected astrocytes contribute to HAD. Overall, 25 genes were identified as differentially expressed in astrocytes as a result of HIV-1 exposure, 15 of these were up-regulated in their expression and 10 suppressed (Su *et al* [2002] and this work). Based on the size of our RaSH-derived EST library (see Materials and methods and text), the number of differentially expressed genes in this system may exceed 100. The magnitude of this HIV-1 effect on astrocyte biology is more remarkable, as it occurs despite relatively inefficient viral expression in these cells (Tornatore *et al*, 1991; Canki *et al*, 2001; Su *et al*, 2002), and it can be reproduced by treatment of astrocytes with isolated HIV-1 envelope glycoprotein (Figures 6, 7). As shown by recent gene array and RaSH analyses, HIV-1 also exerts profound effects on cellular gene expression during infection of T lymphocytes (Geiss *et al*, 2000; Corbeil *et al*, 2001; Simm *et al*, 2001). Notably, in the study of Corbeil *et al* (2001), HIV-1 infection of $CD4^+$ T cell line (CEM) cells was associated with a 30% decline in overall cellular RNA expression, replacement of cellular RNA by viral transcripts, and increased expression of proapoptotic genes and selected caspases; these molecular changes are consistent with the known cytopathic course of HIV-1 infection in T cells that culminates in cell death. In contrast, primary astrocytes are not killed by HIV-1 infection *in vitro* (Tornatore *et al*, 1991; Bencheikh *et al*, 1999) and the major morphological change observed in astrocytes during HIV-1 infection *in vivo* is gliosis (Budka, 1991; Sharer, 1992), which represents activation and possible expansion of the glial tissue. We therefore interpret our results as indicating that exposure of astrocytes to HIV-1 or gp120 may induce long-lasting effects on cell physiology and functions rather than affecting cell viability, as with other HIV-1 host cells. Some of these functions, such as maintenance of ionic equilibrium in the synapse and transport of the neurotransmitter L-glutamate, impact neurons directly and their impairment has been shown to cause neurotoxicity (Choi, 1988; Maragakis and Rothstein, 2001). Recent data also indicate that astrocytes are a critical functional component of the synapse and play a role in signal transmission (Iino *et al*, 2001; Oliet *et al*, 2001; Beattie *et al*, 2002); disruption of these

