A Role of RNA Helicase A in cis-Acting Transactivation Response Element-mediated Transcriptional Regulation of Human Immunodeficiency Virus Type 1

RNA helicase A (RHA) has two double-stranded (ds) RNA-binding domains (dsRBD1 and dsRBD2). These domains are conserved with the cis-acting transactivation response element (TAR)-binding protein (TRBP) and dsRNA-activated protein kinase (PKR). TRBP and PKR are involved in the regulation of HIV-1 gene expression through their binding to TAR RNA. This study shows that RHA also plays an important role in TAR-mediated HIV-1 gene expression. Wild-type RHA preferably binds to TAR RNA in vitro and in vivo. Overexpression of wild type RHA strongly enhanced viral mRNA synthesis and virion production as well as HIV-1 long terminal repeat-directed reporter (luciferase) gene expression. Substitution of lysine for glutamate at residue 236 in dsRBD2 (RHA236E) reduced its affinity for TAR RNA and impaired HIV-1 transcriptional activity. These results indicate that TAR RNA is a preferred target of RHA dsRBDs and that RHA enhances HIV-1 transcription in vivo in part through the TAR-binding of RHA.

RNA helicase A (RHA) catalyzes the unwinding of duplex RNA and DNA in a process coupled with the hydrolysis of NTPs.

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†‡‡ The abbreviations used are: RHA, RNA helicase A; CREB, cAMP-responsive element-binding protein; CBP, CREB-binding protein; pol II, RNA polymerase II; dsRBD, double-stranded RNA-binding domains; HIV-1, human immunodeficiency virus, type 1; LTR, long terminal repeat; TAR, cis-acting transactivation response element; RRE, Rev response element; HEK, human embryonic kidney; HA, hemagglutinin; GST, glutathione S-transferase; P-TEFb, positive transcription elongation factor b; PABP, polyadenylation factor; lkb, kilobase pair; wt, wild type; TRBP, TAR-binding protein; PCR, polymerase chain reaction; ssRNA, single-stranded RNA; PKR, dsRNA-activated protein kinase; RGG, Arg-Gly-Gly; NF-κB, nuclear factor-κB; RSV, Rous sarcoma virus.

(1, 2). We have previously shown that RHA mediates association of the CREB-binding protein (CBP) with RNA polymerase II (pol II) (3) and that RHA links breast cancer-specific tumor suppressor protein (BRCA1) to pol II (4). RHA consists of two type A double-stranded RNA-binding domains (dsRBDs) (5, 6), a classical Walker type NTP-binding site, a DEAH/D helicase domain, and a single-stranded nucleic acid-binding domain characterized by Arg-Gly-Gly (RGG) repeats (7). Trpysin-digested RHA, lacking both dsRBDs and the RGG repeat sequence, has reduced helicase activity, implicating these domains in the unwinding function of RHA (7). Amino acids 1–262 and 255–664 of RHA have proved to be CBP- and pol II-binding sites, respectively (3). RHA shuttles between nucleus and cytoplasm with a cis-acting constitutive transport element in simian retroviruses (8). It has been proposed that RHA is necessary for releasing both constitutive transport element- and HIV-1 Rev response element (RRE)-containing RNA from spliceosomes prior to the completion of splicing (9).

After integration of HIV-1 into the host genome, the nuclear factor-xB (NF-xB) binds to enhancer elements in the HIV-1 long terminal repeat (LTR) and stimulates the expression of the viral genome in a signal-dependent manner (10, 11). HIV-1 expression can be divided into two phases (early and late). In the early phase, the majority of viral mRNA is multiply spliced to produce 2-kb transcripts that encode the regulatory proteins, including Tat and Rev, necessary to activate HIV-1 expression. A transition subsequently occurs to accumulate singly spliced (4-kb) and full-length (9-kb) transcripts encoding the viral structural proteins and providing the genomic RNA (12–14). Regulation of the viral RNA-splicing transition mechanism is reported to involve the protection and transport of full-length HIV-1 RNA by Rev (15, 16).

