**Characterization of the Influence of Mediator Complex in HIV-1 Transcription**

Revised for publication, April 1, 2014, and in revised form, July 18, 2014. Published, JBC Papers in Press, August 6, 2014. DOI 10.1074/jbc.M114.570341

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**Background:** The transcription Mediator complex was suggested to influence HIV infection, but a more accurate study is needed to define this contribution.

**Results:** Nine Mediator subunits are implicated in early and late HIV transcription, although MED14 plays a major role.

**Conclusion:** HIV transcription needs specific Mediator subunits interacting with HIV Tat.

**Significance:** This work enables further studies on cofactors influencing HIV transcription.

HIV-1 exploits multiple host proteins during infection. siRNA-based screenings have identified new proteins implicated in different pathways of the viral cycle that participate in a broad range of cellular functions. The human Mediator complex (MED) is composed of 28 elements and represents a fundamental component of the transcription machinery, interacting with the RNA polymerase II enzyme and regulating its ability to express genes. Here, we provide an evaluation of the MED activity on HIV replication. Knockdown of 9 out of 28 human MED proteins significantly impaired viral replication without affecting cell viability, including MED6, MED7, MED11, MED14, MED21, MED26, MED27, MED28, and MED30. Impairment of viral replication by MED subunits was at a post-integration step. Inhibition of early HIV transcripts was observed by siRNA-mediated knockdown of MED6, MED7, MED11, MED14, and MED28, specifically affecting the transcription of the nascent viral mRNA transactivation-responsive element. In addition, MED14 and MED30 were shown to have special relevance during the formation of unspliced viral transcripts (p < 0.0005). Knockdown of the selected MED factors compromised HIV transcription induced by Tat, with the strongest inhibitory effect shown by siMED6 and siMED14 cells. Co-immunoprecipitation experiments suggested physical interaction between MED14 and HIV-1 Tat protein. A better understanding of the mechanisms and factors controlling HIV-1 transcription is key to addressing the development of new strategies required to inhibit HIV replication or reactivate HIV-1 from the latent reservoirs.

Recent years have seen a significant increase in the understanding of the host determinants of susceptibility to human immunodeficiency virus (HIV) infection and disease progression, driven in large part by candidate gene studies, genome-wide association studies, genome-wide transcriptome analyses, and large scale functional screens (1). The mechanism of HIV transcription has been actively studied, but the participating cellular pathways and elements are not yet fully understood (2). A clear understanding of the viral transcription mechanism is essential for addressing the development of new antiviral drugs as well as therapeutic strategies to eradicate HIV-1 reservoirs in chronic infected patients (3).

After integration of the viral DNA into the host cell genome, HIV provirus can rest transcriptionally silent or start the transcription process leading to the completion of the viral cycle. Transcription of the HIV genome involves RNA polymerase II (RNAP II) working together with viral protein Tat and a number of host proteins (4). The transcriptional transactivator Tat is initially expressed from integrated viral DNA during active infection, and subsequently, RNAP II is recruited to the HIV promoter long terminal repeat (LTR) where the viral transcription starts. In a second phase, viral transcripts are elongated, generating immature mRNAs that will be consequently spliced and translated. Without Tat, RNAP II has been found bound to the HIV-1 LTR but functionally paused, producing only short viral transcripts that contain at their 5′ end an RNA stem-loop structure, the transactivation-responsive elements (TAR). The presence of TAR blocks HIV transcription at the initiation step and does not allow elongation (5).

Different host factors have been linked to HIV transcription, including the human positive transcription elongation factor (P-TEFb) (6–8). Several other proteins have been previously identified (9) but remain to be confirmed, including those of the Mediator complex (10, 11), a large multisubunit complex that regulates RNAP II transcribed genes. In humans, MED is composed of 28 proteins assembled in four distinct modules as follows: the “Head,” which contacts the RNAP II; the “Middle” and “Tail” that transfer regulatory signals after interacting with transcription factors to the Head; and the “CDK8 module,” including MED12 and MED13 that associate less stably to the

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3 The abbreviations used are: RNAP II, RNA polymerase II; MED, Mediator; TAR, transactivation-responsive element; LTR, long terminal repeat; WCL, whole cell lysate; AZT, Zidovudine; NT, nontargeting; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBMC, peripheral blood mononuclear cell; MDM, monocyte-derived macrophage; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-N,N,N′,N′-tetramethylrhodamine; qPCR, quantitative PCR.
The Mediator complex has been attributed to a role on viral transcription, due to its RNAP II-related function (10) or its link to the Tat-mediated transcription through the HIV LTR (11). In this study, we provide a comprehensive evaluation of Mediator complex activity on HIV replication that highlights the role of host factors acting in an orchestrated process with RNAP II during the HIV replication cycle. We identified nine human MED proteins whose inhibition by RNA interference (RNAi) significantly impaired viral replication. Knockdown of MED genes compromised HIV transcription, allowing the identification of a subgroup of proteins especially relevant during early HIV transcription stages as follows: MED6, MED7, MED11, MED14, and MED28. MED proteins play a crucial role during elongation of viral transcripts and transcription of viral genes induced by Tat, with MED6 and MED14 being the ones showing the strongest effect. Co-immunoprecipitation experiments showed physical interaction between MED14 and the HIV-1 Tat protein. Altogether, our data demonstrate the requirement of the Mediator complex for efficient HIV transcription, a dependence that needs the interaction between MED14 and HIV Tat protein.