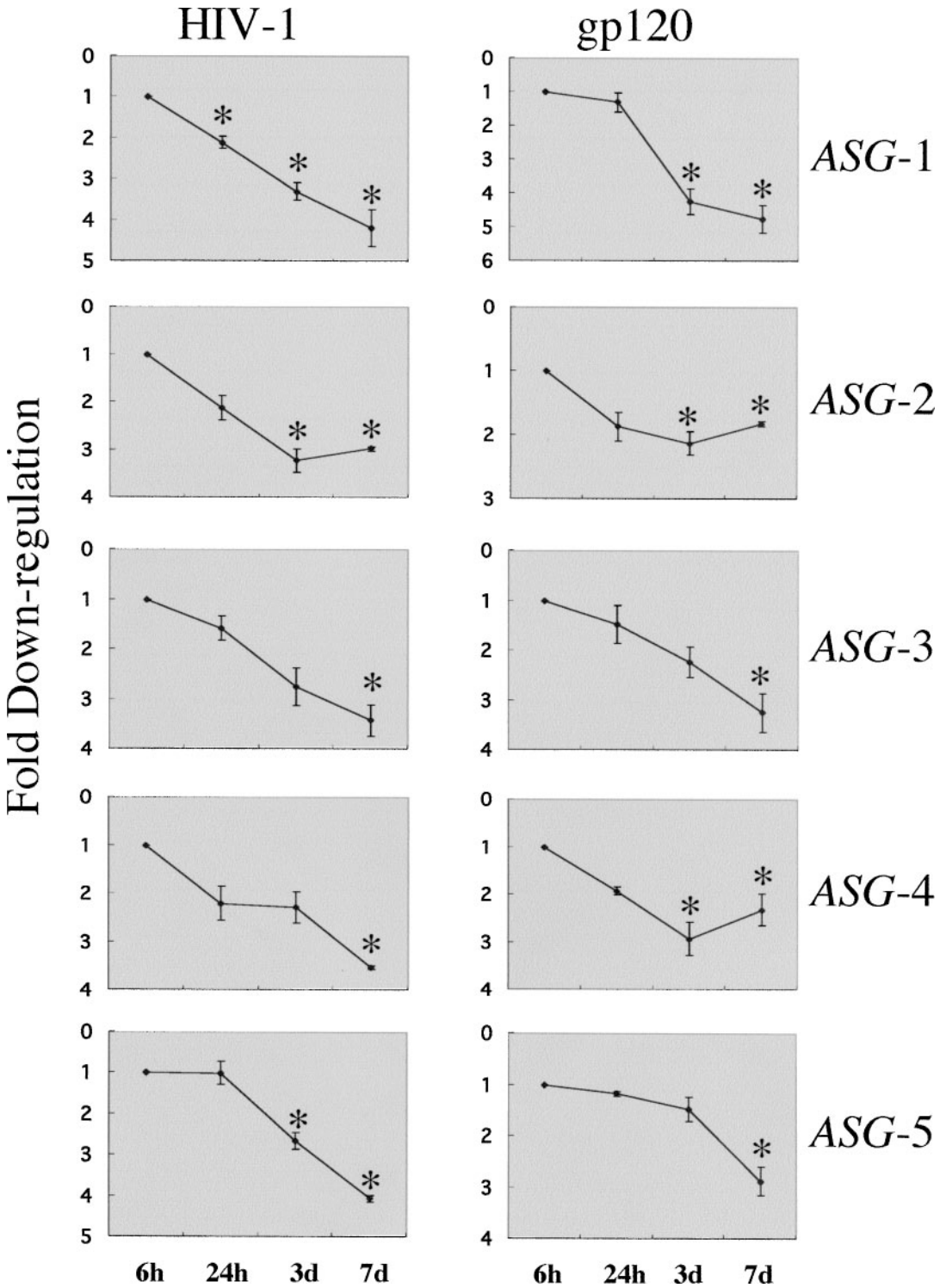

Figure 6 Temporal modulation of *ASG-1* to *ASG-5* expression in normal human fetal astrocytes as a consequence of infection with HIV-1 or exposure to gp120. Data presented reflects values ~ SD obtained after scanning and normalizing (to *gapdh*) the Northern blots from two independent experiments using temporally isolated samples from HIV-1–infected and gp120-treated human fetal astrocytes. Fold-decline in expression was determined as described in Materials and methods and in the legend to Table 1. ˜ Indicates significance with a P value of $<.05$.

