NEAT1 Long Noncoding RNA and Paraspeckle Bodies Modulate HIV-1 Posttranscriptional Expression

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ABSTRACT Most of the human genome is transcribed into protein-noncoding RNAs (ncRNAs), including small ncRNAs and long ncRNAs (lncRNAs). Over the past decade, rapidly emerging evidence has increasingly supported the view that lncRNAs serve key regulatory and functional roles in mammalian cells. HIV-1 replication relies on various cell functions. To date, while the involvement of host protein factors and microRNAs (miRNAs) in the HIV-1 life cycle has been extensively studied, the relationship between lncRNAs and HIV-1 remains uncharacterized. Here, we have profiled 83 disease-related lncRNAs in HIV-1-infected T cells. We found NEAT1 to be one of several lncRNAs whose expression is changed by HIV-1 infection, and we have characterized its role in HIV-1 replication. We report here that the knockdown of NEAT1 enhances virus production through increased nucleus-to-cytoplasm export of Rev-dependent instability element (INS)-containing HIV-1 mRNAs.

IMPORTANCE Long protein-noncoding RNAs (lncRNAs) play roles in regulating gene expression and modulating protein activities. There is emerging evidence that lncRNAs are involved in the replication of viruses. To our knowledge, this report is the first to characterize a role contributed by an lncRNA, NEAT1, to HIV-1 replication. NEAT1 is essential for the integrity of the nuclear paraspeckle substructure. Based on our findings from NEAT1 knockdown, we have identified the nuclear paraspeckle body as another important subcellular organelle for HIV-1 replication.

RESULTS HIV-1 infection enhances NEAT1 expression levels. To examine if the expression of lncRNAs is changed by HIV-1 infection, we...
investigated 83 lncRNAs that have been implicated in diseases ranging from neurodegeneration to cancer in HIV-1-infected T cells. Two T-cell lines, Jurkat and MT4, were infected with HIV-1 NL4-3 and compared to mock-infected counterparts for the expression of lncRNAs. From these analyses, we identified several lncRNAs that were reproducibly up- or downregulated in HIV-1-infected Jurkat or MT4 cells (Fig. 1A). These identifications were based on two criteria: first, all changes were at least 2-fold or more compared to findings for controls; second, we dismissed those lncRNAs whose expression was too low (threshold cycle $C_T > 30$) so as to make their real-time quantification unreliable. Accordingly, 18 lncRNAs were identified from HIV-1-infected Jurkat and MT4 cells. As shown in Fig. 1B, 8 lncRNAs were up- and 5 were downregulated in HIV-1-infected Jurkat cells, while 6 lncRNAs were up- and 5 were downregulated in HIV-1-infected MT4 cells. Six lncRNAs were changed in both Jurkat and MT4 cells (BIC, LIT, MALAT1, NEAT1, PANDA, and SRA, downregulated) (Fig. 1B).

Among the 4 upregulated lncRNAs common to Jurkat and MT4 cells, we pursued the characterization of NEAT1 for several reasons. First, its expression was upregulated consistently among the highest levels in both Jurkat and MT4 cells in all the lncRNAs that were changed by HIV-1 infection (two other lncRNAs were higher than NEAT1 in MT4 cells, but they were not highly upregulated in Jurkat cells); second, in the published literature, NEAT1 has been reported to be an important constituent of paraspeckles, a nuclear substructure that is found in all cultured cell lines and primary cells except for embryonic stem cells (41, 42). Because several cellular proteins that play roles in HIV-1 replication are found in paraspeckles (e.g., PSF, p54nrb, and matrin 3 [43–45]), we were moved to investigate NEAT1 interaction with HIV-1. Consistent with our notion, published studies from mice infected with Japanese encephalitis virus or rabies virus have found increased NEAT1 expression (46), suggesting that NEAT1 may have interactions with these viruses as well as many other viruses.

NEAT1 has two isoforms (47), NEAT1_1 (3.7 kb in humans) and NEAT1_2 (23 kb in humans) (Fig. 2A); the isoforms are also named MENe and MENb. We next confirmed our above profiling results using two primer pairs to quantify NEAT1 RNA isoforms by real-time quantitative reverse transcription-PCR (qRT-PCR). One primer pair recognizes both NEAT1_1 and NEAT1_2, while the second pair is specific for NEAT1_2 only (Fig. 2A). When Jurkat, MT4, THP1, and THP1 cells differentiated with phorbol myristate acetate (PMA) (48) were infected with HIV-1, these primers in qRT-PCR assays verified that NEAT1 expression levels in the infected cells were indeed upregulated by approximately 5- to 8-fold (Fig. 2B to E).