**EXPERIMENTAL PROCEDURES**

**Cells**—TZM-bl and HEK293T cells (AIDS Research and Reference Reagent Program) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Peripheral blood mononuclear cells (PBMC) were obtained using a Ficoll-Paque density gradient centrifugation and used for purification of monocytes using a negative selection antibody mixture (StemCell Technologies). Purity of the population was confirmed by flow cytometry. Monocytes were resuspended in complete culture medium as follows: RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin. Obtained monocytes were differentiated for 4–5 days in the presence of macrophage-colony-stimulating factor (M-CSF, PeproTech) at 100 ng/ml. PBMC were isolated from “buffy coats” of healthy blood donors. Buffy coats were purchased anonymously from the Catalan Banc de Sang i Teixits. The buffy coats received were totally anonymous and untraceable, and the only information given was whether or not they had been tested for disease.

**RNA Extraction**—RNA from TZM-bl cells, MT-4 cells, PBMC, monocytes, and monocyte-derived macrophages (MDM) and commercially available lymph node, adipose, small intestine, and thymus RNAs (First Choice from Invitrogen) were used to analyze MED gene expression. RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel) in accordance with the manufacturer’s instructions. RNA was used for a clustering analysis of all MED genes by using the GEPAS software.

**Plasmids**—Tat-expressing plasmid pCТat has been previously described (21, 22). To generate the p3×FLAG-CMV-Tat, RNA from TZM-bl cells infected with an HIV-1 NL4-3 strain was extracted using the NucleoSpin RNA II kit (Macherey-Nagel) in accordance with the manufacturer’s instructions. After reverse transcription with the High Capacity cDNA reverse transcription kit (Invitrogen), Tat was amplified using the following primers: forward 5′-ATGGAGCCAGTAGATCCT-3′ and reverse 5′-CTATTCCTTGGGCCTGT-3′. PCR products were cloned into the pGEM-T Easy vector (Promega) and transformed into competent cells (One Shot OmniMAX 2 T1 R E. coli, Invitrogen). Plasmid DNA was extracted using HiSpeed kits (Qiagen), and the purified plasmid was then digested with EcoRI before being introduced into the EcoRI-digested p3×FLAG-CMV-7.1 vector (Sigma). Sequence integrity of all plasmids was checked by sequencing.

**siRNA and Plasmid Transfection**—For the MED siRNA screen (Table 1), 12.5 or 50 μM of the corresponding siRNA pool targeting four nonoverlapping mRNA sequences (SMART pool from Dharmacon, Thermo-Scientific) were mixed with Lipofectamine 2000 reagent (Invitrogen) in serum-free medium (Opti-MEM I, Invitrogen) and added onto 2×10⁴ or 8×10⁴ TZM-bl cells for 96-well or 24-well plate format, respectively. The same siRNA retrotransfection protocol was used for HEK293T in co-immunoprecipitation experiments. Four siRNA sequences targeting MED14 were purchased for siRNA target confirmation (FlexiTube Gene Solution, Qiagen) and used as described. Monocytes were transfected with 50 pmol of siRNA using the monocyte Amaxa nucleofection kit (Lonza) as described previously (23). Transfected monocytes were left untreated overnight and then differentiated to macrophages as described above.

For DNA transfections, plasmids were transfected into TZM-bl cells using Lipofectamine 2000 reagent. HEK293T cells were transfected using Lipofectamine 2000 reagent. HEK293T cells were transfected using polyethyleneimine (PEI) as described previously (23). In all cases, 48 h post-transfection the cells were used for gene expression assay (mRNA or protein), Tat transactivation assay, or HIV-1 infections.

**Viability Assays**—For measurement of cell toxicity or viability, methyl tetrazolium-based colorimetric assay (MTT method) was performed in transfected TZM-bl cells as described (24).