TAR, a nascent viral leader RNA transcribed from the R region of the LTR, plays an important role in HIV-1 gene expression and forms a unique stem and loop structure (17, 18). Tat function is mediated by the TAR RNA and requires the recruitment of a complex consisting of Tat and cyclin T1 component of positive transcription elongation factor b (P-TEFb) bound to TAR (19). A defect of Tat-induced transactivation in murine cells was attributed to the lack of a functional cyclin T1 (20, 21). Alternatively, the defect was linked to the reduced abundance of p300 and p300/CREB-associated factor (22). In addition, several cellular cofactors play crucial roles in HIV-1 gene expression through binding to the TAR RNA. For instance, TRBP consists of two type A dsRBDs and another type B dsRBD and activates the HIV-1 LTR (6, 23) through binding of the second type A dsRBD (dsRBD2) to the TAR RNA (24).
PKR contains two type A dsRBDS and functions as a cellular antiviral factor by inhibiting eukaryotic initiation factor 2 via phosphorylation of its α-subunit (25). A similar function of PKR is mediated by an interaction with several kinds of RNA, leading to PKR autophosphorylation (26). Like TRBP and PKR, RHA possesses dsRBDS and may act as a cellular transcriptional regulator. Of particular interest is whether RHA binds to the TAR RNA and influences HIV-1 gene expression. This study shows that RHA acts as a novel TAR-binding cellular cofactor and enhances HIV-1 LTR-directed gene expression and viral production in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney (HEK) 293 (27) and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cells were grown at 37 °C in a humidified 95% air, 5% CO2 atmosphere.

Plasmids—Plasmid pHg LTR-Luc encodes a chimeric gene consisting of the HIV-1 LTR containing two intact eB elements and a luciferase gene (28). The mNF-B LTR-Luc plasmid was constructed by inserting both of the eB element-mutated LTR fragments (14) into the Smal/HindIII site of PCG vaccination containing a luciferase gene without a promoter (Toyo-Inki). The pcDSR/tat plasmid, a mammalian expression vector for HIV-1 Tat, was constructed as described previously (28). The HIV-1 pNL4–3 proviral DNA clone (30) was obtained from the AIDS Research Institute of the National Institutes of Health and was contributed by Malcolm Martin. Wild-type (wt) RHA and RHA(AATP) constructs were prepared as described previously (3). The RHA(AATP) and RHA(WESTRA) constructs contain single amino acid substitutions leading to defects in ATPase/helicase activity and pol II binding ability of RHA, respectively. RHA(BESTRA) containing the lysine to glutamate substitution at residue 236 was generated by PCR. The wt RHA construct and three RHA mutants were tagged by hemagglutinin (HA) for Western blot assays and immunoprecipitation studies. To obtain the RNA probes by in vitro transcription, the pcRHA and ΔoopTAR plasmids were constructed by inserting PCR-generated cDNA fragments (5′-GGGAGCTCTCTGGCTAACTAGGGAACCC-3′) and 5′-GGGTCTCTCTTGTTAGACCAGATGGACGGCGGAGCTCTCTGGCTAACTAGGGAACCC-3′) with HindIII linker into the HindIII site of pcDNA3 (Invitrogen). The plasmids as described above were purified using cesium chloride gradients.

