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Sam68 is absolutely required for Rev function and HIV-1 production

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ABSTRACT

Sam68 functionally complements for, as well as synergizes with, HIV-1 Rev in Rev response element (RRE)-mediated gene expression and virus production. Furthermore, C-terminal deletion/point mutants of Sam68 (Sam68ΔC/Sam68-P21) exert a transdominant negative phenotype for Rev function and HIV-1 production. However, the relevance of Sam68 in Rev/RRE function is not well defined. To gain more insight into the mechanism of Sam68 in Rev function, we used an RNAi (RNA interference) strategy to create stable Sam68 knockdown HeLa (SSKH) cells. In SSKH cells, Rev failed to activate both RRE-mediated reporter gene [chloramphenicol acetyltransferase (CAT) and/or *gag*] expressions. Importantly, reduction of Sam68 expression led to a dramatic inhibition of HIV-1 production. Inhibition of the reporter gene expression and HIV production correlated with the failure to export RRE-containing CAT mRNA and unspliced viral mRNAs to the cytoplasm, confirming that SSKH cells are defective for Rev-mediated RNA export. Taken together, these results suggest that Sam68 is involved in Rev-mediated RNA export and is absolutely required for HIV production.

INTRODUCTION

Sam68 was initially identified as a 68 kDa *Src*-associated protein in mitosis (1,2), and belongs to a family of proteins that contain KH domains. The KH domain is highly conserved in several RNA-binding proteins such as hnRNP-K (3), glycine-rich protein 33 (4), fragile X mental retardation gene FMR-1 (5) and the *Caenorhabditis elegans* germ-line-specific tumor suppressor GLD-1 (6), and as recently reported, other proteins such as SLM-1 (Sam68-like mammalian), SLM-2 (7), and the quaking proteins QKI-5, QKI-6 and QKI-7 (8,9). Some KH proteins are translational regulators (10), while others are thought to mediate alternative splicing (11,12). Using the random homozygous knockout antisense strategy, Sam68 has been implicated in cell proliferation and tumorogenesis (13). However, the exact cellular functions of Sam68 still remain to be established. It has been postulated to play a role in the post-transcriptional regulation of gene expression.

Sam68 binds to the Rev response element (RRE) of HIV-1 *in vitro* and *in vivo*, and can functionally replace and/or synergize with HIV-1 Rev in RRE-mediated gene expression and virus replication (14–17). Furthermore, Sam68 was also shown to enhance the activities of the Rev-like proteins of other complex retroviruses (18). Recently, it has been demonstrated that Sam68 enhances the 3′ end processing of unspliced HIV-1 RNAs to be exported to the cytoplasm (19). KH proteins other than Sam68 (i.e. SLM-1, SLM-2, QKI-5, QKI-6 and QKI-7) also enhance Rev/RRE-mediated gene expression (17,20). However, among the KH proteins tested, only Sam68 was able to activate constitutive transport element [(CTE) (21)]-mediated *gag* gene expression in human cells (20,22). Additionally, Sam68 also enhances the Tap activity in CTE-dependent *gag* gene expression in quail cells (22,23).

To understand the mechanism of action of Sam68 in Rev function, we used an RNAi (RNA interference) strategy to reduce the expression of Sam68 and assessed the effect of depletion of Sam68 on Rev/RRE function. We report here that the stable knockdown of Sam68 significantly inhibited both Rev activation of RRE-mediated gene expression and HIV production. Furthermore, the inhibition of Rev activity in Sam68 knockdown cells correlated with failure to export unspliced RRE-containing mRNAs to the cytoplasm. Therefore, these results provide the first direct evidence that Sam68 is involved in the nuclear export of RRE-containing RNAs and is absolutely required for Rev function in HIV biology.