**HIV Infection and Replication**—HIV-1 stock of NLA-3 was grown in lymphoid MT-4 cells and titrated in TZM-bl cells. The reverse transcriptase inhibitor zidovudine (AZT, Sigma) and the integrase inhibitor raltegravir (Merck) were used at a concentration of 3 and 2 μM, respectively. 24 h after infection, samples were lysed, and DNA was extracted for integrated HIV DNA quantification. 48 h after infection, samples were collected for β-galactosidase assay, and quantification of viral transcripts was obtained. siRNA-treated macrophages were infected 72 h post-transfection with vesicular stomatitis virus-
Role of the Mediator Complex in HIV-1 Transcription

| Gene symbol | Gene ID | Gene accession no. | Sequence |
|-------------|---------|--------------------|----------|
| MED23       | 9439    | NM_015979          | CCGCAAGCGGUGUGUCGUAAC |
| MED23       | 9439    | NM_015979          | GAGCCCCGUGUGUGGAAGGC |
| MED23       | 9439    | NM_015979          | UGGUGUGUGUGUGUGUAAAC |
| MED24       | 9862    | NM_014815          | CAAAGGACGUGUGUGUAGA |
| MED24       | 9862    | NM_014815          | GACGUGUGUGUGUGUAGA |
| MED25       | 81857   | NM_030973          | CCAAAGCAGGACGACGAGA |
| MED25       | 81857   | NM_030973          | CAGGACGUGGACGACGAGA |
| MED25       | 81857   | NM_030973          | UGACGUGGACGACGACGAG |
| MED26       | 9441    | NM_004831          | CGACGUGGACGACGACGAG |
| MED26       | 9441    | NM_004831          | CACAGGACGUGGACGACGAG |
| MED27       | 9442    | NM_004269          | CAACUGUACGACGACGACG |
| MED27       | 9442    | NM_004269          | CAAACUGUACGACGACGAC |
| MED27       | 9442    | NM_004269          | CAAACUGUACGACGACGAC |
| MED28       | 80306   | NM_025205          | CACGACGUGGACGACGACG |
| MED28       | 80306   | NM_025205          | CACGACGUGGACGACGACG |
| MED28       | 80306   | NM_025205          | CACGACGUGGACGACGACG |
| MED29       | 55588   | NM_017952          | CAAAAGGACGUGUGUGUAGA |
| MED29       | 55588   | NM_017952          | CAAAGGACGUGUGUGUAGA |
| MED29       | 55588   | NM_017952          | CAAAGGACGUGUGUGUAGA |
| MED30       | 90390   | NM_080651          | GAAACUGUACGACGACGAC |
| MED30       | 90390   | NM_080651          | GAAACUGUACGACGACGAC |
| MED30       | 90390   | NM_080651          | GAAACUGUACGACGACGAC |
| MED31       | 51003   | NM_016060          | CAACGACGUGGACGACGAG |
| MED31       | 51003   | NM_016060          | CAACGACGUGGACGACGAG |
| MED31       | 51003   | NM_016060          | CAACGACGUGGACGACGAG |
| MED32       | 104     | NM_153450          | ACAAGACGACGUGUGUGU |
| MED32       | 104     | NM_153450          | ACAAGACGACGUGUGUGU |
| MED32       | 104     | NM_153450          | ACAAGACGACGUGUGUGU |
| MED33       | 9888    | NM_004229          | CGGTTTACCTTACCTATAG |
| MED33       | 9888    | NM_004229          | CGGTTTACCTTACCTATAG |
| MED33       | 9888    | NM_004229          | CGGTTTACCTTACCTATAG |
| MED34       | 298     | NM_004229          | CGGTTTACCTTACCTATAG |
| MED34       | 298     | NM_004229          | CGGTTTACCTTACCTATAG |
| MED34       | 298     | NM_004229          | CGGTTTACCTTACCTATAG |

pseudotyped NL4-3-GFP virus. Infection was measured by flow cytometry as the percentage of GFP-positive cells 48 h post-infection.

Tat Transactivation Assay—The transactivation activity of HIV Tat was analyzed by monitoring the production of β-galactosidase after activation of lacZ expression in TZM-bl cells that were previously transfected with siRNA and pcTat, a Tat expression plasmid. In brief, 8 × 10⁴ TZM-bl cells were retrotransfected with siRNA and seeded into 24-well plates as described above; 24 h later, 200 ng of a plasmid encoding HIV-1 Tat (pcTat) was transfected. 48 h after DNA transfection, samples were processed for β-galactosidase assay as described.

β-Galactosidase Detection Assay—β-Galactosidase activity was quantified by a colorimetric assay, as described elsewhere (21, 25). Background from noninfected cells was subtracted from the rest of the samples, and absorbance was expressed as percentage of β-galactosidase activity relative to the control treated without siRNA.