A series of RHA polypeptides (1–262, 255–664, 649–1077, and 1064–1270) were transfected with 100 ng of pHg LTR-Luc reporter, and 2 ng (for HEK 293 cells) or 10 ng (for HeLa cells) of pcDSR/tat (tat) or “empty vector” (–tat), together with various amounts of wt RHA expression vector as indicated. To ensure an equal amount of DNA, the control plasmid was added in each transfection. Luciferase activity was normalized to transfection efficiency via cotransfection with 100 ng of RSV-β-gal control plasmid, 2 or 10 ng of pcDSR/tat, and various amounts of RHA, using the calcium phosphate method as described previously (31). To ensure an equal amount of DNA, “empty plasmids” were added in each transfection. In the reporter gene assays, luciferase activity derived from expression of the centromere DNA and mNF-B LTR-Luc reporter plasmid was inhibited by tobacco LTR TAR plasmid containing the β-galactosidase activity from cotransfected Rous sarcoma virus (RSV)-expression plasmid containing the β-galactosidase gene (RSV-β-gal) (3). Luciferase activity was measured with AutoLumat (Berthold). All experiments were performed in triplicate, and all results were obtained from at least three separate experiments. Equivalent expression of RHA constructs was verified by Western blot analysis using the anti-HA antibody 12CA5 (Roche Molecular Biochemicals).

**RHA Binds to TAR and Enhances HIV-1 Transcription**

**Fig. 1.** The effects of RHA on HIV-1 LTR-directed gene expression. HEK 293 (A) and HeLa cells (B) were transfected with 100 ng of pHg LTR-Luc reporter, and 2 ng (for HEK 293 cells) or 10 ng (for HeLa cells) of pcDSR/tat (tat) or “empty vector” (–tat), together with various amounts of the wt RHA expression vector as indicated. To ensure an equal amount of DNA, the control plasmid was added in each transfection. Luciferase activity was normalized to transfection efficiency via cotransfection with 100 ng of RSV-β-gal control plasmid, 2 or 10 ng of pcDSR/tat, and various amounts of RHA, using the calcium phosphate method as described previously (31). To ensure an equal amount of DNA, “empty plasmids” were added in each transfection. In the reporter gene assays, luciferase activity derived from expression of the centromere DNA and mNF-B LTR-Luc reporter plasmid was inhibited by tobacco LTR TAR plasmid containing the β-galactosidase activity from cotransfected Rous sarcoma virus (RSV)-expression plasmid containing the β-galactosidase gene (RSV-β-gal) (3). Luciferase activity was measured with AutoLumat (Berthold). All experiments were performed in triplicate, and all results were obtained from at least three separate experiments. Equivalent expression of RHA constructs was verified by Western blot analysis using the anti-HA antibody 12CA5 (Roche Molecular Biochemicals).

**TAR Binding Assays—**Total cellular extract from Escherichia coli BL21, expressing a series of RHA polypeptides or full-length TRBP fused to GST protein, was electrophoresed on an SDS gel and transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore). TAR binding assays were performed as reported previously (23). Filters were incubated in binding buffer (20 mM HEPES, pH 7.3, 40 mM KCl, 1.5 mM MgCl2, and 1 mM diethiothreitol) with 10 μg/ml yeast RNA, 10 μg/ml calf thymus DNA, and 200 pmol/ml gel-purified 32P-labeled TAR RNA probe. Filters were washed with binding buffer and then exposed to x-ray film. Comparable expression of GST-fused protein was determined by Western blotting with an anti-GST antibody (Amersham Pharmacia Biotech) and Coomassie Brilliant Blue staining.

**Wild-type TAR, Δoop TAR RNA, and ssRNA were obtained by in vitro transcription using BsnHI-cleaved pTAR or ΔoopTAR plasmid and Xhol-cleaved pcDNA3, respectively. GST and GST (1–262)-polyepitope helixes were incubated in binding buffer with 10 μg/ml calf thymus DNA, 1 pmol/ml 32P-labeled wt TAR RNA, Δoop TAR RNA or ssRNA probe, and 300 pmol/ml unlabeled RNA or yeast tRNA (Sigma) as a competitor. The filters were washed with binding buffer and exposed to x-ray film.

