Heterogeneous Nuclear Ribonucleoprotein A1 Interferes with the Binding of the Human T Cell Leukemia Virus Type 1 Rex Regulatory Protein to Its Response Element*

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The human T cell leukemia virus, type 1 (HTLV-1), Rex protein mediates the nuclear export of unspliced and incompletely spliced viral mRNAs. This post-transcriptional activity is dependent in part on the binding of this protein to cis-regulatory sequences termed the Rex-response element (XRE). We have proposed previously that the decreased functionality exhibited by Rex in human lymphoblastoid Jurkat T cells may be linked to alterations in the Rex/XRE interactions. The analysis of the ribonucleoprotein complexes formed between Jurkat nuclear proteins and XRE-RNA led to the identification of a 36-kDa protein as heterogeneous nuclear ribonucleoprotein (hnRNP) A1. In vitro binding assays revealed that hnRNP A1 proteins were found to interfere with the binding of Rex to XRE, whereas nuclear extracts depleted of these proteins were unable to disrupt Rex-XRE complexes. Furthermore, A1 proteins from Jurkat cells were acting in a concentration-dependent manner, suggesting that the amount of these RNA-binding proteins is a critical parameter in controlling Rex activity. We indeed observed a lower level of hnRNP A1 in in vitro HTLV-1-transformed virus-producing T cells than that detected in Jurkat cells. Likewise, overexpression of hnRNP A1 proteins in 293T cells and in Jurkat cells led to a decrease in the expression of a reporter gene dependent on Rex/XRE interactions. Such a decrease was not observed when the expression of the same reporter gene by cells overexpressing hnRNP A1 was dependent on the interactions of human immunodeficiency virus Rev protein with the Rev-response element. These findings indicate that hnRNP A1 by competing with Rex for the formation of Rex-XRE complexes is specifically involved in the modulation of the post-transcriptional activity of Rex.

The replication of complex retroviruses is controlled at both transcriptional and post-transcriptional levels, by specific regulatory proteins encoded by the viral genome (1–4). Thus, cytoplasmic expression of spliced and unspliced transcripts encoding the gag-pol and env gene products is dependent on a RNA-binding viral protein, the 27-kDa Rex protein of human T cell leukemia virus, type 1 (HTLV-1),1 or the 19-kDa Rev of human immunodeficiency virus, type 1 (HIV-1). The post-transcriptional activity of both proteins is dependent on their interactions with RNA cis-acting sequences, referred to as the Rex-response element (XRE) and the Rev-response element (RRE), respectively. These proteins display two functional domains, an N-terminal basic domain, acting mainly as a sequence-specific RNA-binding domain, and a C-terminal leucine-rich domain termed the activation domain, acting as a nuclear export signal. These leucine-rich sequences are interchangeable between Rex and Rev, underlining that the specificity of each protein relies on the interaction of the RNA-binding domain with its response element (5–8).

We have reported previously (9) that Rex functionality was impaired in lymphoblastoid Jurkat T cells, as shown by the failure of these cells to produce HTLV-1 envelope glycoproteins after transfection with a Rex/REX-dependent env construct together with a Rex expression plasmid. Conversely, synthesis of HTLV-1 envelope glycoproteins was observed in Jurkat cells transfected with a Rev/REV-dependent env construct together with a Rev expression plasmid. These results therefore indicated that the inability of these lymphoblastoid cells to support Rex function is linked to the deficiency of this viral regulatory protein to bind its response element. They in turn pointed to host cell proteins that would interfere with Rex/XRE interactions.

In the present study, we report that nuclear proteins from Jurkat cells are able to bind the XRE sequences. Purification on RNA affinity column and immunoblot analysis showed that one of these proteins with an apparent mass of 36-kDa is hnRNP A1. We then demonstrated that hnRNP A1-containing nuclear extracts are able to compete with Rex for binding to XRE-RNA using in vitro binding assays. Furthermore, nuclear extracts immunodepleted of hnRNP A1 were unable to disrupt Rex-XRE complexes. In these experiments, the amount of these RNA-binding proteins appears to be a critical parameter in controlling Rex activity. We indeed observed a lower level of hnRNP A1 in in vitro HTLV-1-transformed virus-producing T cells than that detected in Jurkat cells. Finally, overexpression of hnRNP A1 in 293T cells was found to correlate with an inhibition of XRE-dependent mRNA accumulation in the cytoplasm of these cells. Collectively, these findings suggest that hnRNP A1 inhibits Rex intervention in the cytoplasmic expression of genomic and singly spliced HTLV-1 mRNAs.