MATERIALS AND METHODS

Plasmids

Plasmids pSam68, pNLRRE-*gag*, pHIV-LTR CAT and pTat have been described previously (14,24,25). We used the SuppressorNeo-IMG-800 RNAi expression vector (Imgenex

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Corp., San Diego, CA) to generate the stable Sam68 and IL10 knockdown HeLa cells. Sam68 (forward primer: 5′-TCGAGGGATGATGAGGAGAATTACGAGTACTGG-TAATTCTCCTCATCATCCTTTTT-3′ and reverse primer: 5′-CTAGAAAAAGGATGATGAGGAGAATTACCAGTACT-CGTAATTCTCCTCATCATCCC-3′) and IL10 (forward primer: 5′-TCGAGCATACTGCTAACCGACTCCGAGTA-CTGGGAGTCGGTTAGCAGTATGTTTTT-3′ and reverse primer: 5′-CTAGAAAAACATACTGCTAACCGACTCCC-AGTACTCGGAGTCGGTTAGCAGTATGC-3′) primers were annealed and cloned into the SalI and XbaI restriction enzyme sites of pIMG-800 to generate pSam68-RNAi and pIL10-RNAi using the standard protocol.

Cells, transfections and chloramphenicol acetyltransferase (CAT) assays

The 293T and HeLa cell lines were maintained in DMEM supplemented with 10% fetal bovine serum. In general, between 1 and 3 μg of DNA was transfected into 293T and/or HeLa cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. PcDNA3 plasmid was used to equalize the amount of DNA for each transfection. Forty-eight hours post-transfection, the cells were harvested, washed with phosphate-buffered saline and then re-suspended in 50–100 μl of 0.25 M Tris, pH 7.8. The cell extracts, CAT assays were performed as described previously (14). To create stable Sam68 knockdown cell lines, HeLa cells were transfected with pSam68-RNAi and placed under G418 selection. The Sam68 expression in G418-resistant colonies was analyzed by western blot analysis using anti-Sam68 antibodies. A similar strategy was used to create HeLa-IL10i control clones.

Western and northern blot analyses

For western blot analysis, total cell extracts (75 μg) were analyzed by SDS–PAGE and electro-transferred to nitrocellulose membrane. The membrane was incubated with antibodies raised against target proteins (Sam68 and/or Rev). The presence of antibody bound to target proteins was detected using an enhanced chemiluminescence western blot analysis kit (Amersham).

For northern blot analysis, nuclear, cytoplasmic or total RNA was isolated using a PARIS™ RNA isolation kit (Ambion Inc., Austin, TX). Approximately 20 μg of RNA was separated on 1% agarose formaldehyde gels by electrophoresis and blotted onto nitrocellulose filters. For the detection of CAT RNAs, the filters were hybridized with ^{32}P-labeled CAT cDNA probe. For the detection of HIV-1 RNAs, the filters were probed with a PCR-amplified probe consisting of nucleotides 9–634 of the 5′ LTR of HxB2 (GenBank accession no. K03455), which hybridizes to all HIV-1 transcripts.

P24 antigen capture assay

Stable Sam68 knockdown HeLa (SSKH) or HeLa-IL10i cells were transfected with HxB-2 proviral DNA. At 24, 48 and/or 60 h post-transfection, cell-free supernatants were collected and subjected to p24 antigen assay (Coulter).

RESULTS

Inhibition of the Sam68 expression by RNAi

The main objective of this study is to deplete intracellular Sam68 and determine whether a reduced expression of Sam68 would impact Rev/RRE function and HIV production. An RNAi strategy was used to reduce the expression of Sam68 in cells. A Sam68-derived oliogonucleotide sequence that met the criteria for effective RNAi (26) was inserted into the RNAi expression vector (Figure 1A). A vector encoding an IL10-derived RNAi oligonucleotide was used as a control.

To produce cell lines with constitutively reduced levels of Sam68 expression, HeLa cells were transfected with pSam68-RNAi or pIL10-RNAi and placed under G418 selection. Three independent SSKH cell lines (SSKH-I, SSKH-II and SSKH-III) and one IL10 (HeLa-IL10i) were generated. The expression of endogenous Sam68 in these cell lines was reduced to 10–30% of the level in HeLa-IL10i cells as assessed by immunoblot analysis (Figure 1B). The successful establishment of SSKH cell lines and the viability of these cells (over 90%) demonstrates that the reduced levels of Sam68 were not toxic.