Quantitative-Polymerase Chain Reaction (qPCR)—For relative mRNA and viral transcripts quantification, RNA was extracted as described above. After reverse transcription with the high capacity cDNA reverse transcription kit (Invitrogen), cDNA was measured for MED gene expression assay by real time qPCR and normalized to GAPDH mRNA expression using...
the ΔΔCt method. Gene expression assays for all analyzed human genes were purchased from Invitrogen.

For viral RNA quantification, the following primers and probe amplifying tat/rev/nef mRNA were used: forward 5′-GGATCTGTCTGTCCTCTCCACC-3′, reverse 5′-ACAGTCAGCTCATAAGTTCTCTATCAAAGCA-3′, and the dual-labeled fluorescent probe FAM 5′-TTCTTCTGG-GGCTGTCGGGTCCC-3′ TAMRA (26). TAR transcripts were quantified with the following primers and probe: forward 5′-GGGGTCTCTGGTAAAA-3′, reverse 5′-GGGTCTCTCTAGTAG-3′, and the probe that is complementary to the TAR loop region of the HIV-1 LTR and also dual-labeled, FAM 5′-GGCTGGAGCTCTTGG-3′ TAMRA (27, 28).

For integrated DNA quantification, total cellular DNA was extracted using the QIAamp DNA extraction kit (QIAamp DNA blood mini kit, Qiagen) as recommended by the manufacturer, and integrated viral DNA quantification was performed as described before (29). Briefly, an Alu-Gag (HIV group-specific antigen) preamplification was performed by using the following primers: forward 5′-GCGCTCCCAAAGTGCTGGATTACAG-3′, and reverse 5′-AGGGTTCTTTGGTCCTTGTG-3′; samples were then followed by a Gag amplification by using the following primers and probe: forward 5′-CAAGCAGCCATGCAAATGTT-3′, reverse 5′-TGCACTGAGTGCATAATCTATCC-3′, and probe FAM 5′-AAAGAGACCATCAATGAGGAACTGCAGA-3′ TAMRA.

Co-immunoprecipitation and Western Blotting—HEK293T cells were harvested, washed in cold PBS, and lysed on ice with lysis buffer as described (23). After centrifugation at 14,000 rpm for 10 min at 4 °C, lysates were collected and aliquots resuspended in loading buffer subjected to SDS-PAGE and transferred to a PVDF membrane. The following antibodies were transferred to a PVDF membrane, and analyzed by immunoblotting: horseradish peroxidase-conjugated secondary antibody (Pierce), anti-MED14 (ab572141, Abcam), and anti-TAT (LS-C68064, Abgent). Lysates from HEK293T cells were incubated with anti-FLAG antibodies covalently attached to agarose (anti-FLAG M2 Affinity Gel, Sigma) overnight at 4 °C on a rocking platform for FLAG-Tat fusion protein immunoprecipitation.

For MED14 immunoprecipitation, MED14 was incubated overnight with anti-MED14 antibody (2 μg per mg of protein) and further incubated with Sepharose A beads (Sigma) for 1–2 h. beads were collected by centrifugation at 3000 rpm for 5 min at 4 °C, extensively washed in lysis buffer, and resuspended in SDS loading buffer. The proteins were separated on a 6 or 15% SDS-polyacrylamide gel, transferred to a PVDF membrane, and analyzed by immunoblotting with the corresponding antibodies. Benzonase at 600 units/ml (Sigma) treatment was performed in cell lysates before the immunoprecipitation by incubating for 2 h at 37°C with 2 mM Mg2+.

Statistical Analysis—For the siRNA screen with all Mediator subunits, siRNA pools were considered for further analysis when HIV replication was inhibited at least 50%. Moreover, selected siRNA might conserve viability values as measured by the MTT method above 70% compared with mock-transfected cells. Data are presented as mean ± S.D. Unpaired Student’s t test was used for comparison between two groups, using the GraphPad Prism software. p values less than 0.05 were considered significant.

RESULTS

RNA Interference of Mediator Complex Subunits Impaired HIV-1 Replication—We first characterized the expression pattern of the different Mediator complex subunits in several primary cells, including PBMC, monocytes, and macrophages, two human cell lines MT-4 and HeLa TZM-bl, and HIV-susceptible tissues like lymph node, thymus, and small intestine, as well as adipocytes to include a non-HIV-susceptible tissue.

Expression profiling analysis indicated the highest MED expression in adipocytes and PBMCs, whereas the lowest expression was found in monocytes and MT-4 cells. HeLa TZM-bl cells and macrophages had a similar mRNA profile with medium levels of expression on average. MED genes were also clustered in different groups to easily distinguish those MED subunits related in terms of mRNA profile (Fig. 1).

The presented MED mRNA profile is informative regarding how similar the analyzed cells and tissues are in comparison with HeLa TZM-bl cells. These cells were chosen for their high susceptibility to RNA interference and HIV infection, as the model for a comprehensive analysis of the relative contribution of each MED subunit in HIV-1 replication by RNAi.

