Eukaryotic elongation factor 1 complex subunits are critical HIV-1 reverse transcription cofactors

Kylie Warren[a,b,1], Ting Wei[a,1], Dongsheng Li[a], Fangyun Qin[c], David Warrilow[d], Min-Hsuan Lin[a,e], Haran Sivakumaran[a], Ann Apolloni[a], Catherine M. Abbott[f], Alun Jones[g], Jenny L. Anderson[h,i], and David Harrich[a,2]

[a]Department of Cell and Molecular Biology, Queensland Institute of Medical Research, Herston, Queensland, 4029, Australia; [b]School of Natural Sciences, University of Western Sydney, Hawkesbury, New South Wales, 2751, Australia; [c]Guangxi Animal Centers for Disease Control, Nanning, Guangxi 530001, People's Republic of China; [d]Public Health Virology Laboratory, Queensland Health Forensic and Scientific Services, Archerfield, Queensland, 4108, Australia; [e]School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia, Queensland, 4072, Australia; [f]Medical Genetics Section, Molecular Medicine Centre, Institute of Genetics and Molecular Medicine, Western General Hospital, University of Edinburgh, Edinburgh EH4 2XU, United Kingdom; [g]Institute for Molecular Bioscience, University of Queensland, St. Lucia, Brisbane, Queensland, 4072, Australia; [h]Centre for Virology, Burnet Institute, Melbourne, Victoria, 3004, Australia; and [i]Department of Medicine, Monash University, Melbourne, Victoria, 3004, Australia

Edited by Stephen P. Goff, Columbia University College of Physicians and Surgeons, New York, NY, and approved April 18, 2012 (received for review March 23, 2012)

Cellular proteins have been implicated as important for HIV-1 reverse transcription, but whether any are reverse transcription complex (RTC) cofactors or affect reverse transcription indirectly is unclear. Here we used protein fractionation combined with an endogenous reverse transcription assay to identify cellular proteins that stimulated late steps of reverse transcription in vitro. We identified 25 cellular proteins in an active protein fraction, and here we show that the eEF1A and eEF1G subunits of eukaryotic elongation factor 1 (eEF1) are important components of the HIV-1 RTC. eEF1A and eEF1G were identified in fractionated human T-cell lysates as reverse transcription cofactors, as their removal ablated the ability of active protein fractions to stimulate late reverse transcription in vitro. We observed that the p51 subunit of reverse transcriptase and integrase, two subunits of the RTC, coimmunoprecipitated with eEF1A and eEF1G. Moreover eEF1A and eEF1G associated with purified RTCs and colocalized with reverse transcriptase following infection of cells. Reverse transcription in cells was sharply down-regulated when eEF1A or eEF1G levels were reduced by siRNA treatment as a result of reduced levels of RTCs in treated cells. The combined evidence indicates that these eEF1 subunits are critical RTC stability cofactors required for efficient completion of reverse transcription. The identification of eEF1 subunits as unique RTC components provides a basis for further investigations of reverse transcription and trafficking of the RTC to the nucleus.

purification | mass spectrometry | cellular factor | siRNA knock down | virus replication

The HIV type-1 (HIV-1) reverse transcriptase (RT) enzyme carries out reverse transcription through DNA polymerase and RNase H activities, which mediate a sequential series of steps that include the initiation of DNA synthesis producing negative strand strong stop DNA (−ssDNA), full-length negative-strand DNA, positive-strand DNA, and intact preintegrative double-strand DNA (reviewed elsewhere 1). The ability of HIV-1 to efficiently produce early reverse transcription products in vitro has been described (2). However, under in vitro conditions, the production of late reverse transcription products is less efficient than that observed in HIV-1–infected cells (3, 4), suggesting that host cellular factors are required. After entry into the host cell cytoplasm, the HIV-1 core, containing the viral genomic RNA, RT, and integrase (IN), reorganizes to form the reverse transcription complex (RTC). The RTC requires both IN and RT to be active (5), and they are believed to recruit cellular factors to facilitate DNA synthesis (6). Although two-hybrid library and genome-wide RNAi screening methods have implicated more than 50 cellular proteins as important for reverse transcription (6, 7), whether any of these cellular proteins are RTC components, or whether they direct the RTC in other ways, is unclear.

Attempts to purify and to identify cellular RTC cofactors by more conventional methods have only partly succeeded (4, 8). Narayan et al. showed that in vitro fusion of avian sarcoma and leukosis virus endosomal particles required the addition of cellular lysates for formation of productive RTCs, which greatly increased early and late products of reverse transcription (8). They reported that the reverse transcription enhancing activity was >5 kDa in mass and required ATP hydrolysis. Warrilow et al. showed that cell lysate made from human and murine cell lines contained an activity that did not affect synthesis of -ssDNA but did improve late DNA synthesis by up to 30-fold (4, 9). The stimulatory activity, isolated by size-exclusion chromatography, purified with an apparent molecular mass of >20 MDa, suggesting that it was associated with a complex of molecules such as aggregated protein, DNA, or RNA. This study aimed to identify cellular factors that stimulate reverse transcription by chromatographic methods.

Here we show that subunits of the eukaryotic elongation factor 1 (eEF1) complex are required to stimulate late stages of HIV-1 reverse transcription in vitro, as depletion of eEF1 subunits eEF1A and eEF1G ablated the ability of active protein fractions to stimulate late steps of HIV-1 reverse transcription. These eEF1 subunits coimmunoprecipitate RTC proteins in vitro, and following cell infection cosediment with RTCs by isopycnography and colocalize with RT as shown by proximity ligation assay. We also observed significant down-regulation of reverse transcription efficiency when eEF1A and eEF1G protein levels were reduced by siRNA treatment that was attributed to reduced levels of RTC in infected cells. Further investigation of the eEF1 and RTC interaction has implications with respect to trafficking and nuclear import of HIV reverse transcription/preintegration complexes (PICs).