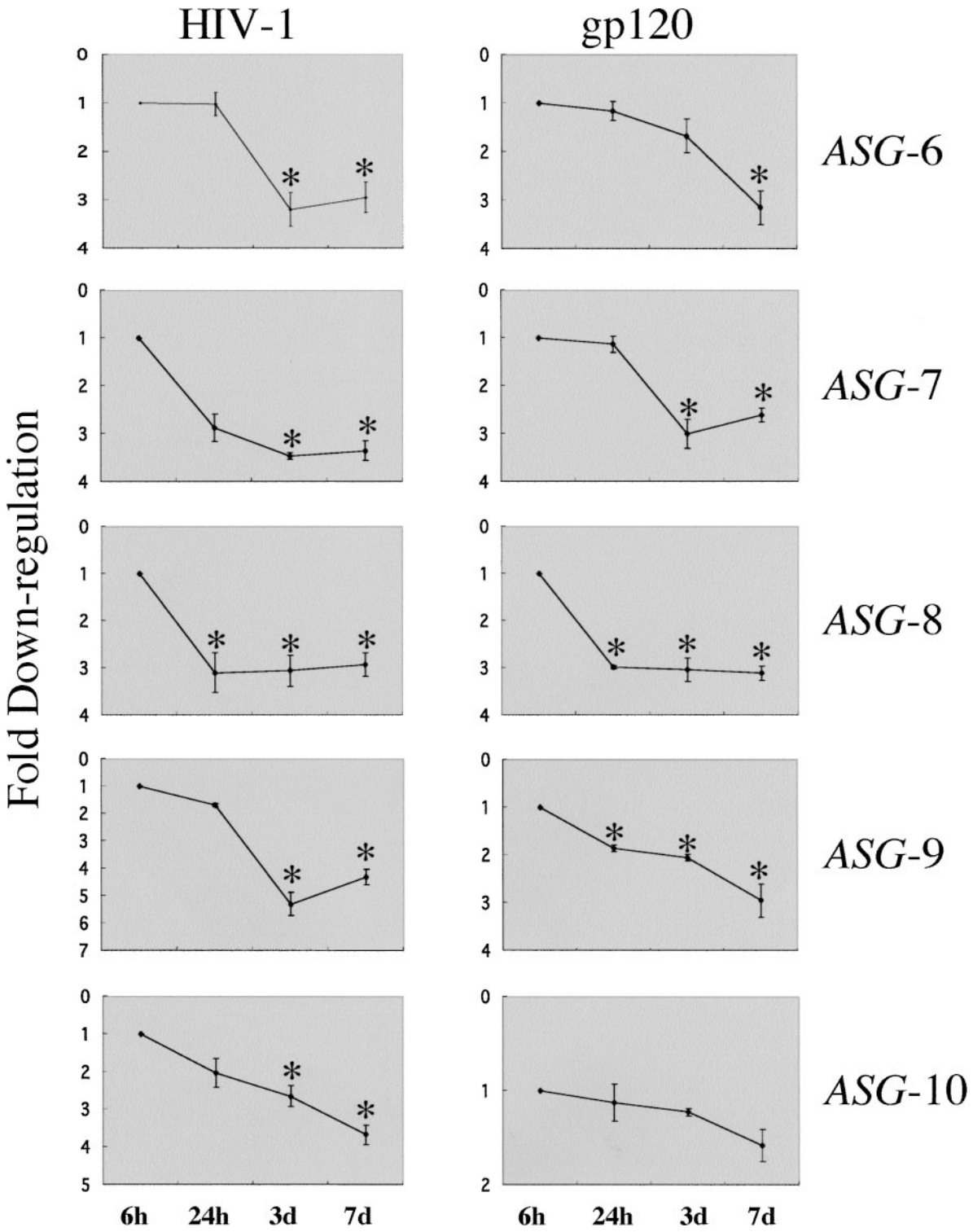

Figure 7 Temporal modulation of *ASG-6* to *ASG-10* expression in normal human fetal astrocytes as a consequence of infection with HIV-1 or exposure to gp120. Data presented reflects values ~ SD obtained after scanning and normalizing (to *gapdh*) the Northern blots from two independent experiments using temporally isolated samples from HIV-1–infected and gp120-treated human fetal astrocytes. Fold-decline in expression was determined as described in Materials and methods and in the legend to Table 1. ~ Indicates significance with a P value of $<.05$.

functions could impair the function of the nervous system and lead to neurodegeneration. Finally, there are indications that astrocytes may serve as immune effector cells in the brain (Dong and Benveniste, 2001); disruption of this function could weaken immune responses against HIV-1, particularly the recruitment of macrophages into the brain (Lipton and Gendelman, 1995).