Therefore, efficient and specific inhibition of each of the different MED subunits was achieved at the mRNA level compared with mock-treated cells (Fig. 2A). siRNA-treated cells were then infected with the NL4-3 HIV-1 strain, and 3 days later HIV-1 replication was assessed (Fig. 2B, white bars). Cell viability was assessed in parallel in uninfected cells (Fig. 2B, black bars). As expected, RNA interference of CD4 or A2T led to decreased HIV replication in the absence of significant cytotoxicity. We selected for further study those siRNAs that inhibited HIV-1 replication by >50% compared with untreated infected cells, and their associated cell viability did not vary more than 30% compared with mock-transfected cells.

The strongest blockade of HIV replication was achieved by siRNA-mediated inhibition of MED14 (β-gal values were reduced by 90% compared with mock-transfected cells, p < 0.005), while partly compromising cell viability in the assayed conditions (reduced viability by 35% of control). The use of confirmatory siRNA targeting MED14 (Fig. 2C) confirmed its specificity and potency in impairing HIV replication in the absence of off-target effects or siRNA-derived toxicity, pointing to MED14 as one of the most relevant MED subunits for HIV replication.

From this work, the siRNA-based Mediator screen showed that silencing of 9 out of the 28 human MED genes (MED6, MED7, MED11, MED14, MED21, MED26, MED27, MED28, and MED30) significantly impaired viral replication. Interferences of these MED genes did not have an effect on cell viability.

We silenced mRNA expression of selected MED in human MDM (Fig. 3A), and HIV-1 infection and cell viability were quantified by flow cytometry in siRNA-treated cells (Fig. 3B). Knockdown of induced the strongest reduction in viral replication (58% of control, p < 0.01), followed by siMED27 cells (63% of control, p = 0.01). Nevertheless, other MED subunits tested did not significantly affect HIV-1 replication in MDM.
Effect of Mediator Complex on Viral Transcription—Because we identified a group of MED subunits whose activity is required for efficient HIV-1 viral replication, we then investigated the role of these subunits before and during viral transcription. For this purpose, the levels of integrated proviral DNA and viral transcripts were quantified by qPCR.

Mediator-interfered (siMED) TZM-bl cells were infected with a NL4-3 HIV-1 strain and viral integration 24 h after infection was quantified (Fig. 4). No differences were observed between siMED and control cells, indicating that Mediator down-regulation did not affect HIV-1 integration or earlier steps in the viral replication cycle. As expected, the inhibitor of HIV-1 retrotranscription AZT and the inhibitor of HIV integration raltegravir effectively inhibited HIV integration.

Evaluation of viral mRNA transcription and formation of TAR was then evaluated. Early viral mRNA transcripts and specifically viral nascent RNA sequences named TAR are formed when HIV promoter elements assemble the pre-integration complex and RNAP II clears the promoter, initiating the transcription of HIV genes (30). Impairment in the formation of TAR forms was found in knockdown cells for MED6, MED7, MED11, MED14, and MED28 (p value range 0.01–0.0001), suggesting that these MED subunits are required particularly during initiation steps in HIV-1 transcription.

We also quantified the unspliced tat/rev/nef mRNA transcripts, transcribed during the elongation process that follows the initial transcription process (26). A reduction in viral elongated transcripts was observed after silencing of all MED subunits (Fig. 4, p value range 0.05–0.0005), compared with cells treated with a nontargeting siRNA pool. AZT-treated as well as raltegravir-treated cells were used as a negative control of infection. siRNA targeting the HIV cofactor zinc ribbon domain-containing 1 (ZNRD1) was used as a control of impaired HIV-1 transcription, as it has been previously shown to have a role in late HIV mRNA formation (31).

Taken together, these results demonstrate a role for Mediator complex in HIV transcription. They also indicate the Mediator subunits implicated in the transcription initiation.

Tat-mediated HIV-1 Transcription Is Modulated by Mediator Subunits—To determine the requirement of MED subunits in Tat-dependent viral expression of the HIV-1 LTR promoter, an HIV-1 Tat transactivation assay was performed in HeLa TZM-bl cells, previously treated with siRNA targeting MED subunits. After successful knockdown expression of Mediator genes (Fig. 5A), a Tat-expressing vector was transfected and β-galactosidase expression under the control of the HIV-1 LTR promoter was monitored. siRNA knockdown of MED6 and MED14 showed significant differences in Tat-mediated HIV-1 transcription levels (up to 40% inhibition, p < 0.05), as well as a more modest inhibition for MED11, MED26, MED27, and MED30 subunits. In contrast, cells with knocked down MED7, MED21, and MED28 cells did not significantly change compared with mock-transfected cells (Fig. 5B). Viability in mock-transfected, siRNA transfected, and siRNA + DNA-transfected cells was monitored by flow cytometry after single or double transfection (Fig. 5C).

