Human T-cell Leukemia Virus Type I p30 Nuclear/Nucleolar Retention Is Mediated through Interactions with RNA and a Constituent of the 60 S Ribosomal Subunit*

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From the †Department of Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Center, Kansas City, Kansas 66160–7420, the ‡Animal Models and Retroviral Vaccines Section, NCI, National Institutes of Health, Bethesda, Maryland 20892–5065, and the §Department of Cell Biology and Anatomy, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064

Human T-cell leukemia virus type I is the etiological agent of adult T-cell leukemia/lymphoma, an aggressive and fatal lymphoproliferative malignancy. The virus has evolved strategies to escape immune clearance by remaining latent in most infected cells in vivo. We demonstrated previously that virally encoded p30 protein is a potent post-transcriptional inhibitor of virus replication (Nicot, C., Dundr, M., Johnson, J. M., Fullen, J. R., Alonzo, N., Fukumoto, R., Princler, G. L., Derse, D., Misteli, T., and Franchini, G. (2004) Nat. Med. 10, 197–201). p30 is unable to shuttle out of the nucleus in heterokaryon assays, suggesting the existence of specific retention signals. Because suppression of virus replication relies on nuclear retention of the tax/rex mRNA by p30, determining the retention features of p30 will offer hints to break latency in infected cells and insights into new therapeutic approaches. In this study, we used live cell imaging technologies to study the kinetics of p30 and to delineate its retention signals and their function in virus replication. Notably, this is the first study to identify p30 nucleolar retention domains. Using mutants of p30 that localized in different cellular compartments, we show that post-transcriptional control of virus replication by p30 occurs in the nucleolus. We further demonstrate that p30 nuclear/nucleolar retention is dependent upon de novo RNA transcripts and interactions with components of the ribosomal machinery.

Nucleoli are the main site of ribosome biogenesis, a highly complex process leading to the production of pre-ribosomal particles, which are then released into the nucleoplasm and exported to the cytoplasm as mature ribosomal subunits (1). In contrast to other cellular compartments, the nucleolus is not membrane-limited; its structure is maintained by a major accumulation of ribosomal rRNA and proteins such as nucleolin and protein B23. Most proteins are only transiently retained in the nucleolus through protein or RNA interactions, and proteins with long resident times usually harbor specific signals (2). Rather than being nucleolar localization signals (NoLS),2 these signals tend to be nucleolar retention signals (NoRS). With very few exceptions (3), these NoRS are generally characterized by arginine- and lysine-rich sequences of highly variable sizes, ranging from five amino acids for angiogenin up to several tens for nucleolin (4, 5). Often, amino acid sequence analysis fails to predict putative NoRS, especially when they overlap nuclear localization signals (NLS). The nucleolus, the site of transient sequestration and maturation of several cellular factors, is critically involved in the control of the cell cycle, DNA repair, aging, and mRNA export. Many viruses encode nucleolar proteins, which are involved in replication of viral genomes (6) as well as in transcriptional and post-transcriptional regulation of gene expression (7).

We found recently that the viral p30 protein is a novel post-transcriptional repressor of human T-cell leukemia virus type I (HTLV-I) replication (8). p30 is a serine/arginine-rich nuclear/nucleolar protein that complexes with and retains the viral tax/rex mRNA in the nuclei of infected cells. Hence, decreased expression of the positive regulators Tax and Rex results in inhibition of virus replication (8). In this study, we investigated the mechanisms underlying p30 retention. Our results show that p30 utilizes multiple strategies and harbors two nucleolar retention domains. Consistent with its role in RNA trafficking, p30 nuclear/nucleolar retention is partially dependent on de novo mRNA transcription. We also report that p30 interacts with a nucleolar constituent of the large ribosomal subunit L18a, a protein shown previously (12, 32–34) to modulate internal initiation of translation.