Results

Purification and Identification of Cellular Proteins in Fractions That Stimulate Reverse Transcription in Vitro. We previously confirmed an activity in a partially purified cytoplasmic lysate derived from a human T cell line (Jurkat), named S100, that increased the

Author contributions: K.W., T.W., D.L., D.W., and D.H. designed research; K.W., T.W., D.L., F.Q., M.-H.L., and A.J. performed research; H.S., A.A., C.M.A., J.L.A., and D.H. contributed new reagents/analytic tools; K.W., T.W., D.L., D.W., M.-H.L., H.S., A.A., C.M.A., J.L.A., and D.H. analyzed data; and K.W., T.W., D.L., D.W., M.-H.L., H.S., A.A., C.M.A., J.L.A., and D.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]K.W. and T.W. contributed equally to this work.

[2]To whom correspondences should be addressed. E-mail: davidH@qimr.edu.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1204673109/-/DCSupplemental.

ability of HIV-1 to generate late endogenous reverse transcription (ERT) products in vitro (4, 9) in a concentration-dependent manner (Fig. S1*A*). We defined reverse transcription efficiency as the ability to synthesize late HIV-1 DNA relative to early DNA product expressed as a percentage. Starting with S100, the activity was fractionated through a CL-4B Sepharose column, where the stimulatory activity, referred to as the "peak of gel filtration" (PGF), was detected only in fractions with a high molecular mass (9). Next, 1 mg PGF protein was applied to a DEAE anion-exchange column that resulted in isolation of reverse transcription stimulatory activity in very few fractions (Fig. 1), and will be hereafter referred to as "DEAE active fraction" (DAF). SDS/PAGE analysis of DAF revealed well-defined, Coomassie-stained protein bands (Fig. S1*B*) that were excised, digested with trypsin, and subjected to liquid chromatography–mass spectrometry (LC-MS/MS) analysis. The protein purification and MS-identification process was repeated four times, and 25 host proteins were identified in at least three of the four experiments (Table S1).

The identified proteins fall into six broad function classes: translation factors, transcription regulators, synthetases, mRNA splicing/transport factors, folding/transport proteins, and cellular organization cytoskeleton proteins. Of particular interest was the identification of translation factors, which are essential not only for translation but for a range of functions throughout eukaryotic cells. Two subunits of the eEF1 complex were detected, eEF1A and eEF1G, in addition to eukaryotic translation elongation factor 2 (eEF2) and the α-subunit of the eukaryotic translation initiation factor 3 (eIF3A). The nonribosomal eEF1 protein complex composed of eEF1A, eEF1G, eEF1B2 (also called, eEF1Bα), eEF1D (also called eEF1δ or eEF1Bβ), and valyl-tRNA synthetase is required for eukaryotic translation (10) (Fig. S2*A*). However, eEF1A has many noncanonical roles in the cell (11) and in virus replication (12–15). In addition, eEF1A was reported to interact with HIV-1 Gag (16) and the cytoskeletal networks (17) that are thought to play crucial role in the completion of HIV-1 reverse transcription (18). Western blot analysis performed using DAF confirmed that eEF1D and eEF1B2 were also present (Fig. S2*B*). Moreover, the immunodepletion of eEF1G codepleted other eEF1 complex protein members (Fig. S2*C*). This indicated that eEF1A, eEF1B2, eEF1D, and eEF1G exist as a complex in DAF.

DAF Reverse Transcription Stimulatory Activity Is Down-Regulated by Immunodepletion of eEF1A and eEF1G. Larger-scale DAF purification was undertaken to obtain larger quantities of protein. Western blot and ERT analysis of DEAE fractions indicated that a peak level of eEF1A and eEF1G in fractions was coincident to a peak of ERT stimulatory activity, although they were present at reduced levels in subsequent fraction (Fig. S3). To determine whether eEF1A or eEF1G were required for stimulatory activity, DAF was incubated with agarose beads covalently coupled to anti–(α)-eEF1A, α-eEF1G, or α-eIF3A antibodies to reduce their concentration in the unbound fraction. DAF was incubated with BSA protein–coupled beads as a control. Western blot analysis of treated DAF showed that the levels of the respective proteins were reduced by ~90%, 70%, and 95%, respectively (Fig. 2*A*). The activity of the depleted DAFs was tested using in vitro ERT reactions in parallel with control reactions containing intact DAF or no added protein (i.e., buffer only). Fig. 2*B* shows that depletion of eEF1A and eEF1G, but not eIF3A, resulted in sharply reduced reverse transcription efficiency in ERT. In all experiments (n = 4), a complete loss of reverse transcription stimulatory activity was observed when eEF1G levels were reduced by 70–90%, whereas it was reduced by three- to fourfold when eEF1A was depleted by 90–95% (Fig. 2*B*). eEF2 was refractory to immunodepletion with the available antibodies. An increase in reverse transcription efficiency observed in these reactions compared with Fig. 1 was consistent with increased quantity of cellular cofactors used in the reactions that increased reverse transcription efficiency in a titratable manner (Fig. S1*A*). Of note, we previously reported separation of ERT-stimulatory activities into S100 (soluble) and P100 (pellet) fractions that had additive effects, most likely because eEF1A and eEF1G were in both fractions (4) (Fig. S4). We conclude that eEF1A and eEF1G translation factors found in DAF, perhaps as part of an active eEF1 complex (Fig. S2), were required to stimulate late HIV-1 reverse transcription in vitro.

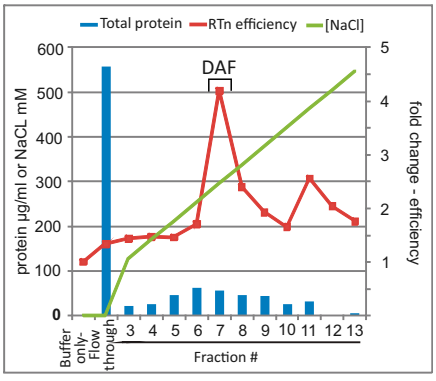

Fig. 1. Purification of cellular factors that enhance late HIV-1 reverse transcription in vitro. PGF was further fractionated using a 1-mL DEAE column that resolved a fraction called DAF that was identified using an ERT assay. A representative purification and mass spectrometry analysis of four experiments is shown (Fig. S1*B* and Table S1).