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1The abbreviations used are: HTLV-1, human T cell leukemia virus type 1; HIV, human immunodeficiency virus; XRE, Rex-response element; RRE, Rev-response element; hnRNP, heterogeneous nuclear ribonucleoprotein; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered solution; PMSF, phenylmethylsulfonyl fluoride; snRNP, small nuclear ribonucleoprotein; DTT, dithiothreitol; nt, nucleotide.
MATERIALS AND METHODS

Cell Culture, Transfection, and CAT Assays—Jurkat lymphoblastoid T cells were incubated at 37 °C in a 5% CO₂ atmosphere in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum and 20 µg/ml penicillin, 20 µg/ml streptomycin. We used in this study three HTLV-1-transformed T cell lines, C91PL (10), MT2 (11), and HUT-102 (12), which were cultured in complete RPMI medium. Cord blood lymphocytes were isolated from freshly collected heparinized cord blood by Ficoll-Paque (Amersham Biosciences) density gradient centrifugation. Adherent cells were removed by incubation of the cells for 1 h at 37 °C on a plastic Petri dish (Falcon). Non-adherent cells seeded at 2 × 10⁶ cells/ml were stimulated with 1 µg/ml phytohemagglutinin (Welcome reagent Ldt) and expanded in a 5% CO₂ atmosphere at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum and 20 µg/ml penicillin, 20 µg/ml streptomycin. These cells seeded at 1.2 × 10⁶ cells per well of a 12-well plate were transfected using the calcium phosphate coprecipitation technique (13). Jurkat cells were transfected by using the X-tremeGENE Q2 transfection reagent (Roche Molecular Biochemicals) according to the procedure described by the manufacturer.

Preparation of Nuclear Protein Extracts—Nuclear extracts from Jurkat cells were prepared according to the procedure of Andrews and Faller (15), derived from the technique described by Dignam et al. (16). The nuclear extracts were then dialyzed against extraction buffer containing 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, and protein concentration was determined by the standard Bradford method. These extracts were stored at −80 °C, until use.

Depletion of hnRNP A1 from the nuclear extract of Jurkat cells was performed through the use of immunomagnetic beads. Briefly, nuclear extracts (15 µg) were incubated with 0.5 µl of either the mouse anti-hnRNP A1 monoclonal antibody 4B10 or a monoclonal antibody isotopic (IgG 2b) control (Immunotech) or of extraction buffer, in a final volume of 30 µl with this buffer. After an incubation for 30 min on ice, immunomagnetic beads (Dynabeads M-450, Dynal) coated with goat anti-mouse IgG (2 × 10⁶ beads/assay) were added. Each sample was then incubated at 4 °C for 30 min. The beads were then removed with a magnet, and Dynabeads M-450 were added again. Another incubation was performed for 30 min at 4 °C. After removing the beads, unbound nuclear proteins were collected, assayed for hnRNP A1 content by Western blot analysis, and immediately used in binding experiments.

Production of Recombinant Rex Protein—The HTLV-1 Rex coding sequence was PCR-amplified with the following oligonucleotide primers: sense 5'-GGGGATCCTGTAAGGAGAGCCG-3' (introducing an NdeI site on ATG) and antisense 5'-GGGGGTTCATCGGGGAATTCTTG-3'. The PCR-amplified fragment was inserted into the vector pET8cHis(6)α (a gift of P. Chavrier, INSERM, Marseille, France). Escherichia coli strain BL21(DE3)Lys containing pET8c-Rex was induced with isopropyl-β-D-galactopyranoside at a final concentration of 0.5 mM during the exponential phase growth (with an A₆₀₀ of 0.5) of the bacterial culture for 4 h at 37 °C. Cells were harvested by centrifugation and then frozen and thawed in 6 µl guanidine hydrochloride, 0.1 µl phosphatase inhibitor repeated three times. The pellet was resuspended by a spin for 15 min at 10,000 × g, the proteins were purified on nickel-nitrilotriacetic acid-agarose column (Chelating resin (Qiagen) according to the manufacturer’s protocol. Recombinant proteins were eluted in buffer containing 50 mM Tris-HCl, pH 8.0, 20 mM KCl, and 500 mM imidazole. After determination of protein concentration by the standard Bradford method, fractions were stored at −80 °C.