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2 The abbreviations used are: NoLS, nucleolar localization signal(s); NoRS, nucleolar retention signal(s); NLS, nuclear localization signal(s); HTLV-I, human T-cell leukemia virus type I; HA, hemagglutinin; LTR, long terminal repeat; GFP, green fluorescent protein; AMD, actinomycin D; FRAP, fluorescence recovery after photobleaching; iFRAP, inverse fluorescence recovery after photobleaching; DFC, dense fibrillar component(s); GC, granular component(s).
MATERIALS AND METHODS

Plasmid Construction and Site-directed Mutagenesis—p30 was cloned into the pEFGP-C1 vector (Clontech) between the HindIII and EcoRI sites. The primers used to generate p30 mutants were as follows: AN100-F (5′-CGC GAG CCA TGG CCG CCT TCT TCT CCG CCC GCC-3′), ΔN200-F (5′-CCC CAA GCT TCC ATG GCC CGT CGC TGC CGC TCA CGA-3′), ΔN280-F (5′-CCC CAA GCG CCA TGG GAA GCC CCC GTG GCA GCC GG-3′), ΔN300-F (5′-CCC CAA GCT TCC ATG CCT CCA CCA GCA GGT CCT CCG GCC GGC-3′), ΔN500-F (5′-CCC CAA GCA TGG TTG GGA CAG TGT CTT TTC GG-3′), and EcoRI-R (5′-CCG AAT TCA GGT TCT CTG GG TGG GGT GG-3′); ΔC200-R (5′-GGG AAT TCA AAG AGC GGA AGG AGA AAG-3′), ΔC250-R (5′-CCA ATT TCA CGA GAA AGC GCC ACC TCC CGG GGC GCA-3′), ΔC300-R (5′-GGG AAT TCA AAG AGC GCC ACC TCG GCC GCC GGC GCC GGC-3′), ΔC400-R (5′-GGG AAT TCA GGT AAT TCG AAT TCG GGC ACG GCC GAT GGC TAT TAT CC-3′), ΔC500-R (5′-GGG AAT TCA CCC TGG GAA GTG GGC TG-3′), ΔC600-R (5′-GGG AAT TCA GCC GGC GCC AGC CGA ACA TAG TCC CCC-3′), and HindIII-R (5′-CCC CAA GCT TCC ATG AGA GAT AGC AAA CCG TCA AGC ACA G-3′) with reverse primers N280-F (5′-CGG CCG GCT GCG AGC GGG CCT TCC AGG GGA-3′), respectively. p30-5RA and p30-5RK were generated with reverse primer 5′-GCC TCA GCA TGC GTT TCC CCG CGA GGT GGC GC-3′ and reverse primer 5′-CCG GCC GGT GCC GGC-3′ with EcoRI and reverse primer 5′-CCG GCT CGA GGA GAG GAA GCC GGC AAA AAA AGA GAG G3′ with HindIII. p30R91-98 was generated by PCR with forward primer 5′-CGG GCC CCT CCA GGG G3′ and EcoRI, and reverse primer 5′-GGA CCC GCC GCA TCA ACG GGA AGG AGA AAG AAA AAA AGA GGC-3′ and HindIII, digested SacII and HindIII, and ligated with the SacII-EcoRI fragment of p30. p30Δ73−78 was generated by PCR with forward primer 5′-CCG GCC CCT CCA GGG G3′ and EcoRI, and reverse primer 5′-GGG AAT TCA AAG AGC GCC ACC TCG GCC GCC GGC GCC GGC-3′ with HindIII and XhoI; and cloned into pEFGP-C1.

Cell Culture, Transfections, and Fluorescence Microscopy—Cos-7 cells were grown on cover slides in Dulbecco’s modified minimal essential medium containing 10% fetal calf serum. Transient transfections were performed using Effectene transfection reagent (Qiagen Inc.) with 1 μg of expression plasmids. Forty hours after transfection, cells were fixed in 4% paraformaldehyde and washed with phosphate-buffered saline. The slides were mounted, and images of p30 fused to green fluorescent protein (GFP) were captured using a Nikon EFD3 microscope (Boyce Scientific, St. Louis, MO) and a Nikon camera with a 100× Eplan (60/0.17) objective. The imaging medium SlowFade was from Molecular Probes (Eugene, OR). The acquisition software Image-Pro Express Version 4 was from Media Cybernetics (Silver Spring, MD). The images presented in this study are representative of a large number of cells observed in three or more independent transfection experiments. For actinomycin D (AMD) and RNase A treatment, COS-7 cells were transfected with 1 μg of GFP-p30 plasmid using Effectene transfection reagent. After 40 h of culture, cells were washed with 1× phosphate-buffered saline and treated for 5 min with saponin (0.1 μg/ml) at 4°C. After being washed, cells were treated with AMD (5 μg/ml) for 30 min, washed, treated with RNase A (1 mg/ml) for 30 min, and fixed as described above.