Depletion of NEAT1 increases HIV-1 production. The above data show that HIV-1 infection increases NEAT1, but we wondered how NEAT1 expression might reciprocally influence HIV-1 replication. To address this question, we employed small interfering RNA (siRNA) knockdown of NEAT1. NEAT1 has been previ-
LncRNA NEAT1 is upregulated in HIV-1-infected cells. (A) Schematic representation of human NEAT1_1 and NEAT1_2. Positions of the sequences amplified by qRT-PCR are indicated with black boxes. (B to E) Jurkat cells (B), MT4 cells (C), THP1 cells (D), or THP1 cells differentiated with PMA (E) were infected with VSV-G-pseudotyped HIV-1 NL4-3. Three days after infection, NEAT1 expression levels were analyzed by qRT-PCR. Results from three separate experiments are shown as mean values ± SD.

Knockdown of NEAT1 increases expression of INS-containing HIV-1 mRNA. We wished to understand which step of HIV-1 expression is affected by the knockdown of NEAT1. HIV-1 transcripts are composed of unspliced, singly spliced, and multiply spliced RNAs (57). Unspliced and singly spliced HIV-1 transcripts are composed of unspliced, singly spliced, and multiply spliced RNAs. The AS approach reduced NEAT1 (Fig. 4C, left) and increased HIV-1 p24 production (Fig. 4C, right), providing results consistent with those from siRNA-mediated knockdown of NEAT1 (Fig. 4A and B).

Knockdown of NEAT1 affects HIV-1 expression. NEAT1 has been reported to be critical for paraspeckle formation (50); we therefore checked to see how our knockdown of NEAT1 affected the number of paraspeckles in cells. PSF and PSP1 are known protein components of paraspeckles (50). Indeed, we could clearly visualize paraspeckles in green fluorescent protein (GFP)-PSF-transfected cells as green puncta (green); similarly, we could also visualize paraspeckles by staining proteins such as PSF have been shown to influence HIV-1 expression (45); however, because they play roles in splicing and transcription in locales outside paraspeckle bodies, it remains unclear whether these factors act within paraspeckles to affect HIV-1 (51, 52). To investigate a direct contribution by paraspeckle bodies to HIV-1 replication, we reasoned that this issue could be addressed in a more targeted manner through depleting NEAT1 lncRNA. Accordingly, we treated HeLa cells first with NEAT1 siRNA or control siRNA, and then we introduced an HIV-1 NL4-3 molecular clone together with a cytomegalovirus (CMV)-GFP expression plasmid into these cells and assessed how NEAT1 siRNA versus control siRNA affected HIV-1 expression compared to results with CMV-GFP expression. HIV-1 expression was checked by Western blotting for the viral p55 and p24 proteins, while GFP expression was checked by blotting using GFP-specific antibody. In NEAT1 knockdown cells, compared to results for control knockdown cells, p55 and p24 expression was increased in the former over that in the latter cells; in contrast, GFP expression was insignificantly changed in both settings (Fig. 4A). We next verified these results using a slightly different approach, infecting HeLa cells transfected with either NEAT1 siRNA or control siRNA with VSV-G-pseudotyped HIV-1. In these infections, we observed that infection of cells with siRNA depletion of NEAT1 lncRNA produced ~3.5-fold more HIV-1 p24 than infection of cells treated with control siRNA (Fig. 4B). Taken together, an interpretation of the results is that the knockdown of NEAT1 lncRNA reduced paraspeckle bodies (Fig. 3), and the reduction in paraspeckles increased HIV-1 expression (Fig. 4).
An HIV-1 LTR luciferase plasmid was cotransfected with a Tat plasmid into NEAT1-AS- or scrambled-AS-treated HeLa cells. As shown in Fig. 5A, AS-mediated NEAT1 knockdown did not measurably affect Tat-activated LTR transcription. We next examined if NEAT1 might affect posttranscriptional regulation of HIV-1 transcripts. Specifically, we assessed how the knockdown of NEAT1 IncRNA influences unspliced HIV-1 INS-containing RNAs. To address this issue, we employed the p37-RRE and p37-RRE M1-10 reporters in expression assays (Fig. 5B). The p37-RRE reporter expresses a Rev response element (RRE)-containing mRNA that encompasses the p17MA and p24CA portions of gag; this transcript retains well-characterized INS sequences that are inactivated by point mutations in the counterpart p37-RRE M1-10 transcript (60). Thus, the p37-RRE transcript is INS containing, while the p37-RREM1-10 RNA is INS free. We cotransfected a Rev-expressing plasmid with either the p37-RRE or the p37 M1-10-RRE reporter into NEAT1-AS- or scrambled-AS-treated HeLa cells. As shown in Fig. 5B, AS-mediated knockdown of NEAT1 increased Rev-mediated p37-RRE expression but did not affect the expression of the non-INS-containing p37 M1-10-RRE mRNA. These results indicate the existence of an activity provided by knockdown of NEAT1 that is specific for INS-containing HIV-1 transcripts.