DNA Constructs and in Vitro Transcription—Plasmids referred to as pKS-XRE and pKS-RRE were constructed by excision with Clal of the complete XRE and RRE sequences, from the two respective variants of PDM138 (17). These sequences were then reinserted into the Clal site of the plasmid BluescriptKS+/ (Stratagene) located between the T3 and T7 promoters. Constructs were verified by DNA sequencing. To generate biotinylated RNAs, pKS-XRE- and pKS-RRE-containing fragments were linearized with HindIII and XhoI, respectively, and were transcribed using the in vitro transcription kit (Promega) in the presence of equal amounts of biotin-21-UTP (CLONTECH) and UTP to ensure incorporation of both biotin molecules per transcript. Biotinylated RNAs were purified by passage through Quick spin columns (Roche Molecular Biochemicals).

Radiolaabeled XRE-RNA probes were obtained by in vitro transcription using T7 RNA polymerase in the presence of [α-32P]UTP. A typical reaction was carried out in 20 µl of T7 polymerase buffer containing 0.2 µg/µl RNA template, ATP, GTP, and CTP, 2.5 mM each, 100 µM UTP, and 50 µCi [α-32P]UTP at 3,000 Ci/mmol (ICN Biomedicals) with 1 µl of RNA polymerase (20 units/µl; Promega). After purification, the labeled RNA was heat-denatured (85 °C for 2 min) and re-natured at room temperature for 20 min before use.

The pFLAG-hnRNP A1 expression vector was generated by PCR, using the primer CGGAGATCTGAGGACTACAAGGATGACGATGACA-ATGTCACAGTCTCTCATAAGA coding for both the FLAG coding sequence and part of the N terminus of hnRNP A1, and the primer CAGGATCCAAATTCCTCCCACCAGAGGATGGTGGTGGAAGCTTATT coding for the vector of the hnRNP A1 cDNA. The PCR-amplified product was digested by BamHI and EcoRI and cloned into plasmid pDNA3.1 (Invitrogen). The truncated hnRNP A1 1C was similarly constructed using a PCR-amplified fragment that represents hnRNP A1 (amino acids 1–245).

The pCAT-XRE and pCAT-RRE plasmids were derived from the reporter plasmid pDM138 containing the chloramphenicol acetyltransferase (CAT) gene and the XRE or the RRE sequences (17). They express, under the control of the cytomegalovirus promoter, a two-oxo, one-intron precursor RNA in which the CAT gene and the XRE or the RRE are located within the intron (see Fig. 6A). The rex or rev expression plasmids, containing the wild type Rex or Rev sequences under the control of the cytomegalovirus promoter, were gifts from B. C. Cullen. The control plasmid pCMV was constructed as described previously (18).

Northwestern Analysis of RNA-binding Proteins—Nuclear proteins (4 µg) were separated by SDS-10% PAGE and transferred to a nitrocellulose membrane (0.22-µm pore size, Schleicher & Schuell). The membrane, first blocked with phosphate-buffered saline (PBS) containing 5% dry milk and 1 mM DTT for 1 h, was washed with a buffer containing 0.1% Tween 20, 1.3 mg/ml NaCl, 20 mM HEPES, pH 7.9, 100 mM KCl, and 0.2 mM EDTA, and protein concentration was determined by the standard Bradford method. These extracts were stored at −80 °C, until use.