Dual-Luciferase Assay, in Vitro Transcription/Translation, and Western Blot Analysis—Firefly and Renilla luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega Corp., Madison, WI) according to the manufacturer’s instructions. After treatment and removal of the incubation medium, cells were washed and lysed by addition of 100 μl of the lysis buffer provided with the kit. Ten microliters of cell lysate were added to 100 μl of luciferase assay reagent II, and firefly luciferase activity was measured for 10 s. One-hundred microliters of Stop & Glo buffer, which contains the Renilla luciferase substrate and stops the firefly luciferase reaction, were added to the same tube, and Renilla luciferase activity was measured for 10 s in a Berthold junior luminometer. Activity was calculated as the ratio of the values of firefly luciferase to Renilla luciferase activities, and results expressed as fold activation are representative of two independent sets of
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experiments. In vitro transcription/translation reactions were performed with pMH-p30-HA using rabbit reticulocyte lysates as reported previously (15). Immunoprecipitation was performed with antibody 12CA5 (Roche Diagnostics). Western blotting was performed using 40 μg of protein lysates from transfected COS-7 cells as described in the figure legends and using anti-HA antibody 3F10 (Roche Diagnostics) according to the manufacturer’s directions.

**Photobleaching**—To probe the degree to which p30 or p30 mutant proteins are mobile within the nucleolus of a living cell, we carried our fluorescence recovery after photobleaching (FRAP) and inverse FRAP (iFRAP). For FRAP experiments, five single scans were acquired, followed by a single bleach pulse of 200–500 ms using a spot 1 μm in radius without scanning. Single section images were then collected at 1.6-s intervals. For imaging, the laser power was attenuated to 1% of the bleach intensity. For iFRAP, experiments were performed on a Zeiss LSM 510 confocal microscope with a 100×1.3 numerical aperture Plan Apochromat oil objective and 3× zoom. GFP was excited with the 488-nm line of an argon laser, and GFP emission was monitored above 505 nm as described previously (9). Cells were maintained at 37 °C with an ASI 400 air stream incubator (Nevtek). The whole nuclear area of transfected cells was bleached, except for a region of one nucleolus, using the 488-nm laser line at 100% laser power. Cells were monitored at 0.5-s intervals for 300 s. To minimize the effect of photobleaching due to imaging, images were collected at 0.2% laser intensity. For quantification, the loss of total fluorescence intensity in the unbleached region of interest was measured using Zeiss software. Background fluorescence was measured in a random field outside of the cells. For each time point, the relative loss of fluorescence intensity in the unbleached region of interest was calculated as follows: \( I_{rel} = (I_0 - BG)/(I_o - BG)(T_B - BG) \), where \( I_0 \) is the background-corrected average intensity of the region of interest during pre-bleaching and \( T_B \) is the background-corrected total fluorescence intensity of a neighboring control cell. Typical measurement errors in all experiments were ~15%.

**Mass Spectrometry**—p30-HA was transcribed in vitro and translated from the T7 promoter of the pMH vector using the TnT quick coupled transcription/translation kit (Promega Corp.). p30-HA was immunoprecipitated overnight at 4 °C using 1 μl (5 μg) of monoclonal antibody 12CA5. As a control, an equal amount of rabbit reticulocyte from the same kit was used and immunoprecipitated overnight at 4 °C with 5 μg of monoclonal antibody 12CA5. Twenty microliters of a 50% protein G-agarose slurry (Invitrogen) were added to both mixtures, followed by incubation at 4 °C for 2 h. The immunoprecipitated complexes were washed three times with 1 ml of radioimmune precipitation assay buffer at 4 °C. The components of the complexes were resolved on 4–20% SDS-polyacrylamide gels (Bio-Rad) and detected by Coomassie Blue staining. The bands specific for the lane containing p30-HA translated in vitro (compared with the control lane) were cut out and analyzed by mass spectrometry.