Effect of stable Sam68 knockdown on Rev/RRE-mediated reporter gene expression

To determine how the reduced levels of Sam68 in SSKH cells affected the function of the Rev/RRE system, the SSKH and HeLa-IL10i (control) cell lines were transfected with pCMV128 alone and with pRev. The amount of CAT activity in the cell lysates was determined as described in Materials and Methods. In control HeLa-IL10i cells, transfection of pCMV128 alone resulted in very little CAT activity (Figure 2A, lane 1). However, co-transfection of pCMV128 and pRev increased the CAT activity 32-fold over basal levels (Figure 2A, lane 2). In the SSKH cell lines, transfection of pCMV128 alone produced CAT levels comparable with that seen in pCMV128-transfected control cells (Figure 2A, compare lanes 3, 5 and 7 with lane 1). However, co-transfection of pRev increased the CAT expression only 2- to 3-fold (Figure 2A, lanes 4, 6 and 8). To determine whether the knockdown of Sam68 expression inhibited cytomegalovirus (CMV) promoter-mediated CAT expression independent of RRE, the HeLa-IL10i and SSKH cell lines were transfected with pCMV-CAT. As shown in Figure 2B, the expression of CAT in SSKH cells transfected with this vector did not differ significantly from the expression in control cells. These results further confirm that Sam68 is required for Rev/RRE-mediated CAT activity.

Effect of Sam68 on RRE-mediated transactivation is not RRE-dependent reporter used

To verify that the requirement of Sam68 for Rev function was not dependent on choice of reporter construct, we investigated the effect of knockdown of Sam68 on Rev/RRE-mediated *gag* gene expression. For these studies, HeLa-IL10i, SSKH-I and SSKH III cells were co-transfected with pNLRRE-*gag* (24) alone, or together with pRev. At 48 h post-transfection, the concentration of p24 antigen in cell-free supernatants was measured. In HeLa-IL10i cells transfected with pNLRRE-gag alone produced very basal levels of p24 antigen. Co-transfection of pRev with pNLRRE-gag into HeLa-IL10i cells yielded high levels of p24 antigen, while SSKH