The *ASGs* (Table 1) and *AEGs* (Su *et al*, 2002) identified thus far can provide leads for investigation of cellular pathways coopted by HIV-1 in astrocytes. One important indicator is the time of gene activation or suppression relative to HIV-1 infection. Early modulation of cellular genes (between 6 and 24 h after HIV-1 infection in our studies) may indicate direct cellular responses to HIV-1 mediated by signal transduction mechanisms activated by virus interaction with cell surface receptors or by disruption of the plasma membrane integrity during virus-cell fusion. Such responses were observed after HIV-1 exposure in T cells (Gupta and Vayuvegula, 1987; Fermin and Garry, 1992; Miller *et al*, 1993), macrophages (Zheng *et al*, 1999; Choe *et al*, 2001), and neurons (Zheng *et al*, 1999). The early-responder genes are represented by *ASG-4*, *-7*, and *-8*, all of which show a decline in expression within 6 to 24 h after HIV-1 infection (Figures 6, 7). *ASG-4* is of particular interest in the context of HIV-1 infection. This gene codes for C3G, a guanine nucleotide–releasing (exchange) protein that was originally identified as one of the two major proteins binding to the *Src* homology-3 (SH3) domain of the Crk adaptor protein (Tanaka *et al*, 1994). The Crk-C3G complex transduces signals from tyrosine-phosphorylated receptors (RTKs) in the plasma membrane to Rap1, a member of Ras-family G-proteins (York *et al*, 1998), and subsequently to Jun kinase, JNK (Tanaka *et al*, 1997). The exact role of the RTK/Crk/C3G/Rap1 signaling pathway has not been fully characterized (Tanaka *et al*, 1997), but published data indicate that it may exert pluripotent effects on cellular gene expression, possibly depending on the initiating signal. For example, Rap1 was shown to antagonize Ras-mediated cell transformation and mitogen-activated protein (MAP) kinase activation in Rat-1 fibroblasts and cloned rat embryo fibroblasts (CREFs) (Cook *et al*, 1993; Su *et al*, 1993). However, conversely, it mediated sustained MAP kinase activation and cell differentiation in response to nerve growth factor (NGF) in PC12 cells (York *et al*, 1998) and C3G-dependent Rap1 activation promoted adhesion of mouse embryonic fibroblasts (Ohba *et al*, 2001). Of note, the Crk/C3G pathway was recently shown to serve as a downstream effector for the latency-associated protein LMP2A of Epstein-Barr virus, a major human pathogen (Engels *et al*, 2001). The observed down-regulation of C3G in astrocytes by HIV-1 may thus disrupt an important cellular signaling pathway and the functions it controls, including, if cell adhesion is affected (Ohba *et al*, 2001), the activity of astrocytes as antigen-presenting cells (Dong and Benveniste, 2001). Of the other two early-response genes in HIV-1–infected astrocytes, *ASG-8* is a novel gene and *ASG-7* is a partially characterized transcription regulation factor that belongs to the zinc finger protein family (Liebhaber *et al*, 1990). Sequence analysis of *ASG-7* suggests that it contains four putative zinc fingers, which may have evolved by duplication of a preexisting two-finger unit (Liebhaber *et al*, 1990). This human cysteine-rich protein gene is highly conserved and was detected in every nucleated tissue and cell line tested (Liebhaber *et al*, 1990). Although further experimentation is necessary, changes in expression of this transcription factor could affect expression of target genes in astrocytes that may contribute to normal astrocyte physiology.

Similar to our *AEGs* series of astrocyte genes (Su *et al*, 2002), the majority of *ASGs* described here appear to be late-response genes, that is, their expression declined maximally only 3 to 7 days after HIV-1 infection. These genes include *ASG-1* (novel), *ASG-3* (platelet-endothelial cell tetra-span antigen 3, or CD151/PETA-3) (Fitter *et al*, 1995; Yáñez-Mó *et al*, 1998), *ASG-5* (neuronatin) (Dou and Joseph, 1996b), *ASG-6* (neuroendocrine differentiation factor) (Wilson *et al*, 2001), *ASG-9* (human mitochondrion genomic DNA, this fragment is homologous to the 952- to 1232-bp region of genomic DNA that encodes 16s rRNA (from the 650- to 1603-bp region of genomic DNA), and *ASG-10* (signal recognition particle, SRP9) (Lütcke, 1995). PETA-3 (*ASG*-3) is a glycoprotein of 253 amino acids that belongs to the tetraspanin family of surface proteins (Fitter *et al*, 1995; Testa *et al*, 1999). PETA-3 RNA is down-modulated 3.4-fold in infected versus uninfected astrocytes (Table 1). Although PETA-3 was originally identified as a platelet surface protein, recent data indicate that it functions as a component of integrin signaling complexes on endothelial cells and it may be involved in regulation of cell motility (Yáñez-Mó *et al*, 1998; Testa *et al*, 1999). Down-modulation of PETA-3 in astrocytes by HIV-1 may affect astrocyte function in maintaining the integrity of the BBB by reducing both the flexibility and adhesion strength of the astrocytic underlayer of the BBB (Morgello *et al*, 1995). Neuronatin (*ASG-5*) is a brain-specific human protein that is selectively expressed during development (Dou and Joseph, 1996a, 1996b) and therefore it is unlikely to play a role in the adult disease such as HAD. The maximum ~4.1-fold down-modulation of neuronatin observed here is of interest because of the proposed function of the protein as a regulator of anion channels (Dou and Joseph, 1996b), an activity that may be functionally related to the observed down-regulation of glutamate transport in these cells (Wang *et al*, unpublished data).