**RESULTS**

**p30 Is Strongly Retained in the Nucleolar Compartment of Living Cells**—For some proteins, nucleolar localization is dependent upon the presence of a NoLS usually characterized by a short stretch of Arg or Lys residues. However, in most cases, nucleolar localization is mediated through a retention mechanism that increases the resident time of a given protein in this cellular compartment. HTLV-I p30 is a nuclear/nucleolar protein. To gain insights into its cellular compartmentalization, mobility, and in vivo kinetics, p30 was cloned in-frame with GFP into the pEGFP-C1 vector and subjected to live cell image analysis, FRAP, and iFRAP. In our FRAP experiments, a defined area of a living cell was bleached irreversibly by a single, high-powered spot laser pulse. The recovery of the fluorescence signal in the bleached area as the consequence of movement of the GFP fusion protein was recorded by sequential imaging scans. The kinetics of recovery are a measure of the mobility of the GFP-fused protein. Interestingly, the experiments showed a rapid and complete recovery of fluorescence in the nucleus (Fig. 1, A and B), indicating a high mobility of GFP-p30 in this cellular compartment. In contrast, a much slower recovery of fluorescence was consistently detected in the nucleolus (Fig. 1, A and B), suggesting that p30 may be specifically retained in the nucleolus. iFRAP as opposed to FRAP is the method of choice for measuring retention kinetics because it provides a relatively direct indication of a protein’s residence time in a structure and because the measurement is independent of the size of the structure. In our iFRAP experiments, the entire nucleus with the exception of the region of interest containing the nucleolus was bleached using a pulsed laser. The loss of fluorescence signal in the nucleolus was then monitored by time-lapse microscopy. The rate of decay is a good approximation for the dissociation kinetics of the observed protein from the region of interest (9, 10). When iFRAP was applied to cells expressing GFP-p30, only a small fraction of the fluorescence signal was lost within the first 5 s after the bleaching event, followed by a slower decline of the nucleolar signal. After 300 s, a plateau representing the dissociation/association equilibrium of the bleached population of molecules was reached. Although the rapid initial loss likely reflects the fraction of loosely bound or diffusing GFP-p30, the slower loss of the majority of protein indicates that GFP-p30 was strongly retained in the nucleolus (Fig. 1C). Typical measurement errors in all experiments were <15%. We then compared the FRAP and iFRAP kinetics of GFP-p30 and nucleolar methyltransferase fibrillarin-GFP. We found that both proteins were strongly retained in nucleoli. The results indicated that p30 exhibited similar albeit slightly faster recovery kinetics compared with fibrillarin (Fig. 1, B and C). With these interesting observations, we next sought to identify the NoLS of p30.

**p30 Contains Two Independent Nucleolar Localization/Retention Signals**—We generated deletion mutants fused to GFP and analyzed their cellular distribution upon transfection into COS-7 cells (Fig. 2A). Serial deletion from the C terminus of p30 showed that p30ΔC200 was mostly nuclear but was excluded from the nucleoli and that p30ΔC250 efficiently accumulated in the nucleoli (Fig. 2B). These results suggest the existence of a nucleolar localization/retention signal located between nucleotides 200 and 250.

We next performed serial deletions from the N terminus of p30 (Fig. 3A). The results showed that GFP-p30ΔN100, GFP-p30ΔN200, and GFP-p30ΔN280 localized to the nucleoli of
transfected cells (Fig. 3B). However, when deletions beyond nucleotide 300 were performed, the resulting fusion proteins, GFP-p30ΔN300, GFP-p30ΔN400, and GFP-p30ΔN500, no longer accumulated in the nucleoli, but were distributed diffusely in the nucleoplasm (Fig. 3B). These results suggest the presence of a second nucleolar localization/retention signal located between nucleotides 280 and 300. Although the above-mentioned domains were reported previously as NLS (11), our results clearly indicate that the sequences are in fact required for nucleolar targeting of p30. This finding is not unusual, as NoLS are often part of NLS. Our results further demonstrate the presence of two previously unidentified NLS in the p30 protein, one in the N terminus between nucleotides 1 and 200 and the other in the C terminus between nucleotides 500 and 750. Both GFP-p30ΔC200 and GFP-p30ΔN500 localized to the nucleus (Fig. 3B), in contrast to the diffuse pattern throughout the cell observed for the insertless pEGFP-C1 vector (Fig. 2B). This finding was also confirmed by the fact that in-frame deletion of the previously identified NLS did not alter the nuclear localization of p30, but rather abolished its nucleolar accumulation (see Fig. 6). In contrast to previous findings, our results indicate that the major role of these arginine-rich sequences in p30 is to act as nucleolar localization/retention signals.