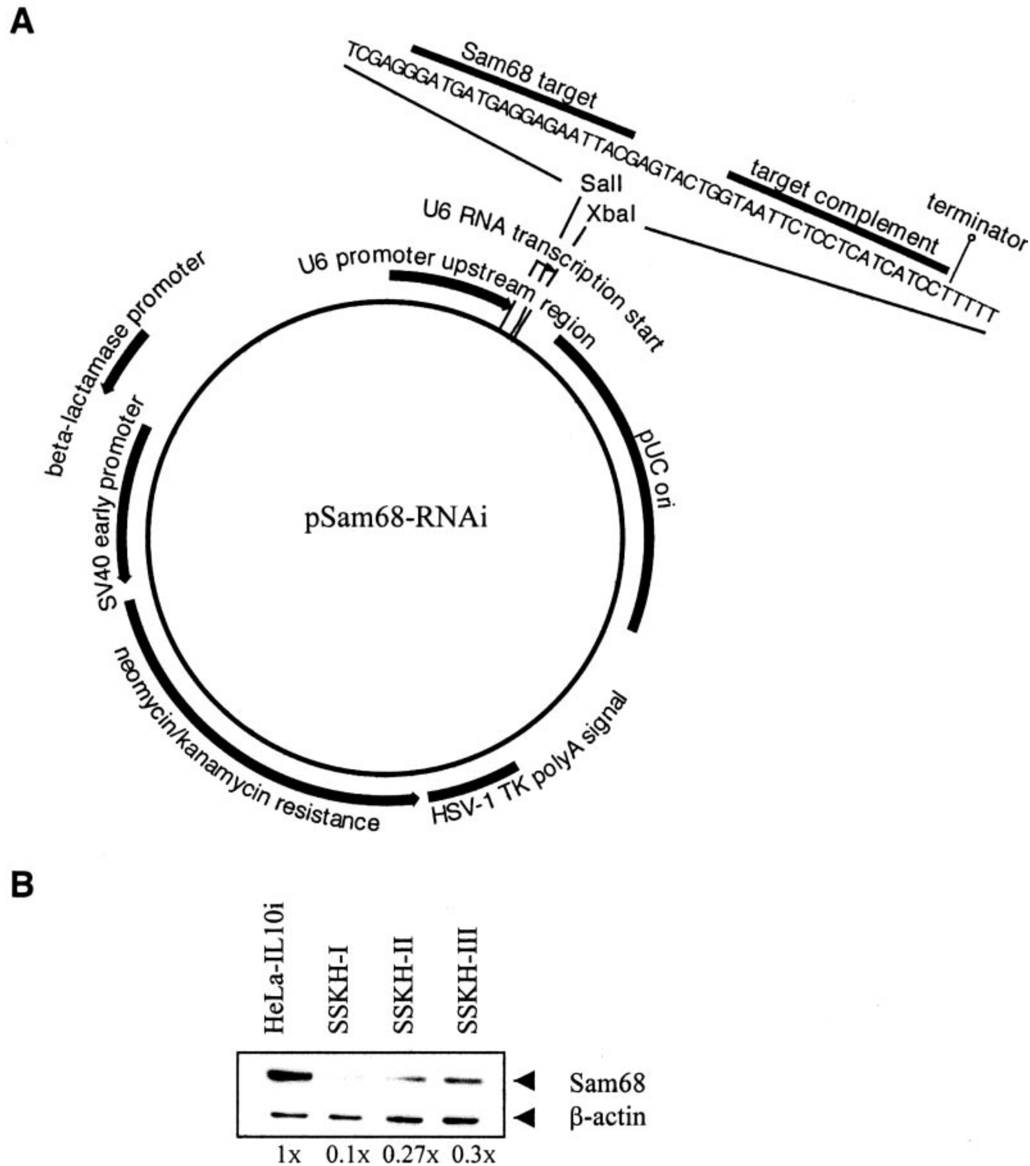

Figure 1. Knockdown of Sam68 expression by RNAi strategy. (**A**) Schematic diagram of Sam68-RNAi expression vector. The Sam68-RNAi plasmid was constructed as described in Materials and Methods. (**B**) Expression levels of Sam68 in stable clones were assessed by western analysis using anti-Sam68 antibodies. Control cells: HeLa-IL10i; Stable Sam68 knockdown HeLa clones (SSKH-I, SSKH-II and SSKH-III). Relative expression level of Sam68 was estimated by densitometric scanning of the blots using β-actin as an internal control.

supernatants had very low levels (Figure 2C, compare lane 2 with lanes 3 and 4). In contrast, reduced Sam68 expression did not affect the CAT gene expression driven by HIV-1 LTR in the presence of Tat (Figure 2D). HIV-1 LTR CAT was used for two reasons, i.e. to serve as an internal control for transfection efficiency as well as for a Rev/RRE-independent gene expression. Thus, these results indicate that the effect of Sam68 on RRE-mediated reporter gene expression is not dependent on the construct used.

Sam68 knockdown cells are defective for Rev-mediated RNA export

To investigate whether the inhibition of Rev transactivation in SSKH cells was due to a failure to export unspliced RRE-containing CAT mRNA to the cytoplasm, HeLa-IL10i and SSKH-1 clones were transfected with pCMV128 alone or together with pRev. At 48 h post-transfection, nuclear and cytoplasmic RNA was isolated and subjected to northern blot analysis with a CAT probe. In HeLa-IL10i cells transfected with pCMV128 alone, the RRE-containing CAT mRNA was found in the nucleus but not in the cytoplasm (Figure 3A, lanes 1 and 4). When pCMV128 and pRev were co-transfected into these cells, CAT mRNA was readily detectable in the cytoplasm and the amount in the nucleus was reduced compared with CMV128 without Rev (Figure 3A, lanes 2 and 5). However, in SSKH cells transfected with pCMV128 and pRev, CAT mRNA was detected only in the nucleus, but was barely detectable in the cytoplasm (Figure 3A, lanes 3 and 6). In the nuclear fraction, besides unspliced RRE-CAT RNA,