The neuroendocrine differentiation factor, NEDF (*ASG-6*), has been recently identified by a yeast two-hybrid screen as a novel intracellular protein that interacts with the insulin-like growth factor

(IGF)-binding protein-related protein-1 (IGFBP-rP1) and proposed to act together with IGFBP-rP1 in inducing neuroendocrine cell differentiation in response to IGF (Wilson *et al*, 2001). *ASG-6* is of interest in the context of HIV-1 infection because IGF-like growth factors appear to be protective during CNS injury (Bondy and Lee, 1993) and HIV-1 disease correlates with defects in the IGF system (Frost *et al*, 1996; Jain *et al*, 1998). Also of note, it has been suggested that the NEDF (*ASG-6*)/IGFBP-rP1 complex acts through the Ras/MAP kinase signaling pathway (Wilson *et al*, 2001), an alternative to the Crk/C3G/Rap1 pathway discussed earlier in the context of observed down-modulation of C3G (*ASG-4*). Thus two genes in astrocytes whose expression is reduced by HIV-1, one early (*ASG-4*) and one late (*ASG-6*) after infection, encode products that transduce signals from RTKs, indicating that this signal transduction pathway is a major target for HIV-1–mediated dysregulation of astrocyte physiology. Experiments are now under way to investigate this possibility.

ASG-10 encodes a signal recognition particle SRP9, a component of *Alu* RNA binding heterodimer SRP9/14 (Lütcke, 1995). SRP targets secretory and membrane proteins to rough endoplasmic reticulum in a complex, cotranslational process that includes a temporary arrest of elongation (Lütcke, 1995). In the CNS, the heterodimer SRP9/14 was found to be an integral part of the brain-specific BC200 RNA, a small nonmessenger RNA that is a constituent of a ribonucleoprotein complex in neurons and that is believed to regulate protein biosynthesis in dendrites (Kremerskothen *et al*, 1998). Down-modulation of SRP9 expression by HIV-1 may impair the formation and function of the SRP9/14 heterodimer and, consequently, affect synthesis of secretory and membrane proteins by astrocytes. The virus could adopt this mechanism as a means for reducing exposure of infected astrocytes to immune recognition, similar in an outcome to the Nef-mediated down-modulation of human leukocyte antigen (HLA) class I in T lymphocytes (Collins *et al*, 1998).

In conclusion, we employed the RaSH approach as an efficient and rapid means of identifying astrocyte genes that display suppressed (*ASG*) (this work) and elevated (*AEG*) (Su *et al*, 2002) expression after exposure of astrocytes to gp120 or infection with HIV-1 *in vitro*. Ten *ASGs* and 15 *AEGs* have been uncovered, providing potential clues for understanding HIV-1 effects on human fetal astrocytes. Further studies are necessary to determine if the presently identified genes are causally related or simply associated with changes induced in astrocyte properties, such as inhibition of glutamate transport, following infection with HIV-1. Although a daunting challenge, these studies are worthwhile and may provide important insights into the process of HAD.

Materials and methods

Human fetal astrocytes, other cells, cell culture conditions

Fetal astrocytes were isolated from second trimester (gestational age 16 to 19 weeks) human fetal brains obtained from elective abortions in full compliance with National Institutes of Health (NIH) guidelines, as previously described (Bencheikh *et al*, 1999; Canki *et al*, 2001; Su *et al*, 2002). Highly homogenous preparations of astrocytes were obtained using high-density culture conditions in the absence of growth factors in F12 Dulbecco's modified Eagle medium (DMEM) (GIBCO-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), penicillin, streptomycin, and gentamycin. Cells were maintained in this medium at $(2–5) \sim 10^4$ cells/cm^2 and subcultured weekly up to six times. For each experiment, a single batch of astrocytes of similar gestational age and passage was used. Cultures were regularly monitored for expression of the astrocytic marker glial GFAP and either HAM56 or CD68 to identify cells of monocyte/macrophage lineage. Only cultures that contained ~99% GFAP-positive astrocytes and rare or no detectable HAM56- or CD68-positive cells were used in these experiments (Canki *et al*, 2001). Other cells used in this study were the human embryonal-kidney epithelial cell line 293T (Graham *et al*, 1977), used for HIV-1 propagation, and MAGI cells, a derivative of HeLa carrying the μ-gal gene under the control of HIV-1 long terminal repeat (LTR) and expressing HIV-1 receptors (Kimpton and Emerman, 1992), used as indicator cells for HIV-1 titration. Both cell lines were cultured in 90% DMEM/10% FBS supplemented with antibiotics and, for MAGI cells, 0.2 mg/ml G-418. Culture media and cells were tested for mycoplasma contamination using the Mycoplasma PCR ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) and found to be negative.