In Vivo Dissociation Kinetics Reveal Two NoRS—Dissociation kinetics measurements by FRAP and iFRAP of live cells can discriminate between an NoLS and an NoRS. To understand the nucleolar retention mechanism of p30 described above (Fig. 1), we used the truncation mutants GFP-p30ΔN280 and GFP-p30ΔC250, with each mutant having only one of the nucleolar localization/retention signals. We measured the dissociation kinetics of GFP-p30, GFP-p30ΔN280, and GFP-p30ΔC250 from the nucleoli. Interestingly, both p30 deletion mutants...
exhibited distinct dissociation kinetics compared with wild-type GFP-p30. The dissociation kinetics of GFP-p30ΔN280 and GFP-p30ΔC250 from the nucleoli were significantly faster than those of wild-type p30 (Fig. 4B). These data indicate that the two mutants are retained in the nucleoli for a significantly shorter period of time compared with the wild-type p30 protein and suggest that these signals may act as NoRS.

To confirm these results and to identify potential amino acid residues critical for NoRS function, each Arg residue present within the two Arg-rich domains was mutated to Ala by site-directed mutagenesis and cloned in fusion with GFP (Fig. 5A). The cellular distribution of these GFP-fused single point mutants was tested upon transfection of COS-7 cells. As shown in Fig. 5B, the single point mutations appeared to be innocuous, as most mutants displayed no substantial change in localization. Our results are similar to those reported previously for herpes simplex virus type 1 US11 protein (3) and angiogenin (13), for which several amino acid substitutions within the NoRS are necessary to abolish nucleolar localization. To confirm the function of each Arg-rich domain of p30, we made internal deletions or multiple Arg replacements. When in-frame deletion of both Arg-rich domains was performed, the resulting GFP-p30Δ73–98 mutant was localized in the nucleoplasm, but excluded from the nucleoli (Fig. 6A). Consistent with the results presented in Figs. 2 and 3, these findings confirmed that the Arg-rich domains are required for efficient nucleolar accumulation of p30 and suggested the existence of additional NLS within the p30 protein. Each nucleolar localization domain was then deleted separately. Although GFP-p30Δ73–78 became nucleolar, excluded from the nucleoli, the cellular distribution of GFP-p30Δ91–98 remained unaffected (Fig. 6A). The results presented here are representative of several transfection experiments, although an occasional nucleolar signal was detected (<10%) for GFP-p30Δ73–98 and GFP-p30Δ73–78 when expressed at high levels (data not shown). Taken together, these results showed the domains could serve as NoLS (Fig. 6A). Although GFP-p30ΔN280 lacked the first Arg-rich domain, it localized to the nucleolus when GFP was fused at the C terminus. We believe that this resulted from the folding and accessibility of the second NoLS because placing GFP in the N terminus prevented nucleolar accumulation and resulted in a nuclear pattern similar to that of GFP-p30Δ73–98 (data not shown). We next confirmed the results obtained with in-frame deletion mutants by mutagenesis replacement of all Arg residues with Ala in the first, second, or both Arg-rich domains of p30. As expected, Arg-to-Ala substitutions yielded results similar to those reported in Fig. 5, and both GFP-p30Δ4RA and GFP-p30Δ9RA were excluded from the nucleoli (Fig. 6, B and C). We also substituted Arg with Lys for amino acid charge conservation. As expected, Arg-to-Ala substitutions yielded results similar to those reported in Fig. 5, and both GFP-p30Δ4RA and GFP-p30Δ9RA were excluded from the nucleoli (Fig. 6, B and C). In contrast, all mutants with Arg-to-Lys substitutions localized similarly to the wild-type protein in the nucleus/nucleolus (Fig. 6C). Quantitative image analysis revealed no significant differences in nucleolar/nucleolus localization between p30–4RK, p30–5RK, and p30–9RK (data not shown).