Role of Mediator Complex in HIV-1 Transcription Is Mediated by Tat-Mediator Interactions—The Mediator complex interacts with a variety of activators/repressors through its Tail module. Received regulatory signals are then transmitted to the RNAP II via the Middle and the Head modules to start tran-
scription of specific genes (32). Based on previous results, where MED14 showed the highest inhibition in HIV replication during early and late stages of viral transcription and in Tat-transactivation assays, we explored the possibility of Tat-MED14 interactions.

To investigate possible interactions between MED14 and the viral LTR-transactivator Tat, a plasmid expressing a fusion protein 3×FLAG-Tat was transfected into HEK293T cells, and Tat was immunoprecipitated using FLAG-specific agarose beads. Interestingly, MED14 co-immunoprecipitated with FLAG-Tat (Fig. 6A, lane 2), but it was not detected when using lysates from mock-transfected cells (Mock, lane 1), cells transfected with untagged Tat (pcTat plasmid, lane 3), or when beads alone were used (lane 4). Intracellular FLAG-Tat or Tat expression did not affect MED14 protein levels compared with the loading control Hsp90 (Fig. 6A, lanes 6 and 7). FLAG-Tat and Tat expression in whole cell lysates (WCL) were verified by immunoblotting analysis and compared with β-actin (Fig. 6B, lanes 6 and 7). Conversely, MED14 immunoprecipitation with anti-MED14 antibodies attached to Sepharose led to the detection of co-immunoprecipitated Tat protein when cells had been transfected with either FLAG-Tat plasmid or untagged Tat (pcTat) (Fig. 6B, lanes 2 and 3, compare with lanes 1 and 4).

FIGURE 2. Inhibition of HIV-1 replication after siRNA-mediated interference of Mediator complex subunits. A, mRNA knockdown efficiency in TZM-bl cells was quantified by qPCR 48 h post-transfection. mRNA expression of each MED gene was normalized to a sample treated without siRNA (Mock). Results from one of three independent experiments are shown. B, TZM-bl cells were transfected with the indicated siRNA and infected 48 h later with HIV-1 NL4-3 strain for 72 h. A nontargeting (NT) siRNA pool and a siRNA targeting CD4 were used as controls. Infection was measured by quantifying expression of β-gal reporter and validated with a dose-response effect of the reverse transcription inhibitor AZT (white bars). Viability of transfected cells was monitored by the MTT method and validated with a DMSO dose-response effect (black bars). Means ± S.D. of at least three independent experiments are shown, *, p < 0.05. C, different siRNA targeting MED14 confirmed the antiviral effect at subtoxic concentrations. HeLa TZM-bl cells were transfected with four different siRNAs targeting MED14 at different molarities, and mRNA levels were quantified by qPCR 48 h later. Transfected cells were then infected with HIV-1 NL4-3 strain, and 72 h later infection was quantified or cells were assayed for siRNA-derived toxicity by the MTT assay. A result of one representative experiment of three is shown.
To rule out that DNA may be the bridging factor mediating MED14-Tat interaction, cell lysates were treated with or without benzonase prior to co-IP. MED14 immunoprecipitated with FLAG-Tat in the absence (Fig. 6C, lane 1) or presence (lane 2) of benzonase, excluding DNA as a bridging factor for the described protein-protein interaction. WCL from FLAG-Tat expressing cells after benzonase treatment showed no differences on Tat or Med14 expression (Fig. 6C, lanes 4 and 5).

FIGURE 3. MED14 and MED27 impaired HIV-1 replication in MDM. A, mRNA knockdown efficiency in MDM cells was quantified by qPCR 72 h post-transfection. mRNA expression of each MED gene was normalized to a sample treated without siRNA (Mock). Means ± S.D. of three independent experiments are shown. B, MDM were transfected with the indicated siRNA and infected (INF) 72 h later with vesicular stomatitis virus-pseudotyped NL4-3-GFP virus. Infection was measured as the percentage of GFP-positive cells in siRNA-treated macrophages and expressed as the percentage to mock-treated cells. AZT was used as a control (white bars). In parallel, cell viability was assayed in uninfected (UN) siRNA-treated macrophages by counting live cells using flow cytometry (black bars). Means ± S.D. of three independent donors are shown. RALT, raltegravir. *, p < 0.05.