**Immunoprecipitation and Slot Blot Assays—**HEK 293 cells were transfected with 3 μg of pHg LTR-Luc or GSlb-Luc (32), 150 ng of pcDSR/tat or 300 ng Gal4-VP16 (33), and 3 μg of each RHA construct. After 24 h of transfection, the cells were lysed in 1 ml of 0.65% Nonidet P-40 lysis buffer (20 mM HEPES, pH 7.3, 150 mM KCl, 1.5 mM MgCl2, 0.65% Nonidet P-40). Cell lysates were pre-cleared with 5 μg of normal mouse IgG (Santa Cruz Biotechnology) conjugated to protein G-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. After a brief centrifugation, the lysates were mixed with 5 μg of normal mouse IgG (Santa Cruz Biotechnology) conjugated to protein G-Sepharose beads. After an overnight incubation at 4 °C, the beads were washed three times with lysis buffer. RNA was extracted from the beads with ISO-GEN (Nippon Gene) and then blotted onto GeneScreen Plus membrane (PerkinElmer Life Sciences) using a slot blotting apparatus (HYBRID-SLOT MANIFOLD, Life Technologies, Inc.). The membrane was hybridized with a 32P-labeled luciferase gene probe generated by PCR.
using the primer pair 5′-GGATGGAAACCGCTGGAGAG-3′ and 5′-GTTTCATAGCTTCTGCCAACCG-3′ and exposed to x-ray film. Equivalency for immunoprecipitation of HA-tagged RHA was verified by Western blot analysis.

p24 Assays—HEK 293 cells were transfected with 10 ng of pNL4-3, 20 ng of PGV-C control plasmid containing the SV40-luciferase gene (Toyo-inki, Tokyo Japan), and various amounts of RHA construct. To ensure an equal amount of DNA, empty plasmid was added in each transfection. After 24 and 48 h of transfection, culture supernatants were collected and tested for HIV-1 p24 antigen levels using a p24 antigen detection kit (Retro-tek, Zepto Metrix Corporation, Buffalo, NY). Equivalent transfection efficiency was verified by luciferase activity derived from the cotransfected PGV-C control plasmid. All experiments were performed in triplicate.

Northern Blot Assays—HEK 293 cells were transfected with 500 ng of pNL4-3, 1 μg of PGV-C control plasmid, and 5 μg of each RHA construct. After 24 h of transfection, polyadenylated RNA was extracted from the transfected cells using an mRNA purification kit (Amersham Pharmacia Biotech), run on a 0.8% agarose gel, and blotted onto GeneScreen Plus membrane. The membrane was hybridized with a 32P-labeled HIV-1 LTR probe and then exposed to x-ray film. The probe was amplified by PCR using the primers 5′-AGTGCTCAAAGTAGTG-GTG-3′ and 5′-GATCTCCCTCGGTTTACCTTT-3′ and pNL4-3 as a template. Equivalent transfection efficiency was determined by measuring the amount of luciferase mRNA derived from the cotransfected PGV-C control plasmid.

RESULTS

The Effects of RHA on HIV-1 LTR-directed Gene Expression—To assess the regulatory effects of RHA on HIV-1 LTR-directed gene expression, several human cell lines were transfected with the pHyg LTR-Luc reporter plasmid. In HEK 293 cells, wt RHA markedly enhanced Tat-induced reporter activity in a dose-dependent manner (Fig. 1A). In contrast, overexpression of wt RHA did not affect HIV-1 LTR-directed luciferase activity in HeLa cells (Fig. 1B) as described previously (9).

Determination of the RHA Regions Required for TAR Binding—Northwestern assays were conducted to determine whether RHA binds to the TAR RNA. First, we divided full-length RHA into four fragments (Fig. 2A) and examined each fragment for their TAR RNA binding activity. Only RHA-(1–262), which contains both dsRBD1 and dsRBD2, could bind to the TAR RNA (Fig. 2B, left panel). Second, we constructed three deletion mutants of RHA-(1–262) (Fig. 2A). Unexpectedly, only RHA-(160–262) (containing dsRBD2) and not RHA-(1–90) (containing dsRBD1) bound to the TAR RNA (Fig. 2B, middle panel). Amino acids 235–249 in the RHA dsRBD2 are well conserved among the dsRNA-binding protein family (Fig. 2C). In particular, the lysine at residue 211 (Lys-211) in the