Both eEF1A and eEF1G Coimmunoprecipitate RT and IN. The observation that eEF1A or eEF1G were required to stimulate reverse transcription suggested they interacted with the HIV-1 RTC. To address this possibility, large-scale ERT reactions containing DAF were immunoprecipitated using α-eEF1A– or α-eEF1G–coated beads. SDS/PAGE and Western blot analysis of the coprecipitated protein using an α–HIV-1 polyclonal antibody revealed two HIV-1 proteins with apparent masses of 51 and 31 kDa (Fig. 3*A, Left*), most likely the p51 subunit of RT and IN, respectively. HIV-1 proteins were not specifically coimmunoprecipitated by α-eIF3A–coated beads (Fig. 3*A, Right*). Whereas the RT p51 subunit was consistently coimmunoprecipitated with α-eEF1G and α-eEF1A antibodies, IN was detected inconsistently, possibly because the avidity or titer of the α-IN antibody component in the anti–HIV-1 polyclonal antibody was low. The coimmunoprecipitation experiments and Western blots were repeated using high-avidity α–HIV-1-RT and α–HIV-1-IN monoclonal antibodies separately. As shown, HIV-1 RT and IN were consistently coimmunoprecipitated with eEF1A or eEF1G, verifying that these cellular proteins associated with enzymatic components of the RTC (Fig. 3*B*), and supporting the hypothesis that a stimulatory activity in DAF was mediated by subunits of the eEF1 complex through an interaction with the viral RTC.

eEF1A and eEF1G Subunits of eEF1 Associate with RTCs in Infected Cells. To test the hypothesis that the eEF1 complex associated with RTC in cells, HEK293T cells were infected with HIV-$1_{pNL4\text{-}3.Luc.R\text{-}E\text{-}}$ pseudotyped with VSV-G or with heat-inactivated virus stock. HIV-1 was incubated with HEK293T target cells at 4 °C for 2 h to allow virus attachment to cells, then at 37 °C for a 4-h infection. Cell lysates were made from the infected and control cells and were fractionated by isopycnography. Fractions collected from the bottom of the gradient were assayed for viral DNA by quantitative PCR and RT, eEF1A and eEF1G by Western blot. The analysis shows the bottom eight fractions that include the sucrose densities of 1.32–

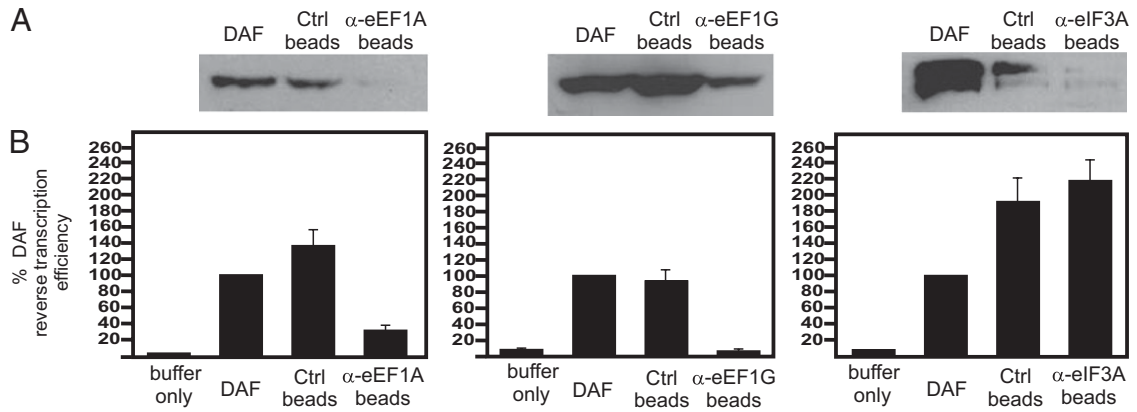

Fig. 2. Immunodepletion of eEF1A and eEF1G from active fractions decreases reverse transcription efficiency in vitro. DEAE active fraction (DAF) was incubated with antibody-coupled (α-eEF1A, α-eEF1G, or α-eIF3A) or BSA control beads. Immunodepletion of target proteins from the unbound fraction was confirmed by Western blot with antibodies specific to target protein. The ability of unbound fractions to stimulate reverse transcription was tested by a large-scale ERT assay using 500 μL DAF. The relative reverse transcription efficiency of treated DAF compared with untreated DAF is shown as a percentage. Experiments were repeated at least four times. Mean values, with the SD of the mean, are shown.

1.34 g/mL reported to contain viral RTCs (19) (Fig. 4, dotted box) that corresponded to fractions 3 and 4. Viral DNA was observed with a peak level in fraction 3 (Fig. 4*A*) in agreement with previous studies (19), whereas very little viral DNA was detected in fractions from the control infection. As previously described (19), fraction 3 also contained a significant amount of RT indicative of RTCs (Fig. 4*B* and Fig. S5*A*). The same Western blot was stripped and probed with a mouse α-eEF1G monoclonal antibody. The process was repeated using a rabbit α-eEF1A polyclonal antibody. As shown in Fig. 4 and Fig. S5, the level of eEF1G (Fig. 4*C* and Fig. S5*B*) and eEF1A (Fig. 4*D* and Fig. S5*C*) was dramatically higher in fractions 3 and 4 compared with the same fractions in the control experiment with the heat-inactivated virus. This outcome supports the hypothesis that the eEF1 complex specifically associates with viral RTC in cells, most likely by interacting with the RT p51 subunit and IN (Fig. 3).