FIGURE 4. Selected Mediator subunits have a role in HIV-1 cycle at the early and late transcription level. Integrated proviral DNA (gray bars), TAR RNA (white bars), and tat/rev/nef late viral transcripts (black bars) were compared by qPCR in siRNA-treated TZM-bl cells infected for 24 h (for integrated proviral DNA) or 48 h (for TAR and late viral transcripts) with NL4-3 HIV-1 strain. Values are expressed as a percentage of the control cells treated with an NT siRNA. The RT inhibitor AZT, the integrase inhibitor raltegravir (RALT), and a siRNA targeting ZNRD1 gene (ZNRD1) were used as controls. UN, uninfected. Means ± S.D. of three independent experiments are shown. *, p < 0.05; **, p < 0.005; ***, p < 0.0005.
Efficacy of DNA removal was controlled by qPCR amplification of genomic RNase P gene and graphed in Fig. 6C.

MED14 is in contact with some other MED subunits into the whole complex that could be, in turn, mediating its interaction with Tat. To test this, apart from MED14, we also knocked down the MED subunits that interact with MED14 either in the same module (MED21 and MED26) or in a different module (MED16). As a negative control, MED12, which is in the CDK8 module, was also knocked down (Fig. 6D). As a negative control, MED12, which is in the CDK8 module, was also knocked down. Despite significant down-regulation of MED14 mRNA and protein by siRNA, the remaining protein could still be immunoprecipitated with FLAG-Tat, reinforcing our results (Fig. 6E).

In all siRNA-treated FLAG-Tat expressing cells, the absence of other MED subunits did not affect the interaction between Tat and MED14 (Fig. 6F). MED14, Tat and Hsp90 protein levels were compared in whole cell extracts (Fig. 6G), corresponding to the lysates used in Fig. 6, E and F. These results indicate that Tat associates with MED14 in cultured cells. They also demonstrate for the first time a possible physical interaction between the viral protein Tat and the Mediator complex.

**DISCUSSION**

A high number of cellular factors modulate HIV infection (1, 33), including Mediator subunits that have been previously identified as potential hits in whole genome siRNA-based screenings (34). Here, we provide a detailed analysis of the role of MED subunits in HIV replication and specifically on viral transcription, demonstrating that Mediator complex is a relevant factor affecting HIV-1 replication.
We have identified nine Mediator subunits whose depletion significantly affects HIV-1 replication confirming previous reports linking Mediator to HIV (11, 34, 35). We could not identify any association between HIV replication or transcription and MED proteins associated with the CDK8 module (34), but we could replicate the effect of four subunits that appeared in at least two siRNA screens before: MED6, MED7, MED14, and MED28 (Table 2). MED subunits identified in this work belong to different functional domains in the whole MED complex, including four head-module factors (MED6, MED11, MED28, and MED30) and five middle module factors (MED7, MED14, MED21, MED26, and MED27).

Moreover, MED subunits selected from our siRNA screen were shown to control HIV-1 transcription by significantly reducing viral mRNA without affecting HIV-1 integration (Fig. 4). Importantly, the four subunits previously identified as host factors affecting HIV replication in the whole genome siRNA screen affected particularly the early stage of HIV-1 transcription, limiting the formation of the TAR element. In contrast, newly identified MED subunits showed prevalent roles in the elongation stage of the transcription of viral genes, probably when P-TEFb recruitment via Tat and TAR takes over and results in abundant HIV replication. This suggests that the Mediator complex may act as an interacting activator of P-TEFb, mediating RNAP II function and increasing rates of initiation as well as elongation of transcription as suggested (30). Moreover, in accordance with previous results showing MED26 as a key element interacting with elongation factors to mediate the elongation phase during transcription (36), we found reduced elongated viral transcripts in cells without MED26, although formation of TAR was not affected during initiation of transcription.

Factors like experimental noise, timing of sampling, or different filtering criteria likely contribute to identify different MED subunits in previously published siRNA screens (9), so a more detailed study with a reduced number of genes is needed to confirm and validate host proteins affecting HIV life cycle, as we demonstrated here.

Of note, we show here that siRNA-mediated interference of MED subunits MED14 and MED27 significantly affects HIV-1 replication in primary cells (macrophages). However, not all the MED subunits identified to affect viral replication in HeLa cells...
appeared to have impact in HIV infection of MDM, suggesting either that additional post-transcriptional control mechanisms may regulate MED function in MDM or that differences at the level of transcriptional regulation between cell types may exist.

In addition to the effect on initiation and elongation steps during HIV transcription, Tat transactivation assays revealed that depletion of the Mediator complex effectively inhibited Tat-mediated activation of the HIV-1 LTR (around 40–60%). In agreement with Zhou et al. (11), Tat-mediated transcription was reduced after RNAi knockdown of MED6 and MED14 and to a lesser extent by MED11, MED26, MED27, and MED30 subunits. Some residual levels of Tat-mediated transactivation were always observed, independently of the basal transcription of the LTR (Fig. 5). This residual transcription could be due to the incomplete mRNA silencing, the compensation by other Mediator subunits (15), or the participation of other host proteins implicated in Tat-dependent HIV transcription (37–40).