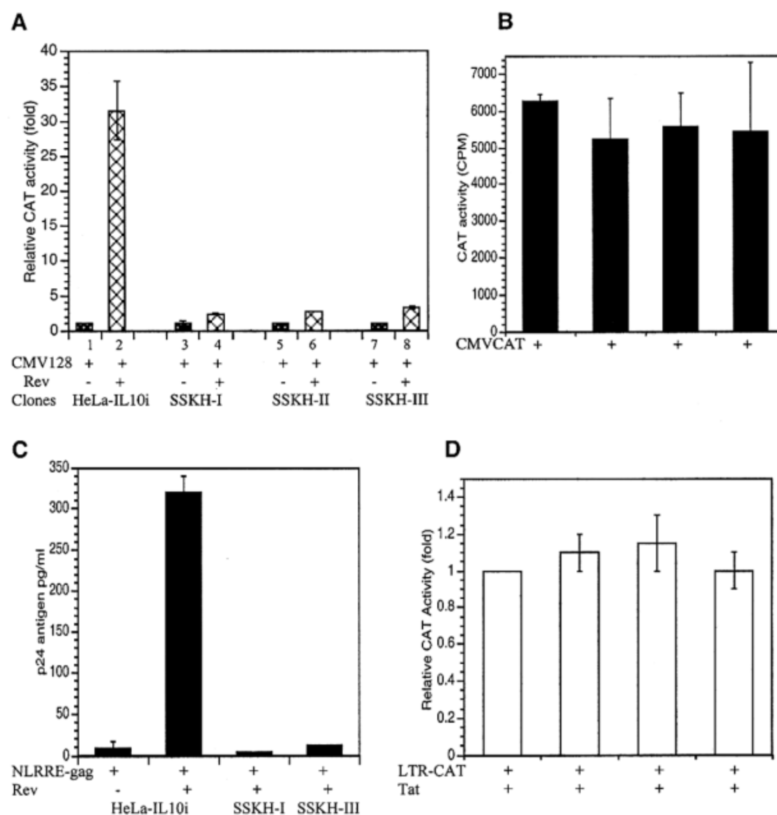

Figure 2. Effect of stable Sam68 knockdown on Rev/RRE function. (**A**) Rev/RRE-mediated CAT gene expression. Cells were co-transfected with pCMV128 (62.5 ng) alone, and with pRev (10 ng). CAT assays were performed as described in Materials and Methods. (**B**) CMV-CAT gene expression. CAT assays were performed on the cells transfected with pCMV-CAT (100 ng) as described above. (**C**) Rev/RRE-mediated *gag* gene expression. Cells were co-transfected with pNLRRE-*gag* (250 ng) alone, or together with pRev (12 ng). For the transfection efficiency, pHIV-1 LTR-CAT (250 ng) with pTat (50 ng) were also added to the transfection mixture. Cell-free supernatants were collected and subjected to p24 antigen capture assay as described in Materials and Methods. (**D**) Cells in (C) were harvested; extracts were made and subjected to CAT activity. The p24 antigen was normalized to the CAT values.

an additional band was also observed, and the nature of this band is not known. A possible explanation is that this band may be either a degradation product of unspliced RNA or spliced RNA trapped in the nuclear fraction. Taken together, these results indicate that Rev failed to mediate export of the RRE-containing CAT mRNA in Sam68 knockdown cells, confirming that Sam68 is required for Rev-mediated nuclear RNA export.