![Fig. 2. Determination of the RHA region required for TAR binding in vitro. A, schematic representation of RHA (top line) and its functional domains (dsRBD, dsRNA binding domain; pol II, RNA polymerase II-binding site; ATP, ATP-binding site; DEAH/D, helicase domain; RGG, Arg-Gly-Gly repeat; CBP, CBP-binding site). Bottom lines represent the portions of RHA deletion mutants used to make the GST fusion proteins. TAR binding activity of each mutant is indicated at right. B, binding of RHA mutants to TAR RNA. Total cellular extract was electrophoresed on an SDS gel and then transferred to polyvinylidene difluoride membrane for Northwestern analysis (TAR binding). The same membrane was used for Western blotting with an anti-GST antibody (α-GST Western), and Coomassie Brilliant Blue (CBB) staining. The clones used are indicated at the top of each lane and correspond to the protein described in A. Negative (GST) and positive (GST-TRBP) controls are the cell lysate from E. coli BL21 cells containing the GST and GST-TRBP fusion proteins, respectively. The arrowheads indicate GST fusion proteins in the panels of CBB and α-GST Western. C, comparison of amino acids sequences between RHA and TRBP dsRBD2.
TRBP dsRBD2 is critical for binding to the TAR RNA (34). Therefore, we constructed two mutants (RHA-(1–262/Δ235–249) and RHA-(1–262/K236E)) that contain the intact dsRBD1 and the mutated dsRBD2. RHA-(1–262/Δ235–249) is a deletion mutant missing amino acids 235–249 from RHA-(1–262). RHA-(1–262/K236E) is also an RHA-(1–262) mutant with lysine at residue 236 (Lys-236) substituted with glutamate. The Lys-236 of RHA corresponds to the Lys-211 of TRBP. Both RHA-(1–262) mutants failed to interact with the TAR RNA (Fig. 2B, right panel), indicating that amino acids 235–249 and in particular residue Lys-236 are essential for TAR binding in vitro.

RHA dsRBD Preferentially Binds the Stem of TAR RNA in Vitro—HIV Tat and cyclin T1 recognize the bulge and loop of TAR RNA, respectively (20). Similarly, it is important to define the region of TAR that interacts with the RHA dsRBD and to confirm its relative specificity. Presumably, RHA dsRBD binds to the stem region of TAR RNA, since dsRBD binds to dsRNA. The Δloop TAR RNA probe illustrated in Fig. 3A was therefore constructed. GST-fused RHA-(1–262) and GST alone (negative control) were blotted onto filters, and the filters were incubated with an equivalent amount of 32P-labeled wt TAR, Δloop TAR RNA, or ssRNA. Interestingly, the binding affinity of Δloop TAR RNA to RHA-(1–262) was significantly stronger than that of wt TAR RNA. ssRNA bound weakly to RHA-(1–262). None of the tested probes bound to GST (Fig. 3B). In competition assay (Fig. 3C), the binding of wt TAR RNA to RHA-(1–262) was almost completely inhibited by wt TAR or Δloop TAR RNA competitor at a concentration of 100-fold higher than that of the 32P-labeled probe. In contrast, the ssRNA competitor and yeast tRNA slightly reduced the probe binding. These results suggest that the RHA dsRBD preferably recognizes the stem of the TAR RNA.