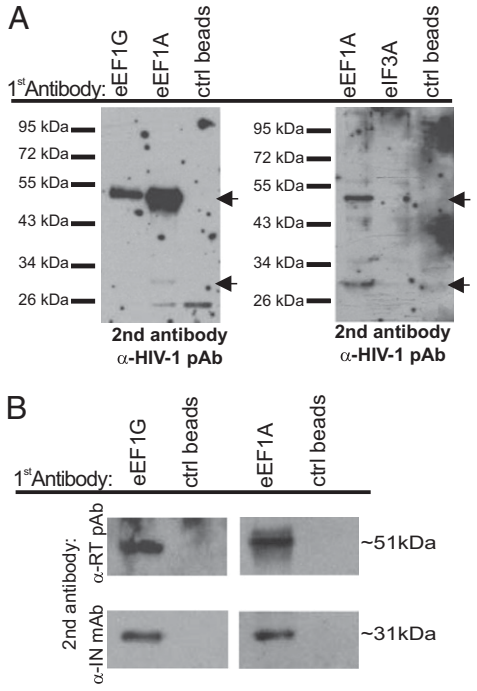

Fig. 3. Coimmunoprecipitation of RT and IN by eEF1 subunits. (*A*) Large-scale (×10) ERT reactions were prepared, and incubated with antibody-coupled (α-eEF1G, α-eEF1A, or α-eIF3A) or BSA control beads. Immunoprecipitated proteins were separated by SDS/PAGE and analyzed by Western blot using α-HIV polyclonal antibody. (*B*) The identity of HIV proteins immunoprecipitated with α-eEF1G and α-eEF1A antibodies was confirmed by Western blot analysis with antibodies specific for HIV-1 reverse transcriptase (RT) and integrase (IN). All data are representative of at least three independent experiments.

To further test the hypothesis that eEF1 complex subunits associated with viral RTCs in infected cells, HeLa cells were infected with VSV-G pseudotyped HIV-$1_{pNL4\text{-}3.Luc.R\text{-}E\text{-}}$. Duolink proximity ligation assays (PLA) (Fig. S6 *A–G*), a modified fluorescent in situ hybridization method, were performed to determine whether HIV-1 RT was closely associated with either translation elongation factor in infected cells. A mouse monoclonal antibody to RT was used in conjunction with rabbit antibodies to eEF1A, eEF1G, or eIF3A individually to stain uninfected or HIV-1–infected cells, and was subsequently processed with oligonucleotide-conjugated detection antibodies, a hybridization linker, and "red" oligonucleotide detector probe. Maximum intensity projections of deconvolved images clearly showed that RT was proximal to eEF1A and eEF1G, but not eIF3A, in infected cells (Fig. 5 and Fig. S6*F*). We noted in pairwise PLA assays performed with infected HeLa cells that the number of red foci generated using α-RT antibody with α-eEF1A or α-eEF1G antibody were very similar (Fig. 5 and Fig. S6*F*). Importantly, very low numbers of foci were detected using the same antibodies in uninfected cells (Fig. 5 and Fig. S6*G*). Given a similar cellular distribution and high abundance of the translation factors generally (Fig. S6*H*), it is extremely likely that proximity signals are highly significant (Fig. 5*;* P = 7.2E-23 and 6.5E-28), favoring the possibility that the eEF1 complex, rather than individual eEF1 subunits, interacted with RT. However, we cannot exclude the possibility that RT or the RTC could bind to eEF1A and to eEF1G individually.

siRNA Down-Regulation of eEF1A and eEF1G in Cells Negatively Affects HIV-1 Reverse Transcription. Finally, cells were independently treated with siRNAs targeting eEF1A or eEF1G, or a control siRNA. In two separate experiments, eEF1A and eEF1G levels were down-regulated by >90% compared with the control (Fig. 6*A*), without affecting cell viability as determined by MTS assays (Fig. S7). Treated or control HEK293T cells were infected with VSV-G pseudotyped HIV-$1_{pNL4\text{-}3.Luc.R\text{-}E\text{-}}$ or heat-inactivated virus at 4 °C for 2 h and then at 37 °C for 4 h, after which

MICROBIOLOGY

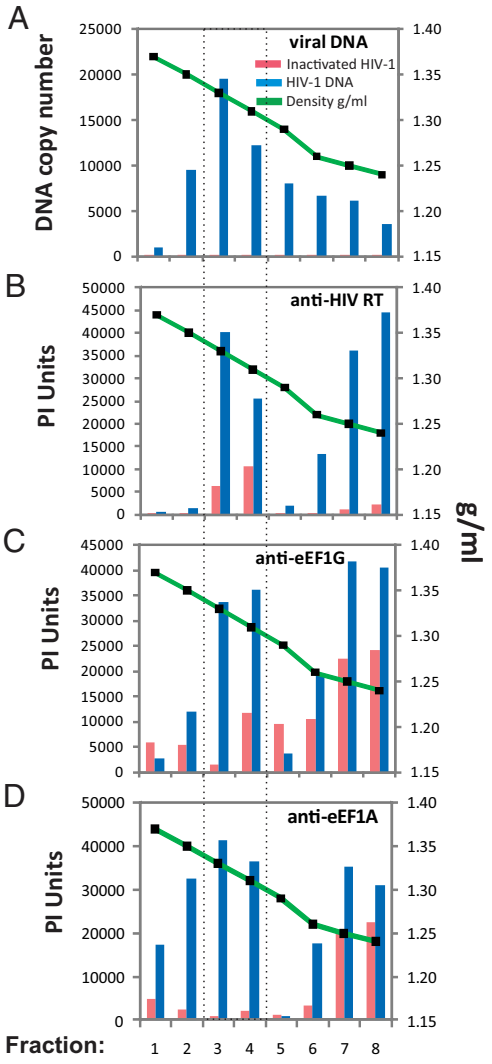

Fig. 4. HIV-1 RTCs from infected cells cosediment with eEF1A and eEF1G. HEK293T cell were infected with VSV-G pseudotyped HIV-$1_{pNL4\text{-}3.Luc.R\text{-}E\text{-}}$ or inactivated HIV-1. Cell lysates were prepared by Dounce homogenization, clarified by centrifugation, and subjected to isopycnography using a sucrose gradient. (*A*) Fractionated gradients were analyzed by quantitative PCR for viral DNA and (*B*) by Western blot for HIV-1 RT. (*C*) Sequential western analysis of the same blot was performed using a mouse monoclonal antibody to eEF1G, (*D*) followed by a rabbit polyclonal antibody to eEF1A. Digital exposures of Western blots (Fig. S5) were analyzed using ImageQuant 5.1. The experiment was repeated three times, and a representative result is shown. The results are expressed as phosphorimager (PI) units.