Nuclear Localization of p30 Is Dispensable for Its Post-transcriptional Repression of Virus Expression—We found previously that p30 is a post-transcriptional negative regulator of viral gene expression (8). This function was related to the ability of p30 to sequester the tax/rex mRNA, encoding positive regulators, in the nuclear compartment. Other nucleolar resident proteins such as La are involved in the protection of RNAs from 3′-exonucleolytic digestion and nuclear retention (14). Many viruses have evolved proteins that localize to the nucleolus to increase or suppress virus replication (6). Because the p30–
The amino acid sequence of the wild-type p30 protein. NoLS and NLS sequences are compared with wild-type p30. However, despite the lack of substitution, p30–9RA was expressed at lower levels than the wild-type protein presumably as a result of arginine erosion blot analysis indicated that p30–9RA migrated a little faster as a surrogate indicator of p30-mediated repression (8). Western blot analysis indicated that p30–9RA migrated a little faster than the wild-type protein presumably as a result of arginine substitution. Of note, p30–9RA was expressed at lower levels compared with wild-type p30. However, despite the lack of nucleolar localization, p30–9RA appeared to be as efficient as wild-type p30 in post-transcriptional inhibition of HTLV-I expression (Fig. 7A). In fact, when p30–9RA and wild-type p30 were normalized for equal expression, no significant difference in post-transcriptional inhibition was found (Fig. 7E). These results strongly suggest that p30-mediated post-transcriptional repression of HTLV-I occurs in the nucleoplasm.

We also tested the transcriptional effect of p30 on the viral LTR. Our data indicated no significant difference between wild-type p30 and p30–9RA, with a 2-fold or less effect on the viral LTR (Fig. 7, B and D).

Nucleolar Retention of p30 Is Partially Transcription-dependent—We next tested whether the GFP-p30 fusion protein localized to the dense fibrillar components (DFC), which are sites of transient accumulation of both elongating and full-length primary transcripts released from the rDNA template, or to the granular component (GC), which is the site of pre-ribosome assembly. To this end, fibrillarin and p19ARF fused to GFP were used as specific markers for DFC and GC, respectively. Fig. 8 shows that p30 localized mainly within the GC compartment.

Because the accumulation of several proteins in nucleoli is transcription-dependent, we determined whether nuclear import and accumulation of p30 in nucleoli are modified by treatment with AMD. The transcriptional inhibition assay is based on the observation that many shuttling proteins accumulate in the cytoplasm when transcription is inhibited (17). COS-7 cells were incubated for 3 h with either 0.05 or 5 μg/ml AMD to inhibit the activity of either RNA polymerase I alone or both RNA polymerases I and II, respectively. The effectiveness of AMD treatment was assessed by reduction in the size and shape of the nucleoli. The localization of GFP-p30 and nucleolin-GFP was not significantly affected at 0.05 μg/ml AMD, suggesting that p30 is not associated with rRNA. However, both proteins appeared to localize more in the nucleoplasm at the higher concentration of AMD (Fig. 9A), suggesting that their nucleolar retention is partly dependent on RNA polymerase II and III transcription. In the same experiment, human immunodeficiency virus Rev-GFP was used as a positive control and was efficiently relocated to the cytoplasm following AMD treat-
We next used live cell imaging to investigate GFP-p30 recovery kinetics in response to AMD treatment. FRAP results indicated a much slower recovery of fluorescence following AMD treatment (Fig. 9B), confirming that GFP-p30 is in fact retained in the nucleoli in a transcription-dependent manner via interaction with less mobile nucleolar components. This was supported by the calculated $t_{1/2}$ for GFP-p30, which was $\sim 9$ s, compared with that for GFP-p30 in cells treated with actinomycin D, which was 77 s.