It is possible that the above NEAT1 knockdown effect is not simply through a reduction of paraspeckle bodies but may also arise from indirect effects of perturbing the levels of the PSF and/or p54nrb proteins. To exclude the latter explanation, PSF and p54nrb protein levels were checked by Western blotting. The amounts of the PSF and p54nrb proteins were not affected by NEAT1 knockdown, verifying that the observed effect on p37-RRE did not arise from changed levels of the PSF or p54nrb proteins (Fig. 5C).

The above results show a specific NEAT1 effect on HIV-1 INS-containing transcripts expressed from the p37-RRE reporter. To better understand this finding in the setting of HIV-1 infection, we infected HeLa cells transfected with control or NEAT1 siRNA with vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1, and then we separated cellular RNAs into cytoplasmic or nuclear fractions. Because unspliced glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (pre-GAPDH) is located exclusively in the nucleus, pre-GAPDH RNA measured using primers spanning GAPDH intronic sequences was used to verify the quality of our cytoplasmic fractionation and the nuclear fractionation. The stringency of our nuclear/cytoplasmic fractionation was confirmed by the amount of pre-GAPDH RNA that measured at least 100-fold more in the nuclear than in the cytoplasmic fraction (Fig. 6A). When unspliced HIV-1 transcripts were analyzed by qRT-PCR, we found that cytoplasmic unspliced viral transcripts were increased by more than 2-fold after knockdown of NEAT1, while the nuclear unspliced HIV-1 RNAs, which are in excess, were commensurately decreased (Fig. 6B). Spliced HIV-1 RNAs in the nucleus and cytoplasm were not changed by NEAT1 knockdown (data not shown). Taken together, the data support an interpretation that knockdown of NEAT1 IncRNA decreases nuclear paraspeckle bodies (Fig. 3), leading to an increase in the nuclear-to-cytoplasm export and expression of unspliced Rev-dependent HIV-1 INS-containing transcripts (Fig. 6 and 7).
DISCUSSION

Here, we report the first evidence of NEAT1 as an lncRNA involved in HIV-1 replication. We show that HIV-1 infection increases NEAT1 RNA expression by 5- to 10-fold over that for uninfected cells (Fig. 2). Using two different approaches, siRNA and antisense DNA, we demonstrated that the knockdown of NEAT1 enhanced virus production by increasing nucleus-to-cytoplasm export of Rev-dependent INS-containing HIV-1 transcripts (Fig. 4 to 6). We anticipate that NEAT1 IncRNA overexpression would produce increased repression of INS-containing HIV-1 mRNAs; however, because NEAT1 IncRNA is more than 23 kb in length, we have been unable to clone and express this moiety intact. To our knowledge, no one else has succeeded in the cloning and expression of full-length human NEAT1 lncRNA.

NEAT1 has been reported by several investigators to contribute a critical scaffolding role in the formation of nuclear paraspeckles (41, 42, 61). A current view is that paraspeckle bodies may represent RNA depots where many RNA binding proteins, including PSF and p54nrb, are found (47). Although the functions of paraspeckles remain incompletely defined, they are suggested to be involved in regulating gene expression through nuclear retention of RNA for export by the Rev-CRM1 complex (66, 67), or to paraspeckles, where they are retained (Fig. 7, middle and right). In this view, paraspeckles may indeed represent the long-postulated nuclear compartment for storing HIV-1 Rev-dependent INS-containing RNAs that are diverted away from splicing (65). We consider that the nuclear pool of unspliced HIV-1 RNAs may contain two subpools, one destined for splicing/degradation and the other destined for storage in nuclear paraspeckles. A small change in the latter pool from downregulation of paraspeckles could have a significant effect on cytoplasmic unspliced RNA levels without dramatically changing overall nuclear unspliced RNA levels (Fig. 6B). We caution that certain experimental assumptions regarding steady-state equilibration kinetics between various subpools of nuclear HIV-1 RNAs (Fig. 7) may need to be revised and renormalized depending on the time of RNA isolation after HIV-1 infection and/or after knockdown of NEAT1 paraspeckle lncRNA.