To ascertain that the inhibition of Rev activity in SSKH cells was not due to the down regulation of Rev expression, HeLa-IL10i and SSKH-I cells were transfected with pRev. At 48 h post-transfection, the cells were harvested and an immunoblot for the expression of Rev protein was performed on cell lysates. An extract of normal HeLa cells was used as a control. As shown in Figure 3B, comparable amounts of Rev were detected in HeLa-IL10i and SSKH-I cells, indicating that the lack of Rev activity in SSKH cells was not due to a failure to produce Rev. These results indicate that the Rev function is severely inhibited in Sam68 knockdown cells due to a reduction in the Sam68 expression.

Sam68 knockdown cells do not support HIV production

To determine whether Sam68 knockdown cells could support HIV-1 production, we transfected SSKH-I, SSKH-III and HeLa-IL10i control cells with HxB-2 proviral DNA. Cell-free supernatants were collected at the times indicated and subjected to p24 antigen assay. At 48 and 60 h post-transfection, as shown in Figure 4A, control cells produced high levels of p24 antigen (400–500 pg). In contrast, p24 production was severely reduced in the SSKH-I clone at all times tested (20–45 pg). These studies indicate that Sam68 is an essential factor required for HIV-1 production.

Next, to correlate whether the inhibition of HIV production in Sam68 knockdown cells is due to the failure of export of unspliced viral RNAs to the cytoplasm, we transfected HxB-2 proviral DNA into SSKH-I or HeLa-IL10i cells. At 48 h post-transfection, the cells were harvested for RNA analysis and cell-free supernatants were collected for p24 antigen. Consistent with the results shown in Figure 4A, HIV-1 did not replicate in SSKH cells (data not shown). For RNA analysis, the cells were divided into two equal portions. Total RNA was isolated from one portion, and cytoplasmic RNA from the other. The RNAs were subjected to northern blot analysis using a ^{32}P-labeled LTR untranslated region probe. In HeLa-IL10i cells transfected with HxB-2, both spliced and unspliced viral mRNAs were readily detectable in total RNA as well as in the cytoplasmic RNA (Figure 4B, lanes 1 and 2). However, in SSKH cells transfected with HxB-2, spliced and unspliced viral mRNAs were transcribed normally (Figure 4B, lane 3), but unspliced RNAs (US) and singly spliced (SS) RNAs failed to be exported to the cytoplasm

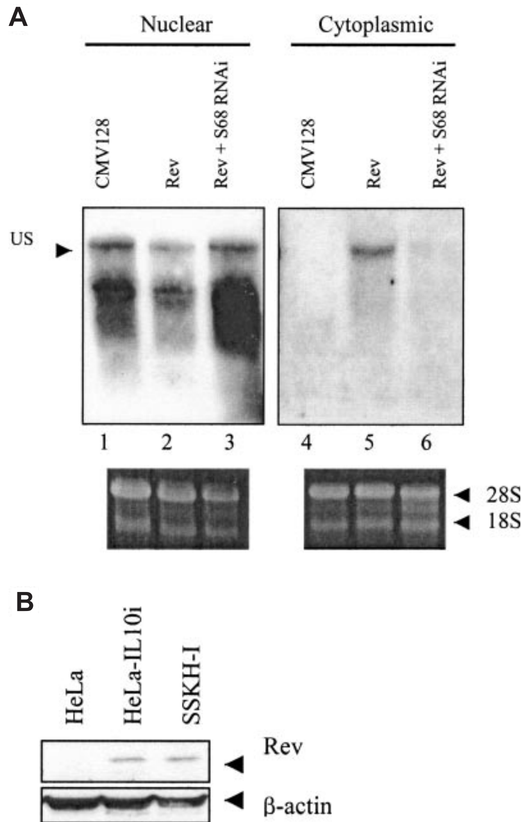

Figure 3. SSKH cells are defective for Rev-mediated nuclear RNA export. (**A**) RRE-containing CAT RNA export. SSKH-I and HeLa-IL10i cells were co-transfected with pCMV128 (250 ng) and pRev (50 ng). Nuclear and cytoplasmic RNA was isolated and subjected to northern blot analysis. Ribosomal RNAs (28S and 18S) served as RNA loading controls. US, unspliced RRE-CAT RNA. (**B**) Rev expression in SSKH clones: cells were transfected with pRev (200 ng), cell extracts were prepared and subjected to western analysis using anti-Rev antibodies. HeLa cells (lane 1), HeLa-IL10i and SSKH-I with Rev (lanes 2 and 3, respectively).