To further demonstrate a role of p30-RNA interactions in the nucleolar retention of p30, we treated transfected cells with RNase A and AMD. Under these experimental conditions, significant amounts of GFP-p30 re-localized to the cytoplasm. We calculated the total fluorescence intensities of the nuclear and cytoplasmic regions in both cells on the presented image. The average total fluorescence intensities of the nuclear and cytoplasmic compartments were almost similar, with 56% of the total GFP signal in the nuclear compartment and 44% in the cytoplasmic compartment (Fig. 9C), thereby demonstrating a significant role of p30-RNA associations in the nucleolar retention of p30. Because GFP-p30 was not completely redistributed to the nucleoplasm subsequent to AMD and RNase A treatment, we hypothesized that, in addition to RNA interactions, p30 retention may also be dependent on interactions with less mobile nucleolar resident proteins.

**p30 Interacts with the Large Ribosomal Subunit L18a**—To identify potential p30-interacting partners, we immunoprecipitated p30-HA transcribed in vitro and analyzed bound proteins by mass spectrometry. Among the various proteins that immunoprecipitated with p30, our attention was drawn to the nucleolar L18a protein (Fig. 10A), a constituent of the 60 S ribosomal subunit (20). L18a was cloned in-frame with a myc epitope to allow detection. Two other constituents of the 60 S ribosomal subunit, L21a and L23a, were also cloned and used as controls. To confirm the presence of p30 and L18a in a protein complex, p30-HA was transfected into 293T cells and immunoprecipitated with anti-HA antibody, and co-immunoprecipitated proteins were analyzed using anti-Myc antibody. Under these experimental conditions, we confirmed that p30 directly or indirectly interacted with L18a. No complexes were detected for p30 and ribosomal proteins L21a and L23a (Fig. 10B) (data not shown), suggesting that protein complex formation between p30

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**FIGURE 6.** p30 mutant localization and nuclear retention. Shown are fluorescence microscope images of COS-7 cells expressing GFP-p30 mutants. A, localization analysis of the p30Δ73–78, p30Δ91–98, and p30Δ73–98 deletion mutants. B, localization analysis of p30–4RA and p30–4RK (Arg) and p30–5RA and p30–5RK (Arg) and p30–9RA and p30–9RK (Arg).

**FIGURE 7.** Transcriptional and post-transcriptional functions of wild-type p30 and p30–9RA. A, luciferase activity in transfected 293T cells from the HTLV LTR-luciferase (Luc) vector (1 μg) driven by the Tax protein produced by pBST (1 μg) in the presence of pMH-p30II (0.25 μg) and pMH-p30II-9RA (0.25 μg). Results were normalized to pRL-TK activity. B, luciferase activity in transfected 239T cells from the HTLV LTR-luciferase vector (1 μg) in the presence of pMH-p30II (0.1–1 μg) and pMH-p30II-9RA (0.1–1 μg). Results were normalized to pRL-TK activity. C, Western blot analysis of pMH-p30 and pMH-p30-9RA at increasing concentrations.

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ment at either concentration, as reported previously (18, 19).
and L18a is not simply due to overexpression of these proteins. Numerous viral proteins have been shown (12, 32–34) to associate with nucleolin, which is the major constituent of the nucleolus. When we transfected a nucleolin expression vector along with p30, we did not detect any interactions between the two proteins (data not shown), again suggesting that protein complexes formed between p30 and L18a are specific.

**DISCUSSION**

HTLV-I is latent in most infected cells in vivo. This feature allows the virus to be amplified with each cellular replication of infected cells while avoiding immune recognition. The notion that HTLV-I is latent in adult T-cell leukemic cells is supported by numerous studies. The expression of viral genes cannot be detected in vivo, but the provirus is rapidly expressed once the cells are cultured ex vivo. Adult T-cell leukemia is characterized by the monoclonal expansion of infected cells, which cannot be explained by a continual de novo infection and immune clearance balance of infected cells. The same proviral integration site can be found several years apart in infected patients (21). It was reported recently that tenofovir administered 1 week after infection of NOD/SCID mice does not reduce the proviral loads, indicating that, after initial infection, clonal proliferation of infected cells is predominant over de novo infection of previously uninfected cells (22).