MED is a co-activator complex acting as a bridge between transcription factors that are mainly linked to the Tail module and the transcription machinery interacting strongly with RNAP II via its Head module factors (41). Although the whole complex changes its conformation and is involved in many other molecular mechanisms, that premise allowed us to select the MED14 as the candidate for our co-immunoprecipitation analysis to prove a possible interaction with the HIV-1 protein Tat that could signal downstream in the MED complex the initiation of HIV transcription, as we showed in Fig. 4. To prove possible interactions of HIV-1 Tat protein and MED14 in cultured cells, FLAG-Tat or Tat were expressed in HEK293T cells and then immunoprecipitated with anti-FLAG antibodies. MED14 was found to co-immunoprecipitate with the Tat HIV protein, and this interaction was not mediated by other interacting MED subunits or DNA.

Conversely, when MED14 was immunoprecipitated with anti-MED14 monoclonal antibodies, Tat was found in the immunoprecipitates. These results indicate for the first time a direct in vitro interaction between the MED14 subunit and HIV-1 Tat. Tat interacts with RNAP II (40) in a context where other cellular proteins exert an influence onto this interaction, with MED14 being a candidate as demonstrated here by its physical interaction with HIV Tat protein, although the presence of other Mediator subunits cannot be excluded. More studies will be needed to define the intricate pattern of interactions within the three main Mediator modules in relation to Tat, although our co-immunoprecipitation study offers the starting point to identify the Mediator cluster mediating the HIV transcription process.

Whether the lack of a Mediator subunit could minimize or prevent expression of a specific set of genes remains to be established, but a growing number of studies have proposed Mediator as a therapeutic target for several diseases such as cancer, cardiovascular disease, and metabolic and neurological disorders (12, 20). Targeting a single Mediator subunit might block a specific pathway but allow a majority of cellular transcription to function normally, and this effect could vary depending both on gene and cell type. Interestingly, MED23, MED14, and MED26 have recently been identified as important proteins for the transcription of early genes of human adenovirus 5 (42) suggesting a broader role for Mediator complex in viral infections and a possible target for intervention.

TABLE 2
Comparison of MED subunits that have been previously linked to HIV-1 replication
Labeled cells show MED subunits identified as “hits” by the indicated papers. Last column shows the MED subunits identified as hits by this work.

| MED subunit | Brass et al. | Zhou et al. | König et al. | This work |
|-------------|--------------|-------------|--------------|------------|
| MED 1       |              |             |              |            |
| MED 4       |              |             |              |            |
| MED 6       |              |             |              |            |
| MED 7       |              |             |              |            |
| MED 8       |              |             |              |            |
| MED 9       |              |             |              |            |
| MED 10      |              |             |              |            |
| MED 11      |              |             |              |            |
| MED 12      |              |             |              |            |
| MED 13      |              |             |              |            |
| MED 14      |              |             |              |            |
| MED 15      |              |             |              |            |
| MED 16      |              |             |              |            |
| MED 17      |              |             |              |            |
| MED 18      |              |             |              |            |
| MED 19      |              |             |              |            |
| MED 20      |              |             |              |            |
| MED 21      |              |             |              |            |
| MED 22      |              |             |              |            |
| MED 23      |              |             |              |            |
| MED 24      |              |             |              |            |
| MED 25      |              |             |              |            |
| MED 26      |              |             |              |            |
| MED 27      |              |             |              |            |
| MED 28      |              |             |              |            |
| MED 29      |              |             |              |            |
| MED 30      |              |             |              |            |
| MED 31      |              |             |              |            |

In established chronic HIV-1 infections, activating specific transcription factors by regulating selective promoters during gene expression could represent a potential tool for eradication strategies. Thus, the identification of cellular collaborators...
Role of the Mediator Complex in HIV-1 Transcription

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Acknowledgments—We thank the National Institutes of Health (AIDS Research and Reference Reagent Program) and the European Union Programme ERA Centralized Facility for AIDS Reagents, National Institute for Biological Standards and Control (NIBSC), United Kingdom, for reagents.

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JOURNAL OF BIOLOGICAL CHEMISTRY 27675

OCTOBER 3, 2014 • VOLUME 289 • NUMBER 40

AASBMB
Role of the Mediator Complex in HIV-1 Transcription

Opaluch, A. M., Caldwell, J. S., Weitzman, M. D., Kuhlen, K. L., Bandyopadhyay, S., Ideker, T., Orth, A. P., Miraglia, L. J., Bushman, F. D., Young, J. A., and Chanda, S. K. (2008) Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell 135, 49–60

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