Based on our current results, we envision the following model (Fig. 7). HIV-1 multiply spliced RNAs that do not contain either INS sequences or RRE are processed by the splicing machinery and exported from the nucleus into the cytoplasm through a Rev-independent pathway (Fig. 7, left). HIV-1 unspliced RNAs are shunted by INS-binding factors (e.g., PSF and p54nrb) away from the splicing machinery to the nucleolus, where they are engaged for export by the Rev-CRM1 complex (66, 67), or to paraspeckles, where they are retained (Fig. 7, middle and right). In this view, paraspeckles serve as retention depots for storing HIV-1 unspliced transcripts, some of which may be subjected to RNA-processing events, such as A-to-I editing. For cellular transcripts, a paraspeckle depot may be a means for stabilizing and maintaining RNAs that might otherwise be degraded. This type of RNA storage followed by release (when required) offers a more rapid and resource-efficient way than de novo RNA biosynthesis for providing needed RNA for rapid use in responding to stress. Thus, HIV-1 seemingly
has co-opted this cellular mechanism to also store its excess unspliced INS-containing viral RNAs in paraspeckle bodies (Fig. 7).

Replication of viruses in mammalian cells is influenced by various subcellular organelles. For example, the replication of several RNA viruses requires cytoplasmic membrane vesicles (68–71), and several reports have implicated cytoplasmic P bodies in HIV-1 expression (35, 72, 73), although this finding has been recently questioned (74). Regarding nuclear bodies that modulate HIV-1 expression, the nucleolus has been suggested as a “positive” nuclear compartment contributing to Rev-dependent expression of HIV-1 INS-containing transcripts (67, 75). Here, based on findings from NEAT1 knockdown, we identified the paraspeckle as a second nuclear body that serves as a “negative” counterbalancing retention depot for HIV-1 INS-transcripts. Additional investigation is needed to understand better how some HIV-1 INS-containing RNAs enter the nucleolus while others go to the paraspeckle (Fig. 7). Moreover, the physiological signals that release HIV-1 INS transcripts from paraspeckles also remain to be determined.

MATERIALS AND METHODS

Plasmids and antibodies. The GFP-PSF plasmid was a gift from B. K. Felber (45). Plasmids p37-RRE and p37-RRE M1-10 were also kindly provided by B. K. Felber (60) and were cloned into pcDNA3 as described previously (43). Rabbit anti-PSP1 (N-terminal) antibody and mouse anti-p54nrb antibody were purchased from Sigma. Mouse anti-GFP was purchased from Santa Cruz Biotechnology.

Cell culture, transfection, and cell fractionation. Jurkat, MT4, and THP1 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS and 2 mM L-glutamine. For THP1 differentiation, cells were treated with 40 ng/ml PMA for 24 h, and then the medium was discarded, the cells were washed twice with phosphate-buffered saline (PBS), and fresh medium was added. For siRNA transfection, HeLa cells were transfected with Lipofectamine RNAiMAX reagent according to the manufacturer’s instructions. For plasmid transfection, X-tremeGENE HP DNA transfection reagent (Roche) was used. For transfection of Jurkat cells, 3×10^6 cells were suspended in 100 μl of solution V of the Nucleofector kit V (Amaxa Biosystems) and then mixed with oligonucleotides (2 μM, final concentration). Transfection was conducted following the manufacturer’s instructions. Cell fractionation was performed to isolate nuclear and cytoplasmic RNAs using a Paris kit (Life Technologies) according to the manufacturer’s instructions.

lncRNA profiling. Jurkat and MT4 cells were mock infected or infected with HIV-1 virus produced from 293T cells. Three days later, infection efficiencies were determined by intracellular p24 staining, and then cells were harvested and total RNA was extracted using Trizol reagent (Invitrogen), following the manufacturer’s instructions. The same amounts of RNA were converted to cDNA using the SuperScript III first-

![FIG 5](image-url)