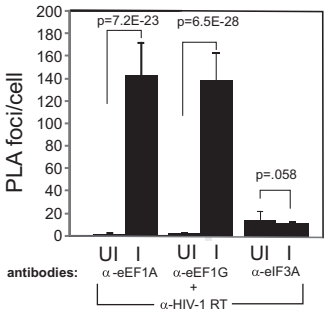

Fig. 5. HIV-1 RT is associated with eEF1A and eEF1G in infected cells. Duolink proximity ligation assays were performed (Fig. S6) using a mouse monoclonal antibody to RT in conjunction with rabbit antibodies to eEF1A, eEF1G, or eIF3A individually with HIV-1 infected (I) or uninfected cells (UI) as indicated (experimental details in Fig. S6). The data sets analyzed were considered to be normal with an α value of 0.05. SDs and *P* values from Welch's two-tailed *t* test are shown comparing experimental and control samples as indicated.

the levels of reverse transcription DNA products in total cellular DNA were measured by quantitative PCR, and the relative efficiency of reverse transcription was calculated. Down-regulation of eEF1A or eEF1G had strong negative effects on reverse transcription efficiency (Fig. 6*B*, bars), and a modest, ~50% decrease in the synthesis of (−) strong stop DNA in cells (Fig. 6*B*, markers) compared with control infections. AZT-treated cells infected in parallel or cells infected with heat-inactivated virus had very low levels of HIV-1 DNA, indicating that the viral DNA synthesis was de novo (Fig. 6*B*). Interestingly, reverse transcription by Moloney murine leukemia virus–like particles was not affected significantly in siRNA-treated cells compared with control cells, but was inhibited by AZT treatment (Fig. S8*A*). Finally, analysis of RTC isolated from control and siRNA-treated cells by isopycnography, as previously described (Fig. 4), showed a four- to fivefold reduction in the level of RT in fractions 3 and 4, to which RTCs sediment, when eEF1A or eEF1G were down-regulated (Fig. 6 and Fig. S8*B*). Early DNA levels were also decreased by about twofold (Fig. 6*B*). These outcomes support the hypothesis that at least two members of the eEF1 complex, which includes eEF1A and eEF1G, associate with and may improve the stability of the HIV-1 RTC, thereby increasing the efficiency of reverse transcription.

Discussion

This report demonstrates a functional role for multiple subunits of the eEF1 complex in HIV-1 early replication by supporting efficient reverse transcription. Given their relative abundance in cells, eEF1 complex subunits are an obvious target for viral subversion. The evidence herein strongly supports the hypothesis that eEF1A and eEF1G associate with the HIV-1 RTC by interaction with RT and/or IN and increase the stability of RTC in the cellular cytoplasm. As IN is also an RT binding protein, the precise contacts between eEF1 subunits and RTC components requires further analysis. It is worth noting that eEF1A was shown to interact with HIV-1 IN in vitro (20, 21), which may be relevant to coimmunoprecipitations of IN with eEF1A or eEF1G observed in this study. Given a tight association between RT and IN (22), it is possible that an IN and eEF1A interaction resulted in coprecipitation of the RT p51 subunit. eEF1A has been implicated as a host cofactor for other RNA viruses (12, 14, 23–25). However, recently, the plant and yeast homologs of eEF1A and eEF1G (called eEF1Bγ) were found to be important for RNA replication of *tomato bushy stunt virus* (TBSV) through interactions with the viral RNA–dependent RNA polymerase (RdRp) and RNA stem-loop structures in the viral genome (15). The RNA binding functions of eEF1A and eEF1Bγ appear to be essential to stimulate RdRp activity, but whether RNA binding is relevant to the activity described here remains to be determined. Although eEF1A and eEF1G can bind cellular and viral RNAs, they are not known to bind DNA. This could indicate that eEF1 subunits act differently in HIV-1 reverse transcription, where the stimulatory activity has a greater effect on late rather than early reverse transcription when the viral genome is mostly or entirely DNA (9). It is possible that eEF1 subunits may contribute to early steps of reverse transcription by an RNA-binding mechanism (9).

The observation that down-regulation of eEF1A or eEF1G by treating cells with siRNA leads to sharply reduced efficiency of reverse transcription, which correlated to significantly reduced levels of RTCs in infected cells, suggests that eEF1 subunits