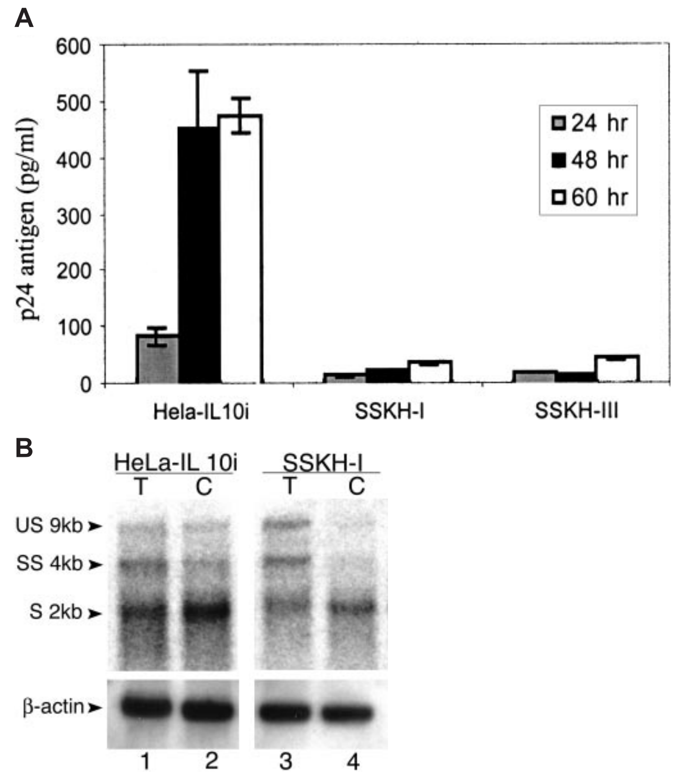

Figure 4. SSKH cells do not support HIV production. (**A**) SSKH-I, SSKH-III or HeLa-IL10i cells were transfected with HxB2 proviral DNA (25 ng). Cell-free supernatant was collected at indicated times and measured p24 antigen. (**B**) Viral RNA export. SSKH-I or HeLa-IL10i cells were transfected with HxB2 proviral DNA (250 ng). At 48 h post-transfection, the cells were harvested and divided into two portions. Total RNA was isolated from one portion, and the cytoplasmic RNA from the remainder. RNAs were subjected to northern blot analysis with a ^{32}P-labeled LTR untranslated region probe. Endogenous β-actin served as RNA loading controls. T, total RNA (lanes 1 and 3); C, cytoplasmic RNA (lanes 2 and 4); US, unspliced 9 kb; SS, singly spliced 4 kb; and S, spliced 2 kb HIV-1 mRNAs.

(Figure 4B, lane 4), indicating that Sam68 is required for HIV-1 production.

DISCUSSION

RRE is a *cis*-acting regulatory element of HIV-1, which acts to retain unspliced and singly spliced HIV-1 RNAs in the nucleus. The HIV-1 Rev protein binds to RRE and facilitates the export of mRNA containing the RRE from the nucleus to cytoplasm. The onset of the Late phase of HIV-1 replication occurs when the levels of the HIV-1 Rev protein (an Early gene product) accumulate to levels high enough to permit export of the unspliced and singly spliced Late mRNAs to the cytoplasm (27,28). Rev-mediated transactivation of Late-gene expression requires binding of Rev to RRE, but the details of Rev function and its interactions with cellular RNA processing and export machinery are unclear. However, we showed previously that Sam68 binds to both Rev and RRE (14). When overexpressed in the presence of Rev, Sam68 synergizes with Rev to substantially increase export of RRE-containing RNAs from the nucleus (14,20). Overexpression of Sam68 in the absence of Rev also facilitates the nuclear export of RRE-containing mRNAs (20). While these studies suggested an important role for Sam68 in Rev function, it was unclear whether Sam68 was actually required for export of RRE-containing RNAs. To address this, we produced cell lines in which Sam68 was depleted by the expression of Sam68-specific interfering RNA (Figure 1).