Knockdown of NEAT1 increases the expression of INS-containing mRNA. (A) HeLa cells were transfected with scrambled AS or NEAT1 AS oligodeoxynucleotides; twenty hours later, cells were cotransfected with HIV-1 LTR-luciferase and Tat plasmids. Twenty-four hours later, luciferase activities of these cell lysates were determined. Results from three separate experiments are shown as the mean values ± SD. (B) HeLa cells were transfected with 100 nM scrambled AS or NEAT1 AS oligodeoxynucleotides; twenty hours later, cells were cotransfected with Rev and INS-containing p37-RRE reporter or codon-optimized (non-INS-containing) p37 M1-10-RRE reporter (ratio, 1:10; Rev, reporter). Cell lysates were analyzed by Western blotting with HIV-1 hyperimmune serum or antitubulin. (C) HeLa cells were transfected with 100 nM scrambled AS or NEAT1 AS oligodeoxynucleotides, and 48 h later, cell lysates were analyzed by Western blotting with antibodies specific for PSF, p54nrb, and tubulin.

PSF were purchased from Sigma. Mouse anti-p54nrb antibody was purchased from BD Transduction Labs. HIV-1 immune serum was obtained from the AIDS Reference and Reagent Program, NIAID. Mouse anti-GFP was purchased from Santa Cruz Biotechnology.

Cell culture, transfection, and cell fractionation. Jurkat, MT4, and THP1 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS and 2 mM L-glutamine. For THP1 differentiation, cells were treated with 40 ng/ml PMA for 24 h, and then the medium was discarded, the cells were washed twice with phosphate-buffered saline (PBS), and fresh medium was added. For siRNA transfection, HeLa cells were transfected with Lipofectamine RNAiMAX reagent according to the manufacturer’s instructions. For plasmid transfection, X-tremeGENE HP DNA transfection reagent (Roche) was used. For transfection of Jurkat cells, 3×10^6 cells were suspended in 100 μl of solution V of the Nucleofector kit V (Amaxa Biosystems) and then mixed with oligonucleotides (2 μM or 10 μM, final concentration). Transfection was conducted following the manufacturer’s instructions. Cell fractionation was performed to isolate nuclear and cytoplasmic RNAs using a Paris kit (Life Technologies) according to the manufacturer’s instructions.

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strand synthesis kit (Invitrogen). The samples were diluted 1:20, and the Disease-Related Human LncRNA Profiler (System Biosciences) was used to profile lncRNAs in the samples according to the manufacturer’s instructions. Eighty-three lncRNAs profiled were as follows: 21A, AAA1, aHIF, AK023948, ANCR, CIMP-D, DDGC5R, DISC2, DLG2AS, EGO, GASS, GOMAFU, H19, H19-AS, HAR1A, HAR1B, HOTAIR, HOTAIRM1, HOTTIP, HOXA1ASA(AA89505, HOXA3AS823151, HOXA3AS8623549, HOXA1AAS, HULC, IPW, IGF2AS, KRASP1, LIPAI6, LIT, LOC285194, LUST, LincRNAVLDLR, LincRNASFMBT2, MALAT1, MEG3, MER11C, NEAT1, NCRM5, NDM29, PANDA, PAR5, PCAT-1, PCAT-14, PCAT-29, PCAT-32, PCAT-43, PCGEM1, PR-AT2, PRINS, PSF-inhibiting RNA, RPFPI, RMRP, ROR, SAF, SCA8, Sox2OT, SRA, ST7OT1, ST7OT2, ST7OT3, ST7OT4, telomerase RNA, TMEVPG1, TU_0017629, TUG1, UCA1, WT1-AS, Y1, Y3, Y4, Y5, ZEB2NAT, 7SK.

RNA inhibition. The siRNA and the AS oligonucleotides (41, 42) used to knock down NEAT1 were synthesized by Integrated DNA Technologies. The sequences are as follows: NEAT1 siRNA sense, 5’-/5Phos/rGrUrGrArGrUrGrArGrUrGrUrGrCrUrUrArGrArArGrUrUrUCC-3’; NEAT1 siRNA antisense, 5’-rGrGrArArGrUrGrArGrUrGrArGrUrGrUrGrCrUrUrArGrArArCrUrUrUCC-3’; nonspecific control siRNA sense, 5’-/5Phos/rGrUrCrArCrGrUrCrUrArArCrUrArArGrArArCrUrUrGTT-3’; control antisense, 5’-mU*mC*mU*mG*mC*T*A*C*T*T*G*G*C*mU*mC*mA*mU*mU-3’; NEAT1 scrambled AS, 5’-mC*mC*mC*mU*mC*T*A*G*T*C*T*T*G*G*C*mU*mC*mA*mU*mU-3’.