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fected cells were lysed in SDS sample loading buffer, and equal amounts of proteins were subjected to 10 or 12% SDS-PAGE. They were subsequently blotted onto nitrocellulose membrane (BA, Schleicher & Schuell). The membrane was then blocked overnight at 4 °C in blocking buffer (PBS and 0.1% Tween 20) supplemented 10% nonfat powdered milk and probed with the appropriate antibody diluted in blocking buffer plus 10% nonfat powdered milk. The following antibodies were used: rabbit polyclonal anti-Rex antibody, mouse monoclonal anti-hnRNP A1, and anti-hnRNP C antibodies (4B10 and 4F4, respectively; gifts from G. Dreyfuss), control murine IgG2b antibody (Immunotech), mouse monoclonal anti-FLAG M2 (Sigma), mouse monoclonal anti-U1 snRNP (H111, gift from R. Lührmann), followed with an anti-rabbit (Immunotech, France) or anti-mouse (Dako) immunoglobulin G-horse-radish peroxidase-conjugated antibody. Blots were then developed using an enhanced chemiluminescence detection system (Renaissance, PerkinElmer Life Sciences). Bands were visualized by using Hyperfilm (Amersham Biosciences).

**Immunofluorescence Staining**—Cells were washed in PBS, fixed in 3% paraformaldehyde for 20 min at room temperature and permeabilized with Triton X-100 as described previously (18). The anti-FLAG or 4B10 antibody was diluted in 5% goat serum and added to cells for 1 h at room temperature. After three washes in PBS, fluorescein-isothiocyanate-conjugated goat anti-mouse antibodies (Dako) were added to cells for 1 h at room temperature. Cells were then washed and examined under a microscope.

**RESULTS**

Nuclear Proteins from Jurkat T Cells Interfere with the Binding of Rex to Its Response Element—To approach the molecular mechanisms underlying the decreased functionality of the Rex protein in Jurkat lymphoblastoid T cells, we investigated whether nuclear proteins from Jurkat cells were able to recognize XRE sequences. To that aim, we elected to use an RNA selection assay in which biotinylated RNA obtained by in vitro transcription was adsorbed to streptavidin-agarose beads and then incubated for 3 h with the recombinant Rex protein. The biotinylated ribonucleoprotein complexes were then isolated on streptavidin-coated beads, and the amount of Rex bound to the RNA probe was assessed by Western blot analysis. Thus, Rex was shown to bind an XRE-RNA probe but not to an antisense XRE-RNA probe (Fig. 1A, compare lanes 1 and 3). The addition of nuclear proteins from Jurkat cells led to a decreased amount of Rex bound to the XRE-RNA probe (compare lanes 1 and 2). Furthermore, we confirmed previous data demonstrating that Rex binds the RRE (19), and we showed that this viral protein interacted with biotinylated RRE-RNA of HIV-1 (lane 5). Interestingly, the formation of Rex-RRE complexes was not altered after addition of nuclear proteins (compare lanes 5 and 6). Since cellular proteins have been shown to bind the RRE (Ref. 20 and data not shown), this observation indicates in turn that they are unable to prevent Rex accessing the RRE.

To characterize further the interference of nuclear proteins with Rex binding to the XRE, biotinylated XRE-RNA was incubated for 3 h with increasing amounts of the recombinant Rex protein (up to 3 ng) either alone or together with an identical amount (1.5 μg) of nuclear proteins from Jurkat cells. Under these conditions, the amount of Rex bound to the XRE probe was found to decrease gradually (Fig. 1B). To note that Rex was stable in the presence of nuclear extract as assessed by Western blot analysis of the biotin supernatant (see Fig. 1B, inset, lane 2). They results clearly underline that XRE-binding nuclear proteins from Jurkat T cells specifically interfere with the binding of Rex to its response element.

hnRNP A1 Is an XRE-binding Nuclear Protein, Which Behaves as an Inhibitor of Rex/XRE Interactions—To identify the XRE-binding nuclear proteins from Jurkat T cells, nuclear extracts were submitted to a Northwestern blotting analysis by using a radiolabeled XRE probe. The autoradiography revealed that, beside several minor proteins, three main proteins with apparent molecular masses of 94, 36, and 30 kDa, respectively, were found to bind the XRE RNA (Fig. 2A).