HIV-1 propagation

The HIV-1 used in this work was NL4-3, a X4-tropic recombinant clone of HIV-1 that expresses all known HIV-1 proteins (Adachi *et al*, 1986). Virus propagation was initiated by transfection of 15 μg of NL4-3 DNA into $1.5 \sim 10^6$ 293T cells as previously described (Bencheikh *et al*, 1999). Culture supernatants were harvested 72 h after transfection, filtered through a 0.45 μm Millipore filters, and stored at ~80°C until use. Cell-free viral stock was tested for HIV-1 p24 core antigen content by enzyme-linked immunosorbent assay (ELISA) using HIV-1 Ag kit according to the manufacturer's instructions (Coulter, Hialeah, FL) and for titers of infectious virus by multinuclear activation of a μ-galactosidase indicator (MAGI) assay (Kimpton and Emerman, 1992). Culture supernatants contained 1 to 2 μg/ml of viral p24 protein and $(1–2) \sim 10^6$ infectious units (IU) per ml. In our hands, a multiplicity of infection (MOI) of one for CD4-positive T cells is approximately 1 pg viral p24

per cell (Canki *et al*, 2001). Virus stocks were also tested for mycoplasma contamination as described above.

HIV-1 infection of astrocytes with HIV-1 or exposure of the cells to gp120, and preparation of samples for cellular RNA analysis
Confluent cultures of human fetal astrocytes in 225-cm^2 culture flasks were exposed to HIV-1 in 10 ml of medium at 1 pg p24 per cell for 2 h at 37˚C, washed three times in warm phosphate-buffered saline (PBS), and cultured in astrocyte culture medium as described. Control astrocytes were treated as described above but without HIV-1. At 6 h, 24 h, 3 days, and 7 days after infection, culture supernatants were removed, and control and infected cells were washed three times in PBS and solubilized by addition of 10 ml of 4 M guanidine isothiocyanate directly to culture flasks. Cell lysates were stored at ~80˚C until further use. To insure preparation of sufficient amounts of RNA for subsequent subtractive hybridization, astrocyte cultures and HIV-1 infections were scaled up to approximately $1 \sim 10^8$ cells per time point (infected or control cells); the RNA yield was 5 to 10 μg per 10^6 cells. Infection of astrocytes with HIV-1 was verified by testing the levels of HIV-1 p24 antigen in culture supernatants by p24 ELISA as described previously (Bencheikh *et al*, 1999; Canki *et al*, 2001). For gp120 treatment of astrocytes, large-scale cultures of cells prepared as described above were treated with gp120 at 1 nM in 10 ml medium for 2 h, and cells were washed, cultured, and extracted for RNA isolation as described for HIV-1 infection. The gp120 used in these experiments was a full-length, glycosylated protein from HIV-1_{MN} produced from baculovirus vector and purified by ImmunoDiagnostics, and provided through the AIDS Research and Reference Reagent Program (Rockville, MD).

RNA isolation and Northern blot analysis
Uninfected, HIV-1–infected and gp120-exposed astrocytes were treated with 4 M guanidinium and total RNA was isolated by the guanidinium/phenol procedure and analyzed by Northern blotting as described previously (Jiang and Fisher, 1993; Kang *et al*, 1998, 2001). Northern blots were quantitated by densitometric analysis using a Molecular Dynamics (Sunnyvale, CA) densitometer. Relative expression of the different *ASGs* versus *gapdh* expression was determined at different time points for HIV-1 infected (H-*ASG*/H-*gapdh*), gp120-treated (G-*ASG*/G-*gapdh*) and control uninfected cultures (C-*ASG*/C-*gapdh*). Fold-increase in expression of each *ASG* at 6 h, 12 h, 24 h, 3 days, and 7 days was then determined for each condition by dividing H-*ASG*/H-*gapdh* by C-*ASG*/C-*gapdh* = fold HIV up-regulation; or by dividing G-*ASG*/G-*gapdh* by C-*ASG*/C-*gapdh* = fold gp120 up-regulation. Poly(A) RNA was purified using Oligo(dT) cellulose columns (GIBCO-BRL). Statistical significance of gene expression level changes between control and treated samples was analyzed using the Student's *t* test. Alterations in gene expression were considered significant if *P* values were $<.05$. An asterisk indicates this level of significance.