Association of RHA with TAR in Vivo—To test for in vivo interaction of the TAR RNA with the RHA complex, communoprecipitation assays were performed on whole cell lysates from HEK 293 cells cotransfected with pHygLTR-Luc or 3 μg of G5b-Luc, 150 ng of pCD-SRat/tat or 300 ng of Gal4-VP16, and 3 μg of wt RHA (HA-wt RHA), RHAK236E (HA-RHAK236E), or empty vector (mock) were immunoprecipitated with normal mouse IgG (IgG) or anti-HA antibodies (α-HA). The extracted RNA from each communoprecipitated complex (Co-IP RNA) was subjected to slot blot assay with luciferase gene-specific probe. Comparable amounts of TAR-containing or TAR-negative luciferase mRNA from each lysate were determined (10% input). Equivalency for immunoprecipitation of HA-tagged RHA with anti-HA antibodies was verified by Western blot analysis (α-HA IP-Western). No-transfectant (No Tf), mock-transfectant (mock), and normal mouse IgG (IgG) were used as negative controls.
RHA precipitant (Fig. 4, upper panel). However, although significantly less mRNA was coprecipitated with RHA_{K236E}. No precipitant was identified in the negative controls (no-transfectant and "mock-transfectant"). Furthermore, the association of wt RHA with the TAR-negative luciferase mRNA was as weak as that of RHA_{K236E} and the TAR-containing luciferase mRNA. These results indicate that the TAR-containing mRNA existed in the RHA complex in vivo and that the complex was formed primarily via the binding of RHA dsRBD2 to the TAR RNA. Although RHA-(1–262/K236E) could not bind to the TAR RNA in vitro (Fig. 2B), the TAR-containing mRNA was weakly associated with RHA_{K236E} complex in vivo. Thus, it cannot be excluded that RHA also binds to mRNA in a TAR-independent fashion, as in the case of RHA binding to ssRNA through the RGG motif (7).

Enhancement of TAR-dependent Gene Expression by RHA—The finding that RHA binds to the TAR RNA in vivo prompted a study of the role of RHA in TAR-mediated gene expression. Transient transfections of the pHgy LTR-Luc reporter plasmid were conducted to analyze the effect of RHA_{K236E} on HIV-1 LTR-directed gene expression. RHA_{K236E}, but neither RHA_{MATT} nor RHA_{N339A}, enhanced the reporter activity (Fig. 5, lanes 7–12), although the effect of RHA_{K236E} was significantly lower than that of wt RHA (≈40%). This reduced effect of RHA_{K236E} may be due to the lack of TAR-binding ability. Alternatively, RHA may be functionally bound to the CBP-NF-κB complex on the κB elements of HIV-1 LTR (35, 36).

To exclude this possibility, transient transfection assays with the mNF-κB LTR-Luc reporter plasmid that contained two mutated κB elements were also performed. Mutation of the κB elements strongly reduced luciferase activity (Fig. 5, lane 4 versus 16), as described previously (11). Wild-type RHA markedly enhanced the Tat-induced reporter activity in a dose-dependent manner (Fig. 5, lanes 16–18), and although both RHA_{MATT} and RHA_{N339A} slightly increased the luciferase activity, RHA_{K236E} had no effect (Fig. 5, lanes 19–24). These results indicate that the association of RHA with the TAR RNA is required for the RHA-induced transactivation in the mutated κB LTR. Conversely, the higher activity of RHA_{K236E} in intact κB LTR compared with mutated κB LTR suggests that RHA can also interact with HIV-1 LTR through the intact κB elements in a TAR-independent fashion.

The Effects of RHA on HIV-1 Production—To elucidate the role of TAR-binding ability of RHA in HIV-1 viral replication, HEK 293 cells were cotransfected with pNL4-3 and different amounts of wt RHA or RHA_{K236E} (Fig. 6A). After 24 h of transfection, HIV-1 p24 production was enhanced 5-fold in the wt RHA transfectants in a dose-dependent manner (Fig. 6A, left panel), as previously demonstrated in HeLa cells (9). The effect of RHA_{K236E} on HIV-1 p24 production was significantly less than that of wt RHA (≈30%), which was consistent with the results in the reporter gene assays (Fig. 5). Similar results were also obtained after 48 h of transfection, except in the cells expressing for higher p24 antigen levels (Fig. 6A, right panel). These results suggest that the association of RHA with the TAR RNA is partly required for the RHA-enhanced HIV-1 gene expression.