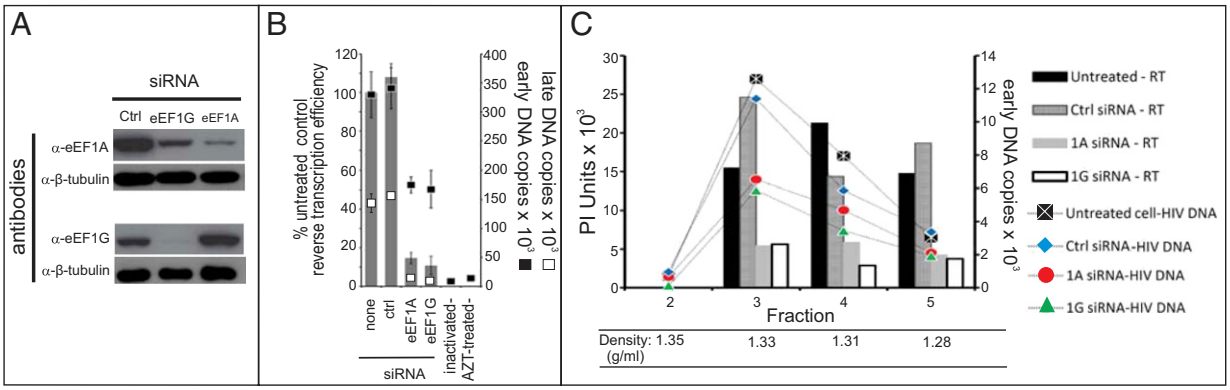

Fig. 6. Down-regulation of eEF1A or eEF1G inhibits HIV-1 reverse transcription. HEK293T cells were reverse transfected with siRNA to eEF1G, eEF1A, or a control siRNA. Untreated, siRNA-treated, or AZT-treated cells were infected with HIV-1 as indicated. (*A*) Western blots of lysates from siRNA-treated cells using antibodies as indicated. (*B*) Reverse transcription efficiency for treated cells was compared with untreated cells where the ratio of late to early DNA was considered as 100%. Early and late DNA levels in total DNA from control and siRNA-treated cells as indicated normalized to the cellular β-globin gene levels in each sample. (*C*) Analysis of fractionated gradients as previously described (Fig. 4) for reverse transcriptase by Western blot analysis (Fig. S8) and early HIV-1 DNA by qPCR. All qPCR reactions were performed in triplicate and the mean value and SDs are shown. Digital exposures of Western blots were analyzed using ImageQuant 5.1. The experiment was repeated three times, with similar results, and a representative data set is shown.

improve RTC stability in the cytoplasm. One possibility is that eEF1 or subunits thereof help to maintain the RTC integrity during the core uncoating process to facilitate late steps of reverse transcription (26, 27). Whether eEF1 subunits support RTC activity directly, protect RTCs from degradation, or both remains to be determined. Preliminary experiments to reconstitute a stimulatory effect in ERT assays with highly purified recombinant eEF1A and eEF1G have not succeeded, indicating, among the many possibilities, that other factors in DAF may contribute to the stimulatory activity or perhaps posttranslational modifications of eEF1 subunits may be required. Determination of the minimum components required to stimulate late steps of reverse transcription is an important long-term goal. Of the listed host factors known to associate with HIV-1 proteins, only a few directly interact with RT or IN or affect reverse transcription. Six of the 25 listed proteins (Table S1) have been demonstrated to interact with components of the HIV-1 RTC or have been implicated in retroviral reverse transcription. Like eEF1A and eEF1G, some of these are abundant cellular proteins. HSP90AB1, an isoform of the heat shock 90 protein family, was also identified and has been linked to reverse transcription of hepatitis B virus and the RNA replication of hepatitis C and influenza viruses (28–30). Similarly, the ATP-dependent RNA helicase A (DHX9) has been hypothesized to play a role in HIV-1 reverse transcription (31) by facilitating tRNA annealing onto the viral genomic RNA (32). Also detected was XRCC6 (also known as Ku70), a subunit of the ATP-dependent complex that protects HIV-1 integrase from the host ubiquitin-proteasome system and, together with PRKDC, associates with HIV-1 preintegration complexes (33, 34). Finally, our mass spectrometry analysis may have missed some important factors, as proteins smaller than 30 kDa were not included due to poor protein resolution following SDS/PAGE. For example, DAF contains all subunits of eEF1 complex, including eEF1B2 (25 kDa) and eEF1D (31 kDa) (Fig. S2), but these were not detected by mass spectrometry analysis. Experiments to determine whether other cellular proteins identified in DAF contribute to the stimulatory activity are proceeding.

Further analysis of eEF1:RTC interactions could lead to novel antiviral strategies; indeed, novel flavonoid drugs have recently been shown to target eEF1A1 (35). However, compounds targeting RT that block interaction with eEF1 may be effective HIV-1 inhibitors. Many questions remain, such as whether cellular factors in DAF other than eEF1 subunits are required for stimulating reverse transcription in vitro and in cells, and determining precisely how eEF1 interacts with RT and IN. Experiments addressing this possibility are likely to provide keys to understanding HIV-1 reverse transcription and transport of the RTC/PIC from the cytoplasm to the nucleus.

Methods

Cell Lines and Viruses. HeLa, HEK293T and Jurkat cells were cultured as previously described (4). AmphoPhoenix cells were maintained as recommended by the supplier (ATCC). Stocks of $HIV_{NL4.3}$ were generated by transfection of HEK293T cells with proviral DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stocks of HIV-$1_{pNL4\text{-}3.Luc.R\text{-}E\text{-}}$ were pseudotyped by cotransfecting proviral plasmid with an expression plasmid encoding the VSV-G protein. Stocks of MLV-A were made by transfecting AmphoPhoenix cells with the retroviral vector pSAMEN-EGFP (36).

Lysate Preparation and Protein Fractionation by Column Chromatography. S100 lysate and PGF were prepared from 10^{11} Jurkat cells as previously described (4, 9). Briefly, the S100 lysate was fractionated by gel filtration through CL-4B size exclusion matrix (Sigma Aldrich) collecting the void volume protein that is called PGF (9). To obtain DAF, PGF protein of 1 or 10 mg was fractionated by using a 1-mL or 5-mL HiTrap DEAE FF column (GE Healthcare), respectively. The bound proteins were eluted from the column with a NaCl gradient (0–1 M) in S100 buffer in 20 fractions of 1 mL or 5 mL each, respectively. Each fraction was dialyzed using 3.5-kDa MWCO dialysis units against S100 dialysis buffer and 0.1 M PMSF. The activities of dialyzed fractions were tested in ERT assays (4).

ERT Assay. ERT assays were performed using partially purified HIV-1 as previously described (4, 9). Briefly, 25 μL of the chromatography fractions or cell lysate containing specified amounts of protein were added to 50-μL ERT reactions as indicated, and 0.2 mM Triton X-100 was the last component added. A no-DNA control was always included. Standard primer sets used for amplification of strong-stop DNA, and for second-strand transfer DNA were previously described (9).