The studies presented here show that knockdown of Sam68 by RNAi dramatically reduced the expression of two Rev-dependent reporter constructs (Figure 2A and C). Most significantly, we found that the reduction of Sam68 expression led to a dramatic inhibition of HIV-1 production (Figure 4A). This inhibition correlated with the failure to export RRE-containing CAT RNA and viral mRNAs to the cytoplasm in the Sam68 depleted cells (Figures 3 and 4B). Therefore, we conclude that Sam68 is essential for Rev function and HIV-1 production.

These observations are in agreement with earlier studies that employed less specific methods of reducing Sam68 activity. By expressing essentially full-length Sam68 antisense RNA, Li *et al.* (29) obtained 80–86% reductions in HIV-1 replication. However, whether this effect was entirely due to antisense inhibition of Sam68 expression is uncertain. Other KH

proteins have varying degrees of sequence similarity to Sam68 and their expression might be affected by antisense Sam68. As we have shown elsewhere (20), some of these KH proteins can enhance Rev/RRE-mediated gene expression. Therefore, it was uncertain whether the inhibitory effect of Sam68 antisense RNA was due to an effect on Sam68 alone. Conversely, residual HIV-1 replication in the presence of Sam68 antisense RNA might be due to the ability of these KH proteins to complement Sam68. Also, full-length antisense RNA may have broad and complicating effects beyond the selective silencing of gene of interest when introduced into cells. For example, antisense expression vectors were reported to modulate the effects of interferon-gamma (30), which in turn may affect the functions of cellular machinery. In contrast, the effects of Sam68-RNAi are expected to be far more specific due to its smaller size in comparison with Sam68 antisense RNA (19 bases versus 1.3 kb). Furthermore, Sam68 RNAi lacks required homology with KH mRNA, and thus it is unlikely that it targets the expression of KH proteins. Also, the KH proteins do not bind RRE RNA (20), and therefore these proteins fail to complement for Sam68 knockdown in RRE-mediated gene expression directly. Thus, Sam68-RNAi proved to be more potent than antisense RNA, inhibiting HIV-1 post-transcriptional gene expression by 98–99%.

Recently, Coyle *et al*. (23) reported that Sam68 had only a modest effect on Rev/RRE-mediated gene expression in a system that employed an RRE-*gag* reporter. It was suggested that more powerful effects of Sam68 on Rev/RRE that we and others had observed previously (14–17) were due to leakiness in the RRE-CAT reporter system. However, our current study, which employed two reporter systems (RRE-CAT and RRE-*gag*), reaffirms and extends our earlier results. Moreover, in support of our studies, Soros *et al*. (17) reported that besides Sam68, SLM1 and SLM2 were also found to stimulate Rev activity using three different reporter constructs (i.e. CAT-, gp120- and Gag-based reporter assays). The reason for the discrepancy between the report by Coyle *et al*. (23) and these other studies is unclear.

Sam68 is a nuclear protein and does not seem to shuttle between the nucleus and the cytoplasm (17). This raises the question of how a protein localized in the nucleus can affect RRE-mediated RNA export. A yeast nuclear protein, Yrb2p, exemplifies a possible mechanism (31). This protein, which also does not shuttle, mediates protein export from the nucleus through a nuclear export sequence receptor, Xpo1p (31). In the case of HIV, Sam68 interacts with Rev (14,29), and it is conceivable that Sam68 may associate with RNA export components (CRM1, Nup98 and Nup214) in the nucleus, either directly or indirectly, and promote the intra-nuclear transport of viral RNA cargo to the nuclear pore complex and be removed from the complex before export. In light of these findings, it would be important to determine which of the nuclear pore proteins associate with Sam68 and play a role in the nuclear export of viral mRNAs.

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