qRT-PCR. Extracted RNA was analyzed by qRT-PCR using an iScript One-Step RT-PCR kit with SYBR green (Bio-Rad). Primers were as follows: NEAT1#1 forward, 5’-CTTCCTCCCTTTAACACTTATAC-3’; NEAT1#1 reverse, 5’-CAGTTAGTTATCAGTCTTCCTACCA-3’; NEAT1#2 forward, 5’-CGCTCCTCCTAAGCTGATCACC-3’; NEAT1#2 reverse, 5’-GTGTTGTCGTC-ACCTTACCT-3’ (41); unspliced transcripts forward, 5’-CTTCCTCCTACTAGGACGACG-3’; unspliced transcripts reverse, 5’-CTTCCTCCTACTAGGACGACG-3’; multiply spliced transcripts forward, 5’-GTGTTGTCGTC-ACCTTACCT-3’; multiply spliced transcripts reverse, 5’-CTTCCTCCTACTAGGACGACG-3’; multiply spliced transcripts reverse, 5’-CTTCCTCCTACTAGGACGACG-3’; multiply spliced transcripts reverse, 5’-CTTCCTCCTACTAGGACGACG-3’; multiply spliced transcripts reverse, 5’-CTTCCTCCTACTAGGACGACG-3’; multiply spliced transcripts reverse, 5’-CTTCCTCCTACTAGGACGACG-3’; multiply spliced transcripts reverse, 5’-CTTCCTCCTACTAGGACGACG-3’; multiply spliced transcripts reverse, 5’-CTTCCTCCTACTAGGACGACG-3’.

FIG 6 Knockdown of NEAT1 increases cytoplasmic levels of unspliced HIV-1 RNA. HeLa cells were transfected with 50 nM control siRNA or NEAT1 siRNA. Twenty hours later, cells were infected with VSV-G-packaged HIV-1 NL4-3 produced in 293T cells. Thirty hours postinfection, RNAs were isolated from nuclear and cytoplasmic fractions, respectively, as described in Materials and Methods. (A) To assess the quality of fractionation, the unspliced GAPDH pre-mRNA was measured by qRT-PCR. The cytoplasmic RNA level was set as 1. Representative results from three separate experiments are shown as the means ± SD. (B) Unspliced HIV-1 transcripts in the cytoplasm and the nucleus were determined by qRT-PCR analysis using specific primers. The cytoplasmic unspliced HIV-1 RNA levels in control siRNA-treated cells were set as 1. Results from three separate experiments are shown as the mean values ± SD.

FIG 7 A model of nuclear events that regulate expression of spliced and unspliced HIV-1 RNAs. Spliced viral RNAs exit the nucleus in a Rev-independent route (left). Unspliced HIV-1 RNAs that contain INS sequences transit to the nucleolus (middle) or the paraspeckle (right). See the text for further discussion.
Western blotting. The cells were washed twice with ice-cold PBS and lysed with 1× SDS protein loading buffer (50 mM Tris, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.1% bromophenol blue). Then, samples were boiled at 95°C for 10 min. The lyses were resolved by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). Then, the membrane was probed with the primary antibodies, followed by incubation with alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich). Finally, signals were detected using a chemiluminescence substrate (Applied Biosystems).

Immunofluorescence and confocal microscopy. Cells were washed twice with ice-cold PBS and then fixed in 4% paraformaldehyde at room temperature, washed two times with PBS, and then blocked with 3% PBS for 30 min at room temperature. After three washes with PBS, cells were permeabilized in PBS containing 0.2% Triton X-100 for 4 min at room temperature, washed two times with PBS, and then blocked with 3% bovine serum albumin (BSA) for 2 h at 4°C. Cells were incubated with P1 antibody (1:1,000) overnight at 4°C and then washed three times with PBS. Alexa-conjugated secondary antibodies (Invitrogen) were applied for 1 h at room temperature, and then cells were washed twice with PBS. DNA was counterstained with Hoechst 33342 stain (Sigma-Aldrich). Coverslips were mounted in ProLong Gold antifade reagent (Invitrogen), and fluorescence signals were visualized using a Leica TCS SP5 microscope.

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