Mass Spectrometry. Active chromatography fractions were precipitated with 20% (vol/vol) trichloroacetic acid, resuspended in protein sample buffer, and separated by SDS/PAGE. Following electrophoresis, gels were stained with Bio-safe Coomassie stain (Bio-Rad). In-gel tryptic digest was performed on excised protein bands and extracted peptides were resuspended in 5% (vol/vol) formic acid. Samples were analyzed by LC-MS/MS on a Nano HPLC (Shimadzu) coupled to a QStar Elite mass spectrometer (AB SCIEX) with a nano electrospray source. The data were acquired and processed using Analyst QS 2.0 software (AB SCIEX). Proteins were identified by database searching using Protein Pilot 3.0 (AB SCIEX) against the UniProt_Sprot_20110325 database (105,198 entries searched). Proteins were considered identified if there were two or more peptides identified with a 99% confidence and a 1% global false discovery rate.

Immunodepletion, Coimmunoprecipitation, and Western Blot Analysis. The immunodepletion and coimmunoprecipitation experiments used large-scale ERT reactions (×10). Antibodies to translation factors (1–10 μg) or BSA (10 μg) were cross-linked to beads (Pierce). For each immunodepletion, 500 μL dialyzed DAF was incubated with antibody or BSA-linked beads for 2 h at 4 °C and unbound fractions were collected. The unbound fractions and untreated DAF were precipitated as previously described before separation by SDS/PAGE. Western blots were probed with relevant primary antibodies α-eEF1A (Santa Cruz), α-eEF1G (Abcam), or α-eIF3A (CST) followed by appropriate HRP-conjugated secondary antibodies and detection by chemiluminescence. Unbound fractions were also tested in large-scale ERT reactions. The immunoprecipitated proteins were assayed by Western blot using human α-HIV, rabbit α-RT, or mouse α-IN mAb (Diatheva) and detected by chemiluminescence.

RTC Purification and Western Blot Analysis. Purification of HIV RTC was carried out as previously described (19), with minor modifications, using HEK293T and HIV-$1_{pNL4\text{-}3.Luc.R\text{-}E\text{-}}$ (or heat-inactivated virus, 65 °C, 20 min) containing 1 μg CAp24 at 4 °C for 2 h with polybrene (80 μg/mL) and then incubated at 37 °C for 4 h. The cell lysate were placed on top of a linear sucrose gradient (20–70%, wt/wt) and subjected to centrifugation at 145,000 × *g* for 18 h at 4 °C. Twelve fractions were collected from the tube bottom and assayed by qPCR for HIV-1 strong stop DNA as previously described (9) using 0.2 μL of each fraction. Protein was extracted from each fraction using chloroform and methanol in the presence of 1 μg BSA. The recovered protein was subjected to SDS/PAGE and Western blot analysis, as indicated.

PLA. HeLa cells were incubated with VSV-G pseudotyped HIV-$1_{pNL4\text{-}3.Luc.R\text{-}E\text{-}}$ (2 h, 4 °C) to allow virus attachment. Cells were then incubated (4 h, 37 °C) to permit virus fusion and at least partial core uncoating and reverse transcription. Duolink proximity ligation assays (Olink) were performed according to the manufacturer's instructions using infected and uninfected HeLa cells with a mouse monoclonal antibody to detect HIV-1 RT in conjunction with rabbit antibodies to eEF1A (Santa Cruz), eEF1G (Sigma), or eIF3A (CST). DAPI stain was used to visualize the nuclei. Both infected and uninfected cells were visualized using a DeltaVision Core imaging system. Maximum-intensity projections of deconvolved images were analyzed using Duolink Image Tool software. Equal numbers of cells were analyzed from at least three fields containing >10 cells.

siRNA Experiments. siRNA (Sigma-Aldrich) targeting eEF1A (siRNA ID: SASI_Hs02_00331773) and eEF1G (siRNA ID: SASI_Hs02_00331781) or negative control siRNA (SIC001) were applied to HEK293T cells by large-scale reverse transfection using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen). The treated and control cells were seeded onto 10-cm dishes, six dishes per treatment, and incubated at 37 °C for 48 h. The transfected cells were infected in duplicate with VSV-G pseudotyped HIV-$1_{pNL4\text{-}3.Luc.R\text{-}E\text{-}}$ (200 ng CAp24) or with inactivated virus at 4 °C for 2 h with addition of polybrene (80 μg/mL) and then incubated at 37 °C for 5 h. Separate plates were used for the following: (*i*) Western blot assays as described, and cell viability measured using CellTiter 96Aqueous MTS reagent (Promega) (one plate); (*ii*) DNA extraction (QIAamp DNA Mini kit; Qiagen) for TaqMan qPCR of HIV-1 early and late DNA products as previously described (one plate); and (*iii*) RTC analysis as previously described (four plates). Untreated cells were incubated with AZT (Sigma-Aldrich) at 30 μM and incubated overnight before infection. Forward and reverse oligonucleotide primers used for PCR of HIV-1 samples and cycling conditions were as previously described (9). The TaqMan probe used for HIV-1 quantitative PCR was 5′(6FAM)CAGAGAGCTCCCAGGCTCAGATC(TAM). Oligonucleotides for MLV qPCR are described elsewhere (Fig. S8).

ACKNOWLEDGMENTS. This work in the D.H. laboratory was sponsored by National Health and Medical Research Council (NHMRC) Project Grant 1002440. T.W. is sponsored by an NHMRC Biomedical Training Fellowship. K.W. was sponsored by a University of Western Sydney Post-Graduate Research Award. M.-H.L. is sponsored by a University of Queensland Research Scholarship and a University of Queensland International Research Tuition Award. D.H. was funded by an Australian Research Council Future Fellowship.