Finally, to elucidate the role of RHA in transcriptional regulation of HIV-1, HEK 293 cells were transfected with pNL4-3 and each RHA construct. Fig. 6B demonstrates that three different lengths of HIV-1 mRNA (≈2-, 4-, and 9-kb transcripts) were detected by Northern blot assay. Equivalent transfection efficiency was confirmed by the amount of luciferase mRNA derived from each cotransfected PGV-C control plasmid (data not shown). The amounts of all HIV-1 mRNA transcripts were increased by wt RHA coexpression (Fig. 6B). No other RHA mutants affected HIV-1 mRNA synthesis or p24 production. These results strongly support a functional role of RHA in the transcriptional activation of HIV-1.

**DISCUSSION**

RHA belongs to the dsRNA-binding protein family, which includes TRBP and PKR. These proteins display variable RNA-binding characteristics. For instance, the PKR dsRBD1 displays higher affinity than the PKR dsRBD2 for several kinds of RNA, including the TAR RNA (37, 38). The TRBP dsRBD2 alone can bind to TAR RNA independent of other dsRBDs (39). Two polypeptides containing each of the RHA dsRBDs bind to poly(rI·rC) dsRNA with similar affinity, and it has been considered that the two RHA dsRBDs cooperate to interact with dsRNA, such as with poly(rI·rC) (7). The results presented here show that only dsRBD2, and not dsRBD1, could bind to TAR RNA in vitro (Fig. 2B). This RNA-binding property of RHA is similar to that of TRBP. Gel mobility shift assays with polypep-
cells were transfected with 500 ng of pNL4-3, and the luciferase activity derived from cotransfected PGV-C plasmid. All p24 antigen levels. Equivalent transfection efficiency was verified by comparing the amount of RNA in each lane, the replica membrane hybridized with the $^{32}$P-labeled HIV-1 LTR probe. To demonstrate that RHA enhances HIV-1 LTR-directed gene expression in a $\kappa$B element-dependent and -independent fashion, NF-$\kappa$B functionally binds to the $\kappa$B elements in the HIV-1 LTR (10, 11) and associates with CBP (35, 36). Furthermore, RHA was shown to mediate the association of CBP with pol II (3). The RHA $K_{236}$ mutant was capable of binding to CBP in GST pull-down assays (data not shown). Therefore, the certain activity of RHA $K_{236}$ in the reporter assays using intact $\kappa$B elements may indicate that RHA also mediates the association of CBP with pol II on the $\kappa$B-dependent HIV-1 preinitiation complex. Conversely, it was shown that RHA enhances HIV-1 gene expression, at least in part, through its TAR-binding activity and pol II binding ability of RHA contribute to the CREB-dependent transcription (3). It can be proposed from the current studies that these functions of RHA may also be important for HIV-1 transcription. Moreover, the data demonstrate that RHA enhances HIV-1 LTR-directed gene expression in a $\kappa$B element-dependent and -independent fashion. NF-$\kappa$B functionally binds to the $\kappa$B elements in the HIV-1 LTR (10, 11) and associates with CBP (35, 36). Furthermore, RHA was shown to mediate the association of CBP with pol II (3). The RHA $K_{236}$ mutant was capable of binding to CBP in GST pull-down assays (data not shown). Therefore, the certain activity of RHA $K_{236}$ in the reporter assays using intact $\kappa$B elements may indicate that RHA also mediates the association of CBP with pol II on the $\kappa$B-dependent HIV-1 preinitiation complex. Conversely, it was shown that RHA enhances HIV-1 gene expression, at least in part, through its TAR binding. TAR RNA is required for the recruitment of a complex consisting of Tat and the cyclin T1 component of P-TEFb (19–21). It is possible that both the ATPase/helicase activity and pol II binding ability of RHA may be required for the unwinding of highly structured RNA, such as TAR RNA, and following HIV-1 transcription.

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