HTLV-I has evolved multiple strategies to prevent apoptosis and to extend the life span of infected cells (23–27). In turn, this allows accumulation of genetic mutations and disease progression. Because HTLV-I is very immunogenic and has a low variability, reducing its expression is key in virus maintenance in vivo. We reported previously that p30 is able to potently suppress virus expression and thus may be involved in silencing in vivo (8). The mechanism by which p30 prevents HTLV-I expression is not fully resolved, but it may result from inhibition of Tax-mediated LTR activation (28) along with interaction and nuclear retention of the tax/rex mRNA (8). Therefore, mechanisms of p30 nuclear/nucleolar retention are essential to its suppressive function.

In this study, we have identified two arginine-rich domains (located between amino acids 73 and 78 and amino acids 91 and 98) essential for HTLV-I p30 nucleolar localization/retention. In addition, our results revealed the existence of two previously unidentified NLS in the N- and C-terminal regions of the protein. These NLS were responsible for the nuclear localization of p30/H9004C2 and p30/H9004N5. The mRNA encoding p30 also encodes p13 in the same open reading frame, and the p13 amino acid sequence corresponds to the C terminus of p30. There is a discrepancy in the published literature concerning the mitochon-
drial nuclear localization of p13 in transfected cells (29, 30). The p30ΔN5 protein described in this study corresponds to the p13 protein with the mitochondrial targeting signal deleted. The localization of this mutant in the nuclear compartment reveals the presence of an NLS, which may be used under specific conditions when the mitochondrial targeting signal is masked. Multiple replacement of arginine with alanine in the NoRS allowed us to generate a nuclear p30 mutant (p30–9RA) excluded from the nucleoli. We then tested whether post-transcriptional inhibition of HTLV-I expression is dependent on the nucleolar localization of p30. Our data demonstrate that post-transcriptional repression occurs in the nucleoplasm because no difference was found between wild-type p30 and the p30–9RA mutant.

The nucleolus is morphologically separated into three distinct components, which reflect the vectorial process of ribosome biogenesis. Fibrillar centers are surrounded by dense fibrillar components (DFC), and GC radiate out from the DFC (31). The observations that HTLV-I p30 is localized mainly to the GC (Fig. 8) and that p30 associates with viral RNA complexes prompted us to investigate whether p30 interaction with RNA and/or proteins may be involved in its nucleolar retention. Our results clearly indicate that p30 retention is associated with RNA polymerase II- and III-dependent transcription of nascent RNA polymerase II- and III-dependent transcription of nascent RNA inasmuch as treatment with AMD and RNase released a significant fraction of p30, which diffused to the cytoplasm. However, the existence of additional retention mechanisms was also suggested by the fact that, under these experimental conditions, only about half of GFP-p30 remained localized in the nucleus/nucleolus (56%). It is unclear however how RNase treatment may alter the nuclear export machinery. Many viral factors interact with nucleolar proteins. To identify p30 partners, we performed mass spectrometry. The results revealed the ribosomal protein L18a, a nucleolar protein, as a putative partner. Transient transfection assays confirmed the ribosomal protein L18a, a nucleolar protein, as a putative partner. Under the same experimental conditions, no binding was found between p30 and L18a is specific. We cannot conclude whether these interactions are direct or indirect in absence of purified p30 protein. Our data are consistent with the fact that L18a is part of the pre-ribosomal subunit, which assembles in the GC compartment. Several studies have shown that the ribosomal protein L18a interacts with viral protein and eukaryotic initiation factor-3 and facilitates internal re-initiation of translation in cytomegalovirus and hepatitis C virus (12, 32–34). Our results indicate that this phenomenon is conserved in HTLV-I. Although p30 is a non-shuttling protein strongly associated with the nucleoli, it is possible that a fraction of loosely bound protein may diffuse to the cytoplasm or may be redistributed during the cell cycle and breakdown of nucleoli, allowing additional cytoplasmic functions. Interestingly, the p30-encoding mRNA completely overlaps two viral gene open reading frames, viz. p12 and p13. Whether p30 expression may directly or indirectly modulate internal initiation to increase expression of p12 and/or p13 warrants further studies.

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