1. Basu VP, et al. (2008) Strand transfer events during HIV-1 reverse transcription. *Virus Res* 134:19–38.
2. Yong WH, Wyman S, Levy JA (1990) Optimal conditions for synthesizing complementary DNA in the HIV-1 endogenous reverse transcriptase reaction. *AIDS* 4:199–206.
3. Hooker CW, Harrich D (2003) The first strand transfer reaction of HIV-1 reverse transcription is more efficient in infected cells than in cell-free natural endogenous reverse transcription reactions. *J Clin Virol* 26:229–238.
4. Warrilow D, et al. (2008) Cell factors stimulate human immunodeficiency virus type 1 reverse transcription in vitro. *J Virol* 82:1425–1437.
5. Zhu K, Dobard C, Chow SA (2004) Requirement for integrase during reverse transcription of human immunodeficiency virus type 1 and the effect of cysteine mutations of integrase on its interactions with reverse transcriptase. *J Virol* 78:5045–5055.
6. Warren K, Warrilow D, Meredith L, Harrich D (2009) Reverse transcriptase and cellular factors: Regulators of HIV-1 reverse transcription. *Viruses* 1:873–894.
7. Warrilow D, Tachedjian G, Harrich D (2009) Maturation of the HIV reverse transcription complex: Putting the jigsaw together. *Rev Med Virol* 19:324–337.
8. Narayan S, Young JA (2004) Reconstitution of retroviral fusion and uncoating in a cell-free system. *Proc Natl Acad Sci USA* 101:7721–7726.
9. Warrilow D, Warren K, Harrich D (2010) Strand transfer and elongation of HIV-1 reverse transcription is facilitated by cell factors in vitro. *PLoS ONE* 5:e13229.
10. Mansilla F, et al. (2002) Mapping the human translation elongation factor eEF1H complex using the yeast two-hybrid system. *Biochem J* 365:669–676.
11. Mateyak MK, Kinzy TG (2010) eEF1A: Thinking outside the ribosome. *J Biol Chem* 285: 21209–21213.
12. Qanungo KR, Shaji D, Mathur M, Banerjee AK (2004) Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. *Proc Natl Acad Sci USA* 101:5952–5957.
13. Davis WG, Blackwell JL, Shi PY, Brinton MA (2007) Interaction between the cellular protein eEF1A and the 3′-terminal stem-loop of West Nile virus genomic RNA facilitates viral minus-strand RNA synthesis. *J Virol* 81:10172–10187.
14. Li Z, et al. (2009) Translation elongation factor 1A is a component of the tombusvirus replicase complex and affects the stability of the p33 replication co-factor. *Virology* 385:245–260.
15. Sasvari Z, Izotova L, Kinzy TG, Nagy PD (2011) Synergistic roles of eukaryotic translation elongation factors 1Bγ and 1A in stimulation of tombusvirus minus-strand synthesis. *PLoS Pathog* 7:e1002438.
16. Cimarelli A, Luban J (1999) Translation elongation factor 1-alpha interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. *J Virol* 73: 5388–5401.
17. Kim S, Coulombe PA (2010) Emerging role for the cytoskeleton as an organizer and regulator of translation. *Nat Rev Mol Cell Biol* 11:75–81.
18. Bukrinskaya A, Brichacek B, Mann A, Stevenson M (1998) Establishment of a functional human immunodeficiency virus type 1 (HIV-1) reverse transcription complex involves the cytoskeleton. *J Exp Med* 188:2113–2125.
19. Fassati A (2009) Methods of preparation and analysis of intracellular reverse transcription complexes. *Methods Mol Biol* 485:107–119.
20. Allouch A, Cereseto A (2011) Identification of cellular factors binding to acetylated HIV-1 integrase. *Amino Acids* 41:1137–1145.
21. Parissi V, et al. (2001) Functional interactions of human immunodeficiency virus type 1 integrase with human and yeast HSP60. *J Virol* 75:11344–11353.
22. Dobard CW, Briones MS, Chow SA (2007) Molecular mechanisms by which human immunodeficiency virus type 1 integrase stimulates the early steps of reverse transcription. *J Virol* 81:10037–10046.
23. Li Z, et al. (2010) Translation elongation factor 1A facilitates the assembly of the tombusvirus replicase and stimulates minus-strand synthesis. *PLoS Pathog* 6: e1001175.
24. Johnson CM, Perez DR, French R, Merrick WC, Donis RO (2001) The NS5A protein of bovine viral diarrhoea virus interacts with the alpha subunit of translation elongation factor-1. *J Gen Virol* 82:2935–2943.
25. Yamaji Y, et al. (2006) In vivo interaction between Tobacco mosaic virus RNA-dependent RNA polymerase and host translation elongation factor 1A. *Virology* 347: 100–108.
26. Arfi V, et al. (2009) Characterization of the behavior of functional viral genomes during the early steps of human immunodeficiency virus type 1 infection. *J Virol* 83: 7524–7535.
27. Hulme AE, Perez O, Hope TJ (2011) Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription. *Proc Natl Acad Sci USA* 108: 9975–9980.
28. Hu J, Seeger C (1996) Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. *Proc Natl Acad Sci USA* 93:1060–1064.
29. Momose F, et al. (2002) Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. *J Biol Chem* 277:45306–45314.
30. Okamoto T, et al. (2006) Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J* 25:5015–5025.
31. Roy BB, et al. (2006) Association of RNA helicase a with human immunodeficiency virus type 1 particles. *J Biol Chem* 281:12625–12635.
32. Xing L, Liang C, Kleiman L (2011) Coordinate roles of Gag and RNA helicase A in promoting the annealing of formula to HIV-1 RNA. *J Virol* 85:1847–1860.
33. Zheng Y, Ao Z, Wang B, Jayappa KD, Yao X (2011) Host protein Ku70 binds and protects HIV-1 integrase from proteasomal degradation and is required for HIV replication. *J Biol Chem* 286:17722–17735.
34. Daniel R, Katz RA, Skalka AM (1999) A role for DNA-PK in retroviral DNA integration. *Science* 284:644–647.
35. Yao N, et al. (2011) Novel flavonoids with antiproliferative activities against breast cancer cells. *J Med Chem* 54:4339–4349.
36. Chuah MK, Vandendriessche T, Morgan RA (1995) Development and analysis of retroviral vectors expressing human factor VIII as a potential gene therapy for hemophilia A. *Hum Gene Ther* 6:1363–1377.