RaSH procedure
Primer design: The sequences of oligonucleotides that were used are as follows: XDPN-18, CTGATCACTCGAGAGATC; XDPN-14, CTGATCACTCGAGA; XDPN-12, GATCTCTCGAGT. The adapters formed from the two sets of oligonucleotides contained an *Xho*I recognition site.

Preparation of PCR-based cDNA libraries: To clone cDNAs expressed at reduced levels in early passage human fetal astrocytes, 1 μg of poly(A) RNA from temporally spaced (6 h, 12 h, 24 h, 3 days, and 7 days) uninfected astrocytes (tester) or temporally spaced (6 h, 12 h, 24 h, 3 days, and 7 days) HIV-1 infected astrocytes (driver) prepared as described above was used for double-stranded cDNA synthesis using standard protocols (Gubler and Hoffman, 1983). The cDNAs were digested with *Dpn*II (New England Biolab, Beverly, MA) at 37˚C for 3 h followed by phenol/chloroform extraction and ethanol precipitation. The digested cDNAs were mixed with primers XDPN-14/XDPN-12 (final concentration 20 μM) in 30 μl of 1~ ligation buffer (GIBCO-BRL), heated at 55˚C for 1 min, and cooled down to 14˚C within 1 h. After adding 3 μl of T4 ligase (5 U/μl) (GIBCO-BRL) to the mixtures individually, ligation was carried out at 14˚C overnight. The mixtures were diluted to 100 μl with Tris-EDTA (TE) buffer (pH 7.0), and at least 40 μl of the mixtures were used for PCR amplification. The PCR mixtures were set up as follows: 1 μl of the cDNA mixture, 10 μl 10~ PCR buffer, 1 mM $MgCl_2$, 0.4 mM dNTPs, 1 μM XDPN-18, and 1 U Taq polymerase (GIBCO-BRL). The parameters for PCR were one cycle for 5 min at 72˚C followed by 25 cycles for 1 min at 94˚C, 1 min at 55˚C, 1 min at 72˚C followed by one cycle for 3 min at 72˚C. The PCR products were pooled and purified using Centricon columns (Amicon, Bedford, MA). Ten micrograms of the tester PCR products were digested with *Xho*I followed by phenol/chloroform extraction and ethanol precipitation.

Subtraction hybridization and generation of subtracted libraries
One hundred nanograms of the tester cDNA were mixed with 3 μg of the driver cDNA in 10 μl of a hybridization solution (0.5 M NaCl, 50 mM Tris pH 7.5, 0.2% sodium dodecyl sulfate [SDS], 40% formamide), and after boiling for 5 min, incubated at 42˚C for 48 h. The hybridization mixture was phenol/chloroform extracted, ethanol precipitated, and dissolved in 20 μl of TE buffer. One microliter of the mixture was ligated with 1 μg of *Xho*I-digested, calf intestinal alkaline phosphatase (CIP)-treated pCRII plasmids, overnight at 14˚C, and transformed into Shot-1 bacteria.

Colony screening

Bacterial colonies were randomly picked and PCR amplified. The PCR products were blotted onto filters and reverse Northern blotting was performed to identify cDNAs displaying differential expression in HIV-1–infected versus uninfected early passage human fetal astrocytes (Kang *et al*, 1998; Huang *et al*, 1999b; Jiang *et al*, 2000). cDNAs displaying reduced expression in HIV-1–infected fetal astrocytes versus uninfected fetal astrocytes were designated *ASGs* with a clone number of 1 to 10. Appropriate expression of the *ASGs* clones identified by reverse Northern blotting was confirmed by Northern blotting. The sequences of these clones were determined using automated cycle sequencing at the DNA facility of Columbia University.

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