We focused our attention on the 36-kDa protein, mainly because of the presence in the XRE of a UAGGUA sequence related to the consensus hnRNP A1-binding site (21). RNA affinity chromatography was performed in which Jurkat nuclear proteins were incubated with biotinylated XRE-RNA immobilized on beads. Analysis of bound proteins from this RNA affinity column was performed by Sypro-red staining of an SDS-electrophoresis gel and revealed the presence of four main bands, with an apparent molecular mass of 95, 60, 55, and 36 kDa (Fig. 2C). Western blot analysis of these proteins in the presence of the 4B10 monoclonal antibody to hnRNP A1 clearly showed that the 36-kDa protein was indeed reactive (Fig. 2B, lane 1) with that antibody but not with the hnRNP C antibody (lane 2). These observations clearly indicate the presence of hnRNP A1 on the XRE-RNA.

Next, in vitro binding assays using biotinylated XRE-RNA were performed, in which a constant amount of recombinant Rex was incubated with different amounts of proteins. Nuclear extracts was incubated with biotinylated XRE-RNA immobilized on beads. Under these conditions, the amount of Rex bound to the XRE probe was gradually reduced, after incubation with two different amounts (1 and 10 μg) of Jurkat nuclear proteins (Fig. 3). This decrease was found to correlate with an increase in the amounts of hnRNP A1 in RNP complexes. These results underscore the interactions of hnRNP A1 with the XRE and propose that hnRNP A1 is able to compete with Rex in binding the XRE.

To ascertain further that hnRNP A1 is affecting the formation of Rex-XRE complexes, we proceeded to the immunodepletion of hnRNP A1 from nuclear extracts of Jurkat cells. We first determined that 15 μg of nuclear proteins were found to inhibit the binding of 10 ng of recombinant Rex to XRE (Fig. 4A). Consequently, this amount of nuclear proteins was immunodepleted of hnRNP A1 by incubation for 1 h in the presence of the murine monoclonal antibody to hnRNP A1 (4B10). In parallel control experiments, incubations were performed either without antibody (mock-treated) or with a murine isotype-matched antibody. Each extract was then incubated with anti-mouse immunoglobulin-coated magnetic beads. After removal of the beads, the Western blot analysis of nuclear proteins confirmed that treatment with the hnRNP A1 antibody led to the partial depletion of that RNA-binding protein (Fig. 4B, lane 2). On the contrary, hnRNP A1 was still present among nuclear proteins either mock-treated or incubated with the murine isotype-matched antibody (lanes 1 and 3). Then each of these three nuclear extracts was incubated with biotinylated XRE-RNA together with recombinant Rex. The Western blot analysis performed to verify the presence of Rex on the XRE probe showed that a significant amount of Rex bound to XRE was detected but only after incubation with the hnRNP A1-depleted nuclear proteins (Fig. 4C, compare lane 2 to lanes 1 and 3). These results confirm that hnRNP A1 behaves as an in vitro inhibitor of Rex/XRE interactions.

The above results imply that hnRNP A1 by interfering with Rex/XRE interactions is able to inhibit the post-transcriptional activity of Rex. As the production of viral particles by in vitro HTLV-1 transformed T cells is attesting to an efficient Rex activity in these cells, we speculate that the amount of hnRNP A1 in these cells should be lower than that found in Jurkat
lymphoblastoid T cells. To verify this hypothesis, the relative abundance of hnRNP A1 in nuclear extracts from three HTLV-1-transformed (C91PL, HUT-102, MT2) cells and from Jurkat cells was measured by a semi-quantitative Western blot analysis using increasing amounts of nuclear proteins (from 31 to 125 ng). Membranes were then probed with anti-hnRNP A1 (4B10) and anti-U1-snRNP (H111) antibodies (Fig. 5, A and C, respectively). The content of hnRNP A1 in each cell line was then expressed as a ratio to that of U1 snRNP. This analysis indicated that the level of hnRNP A1 expressed in the three HTLV-1-transformed T cell lines was from 5.4- to 3.4-fold lower than that in Jurkat T cells (Fig. 5B). To ascertain that this protein is not present in the extracts because of association with HTLV-1 RNA, nuclear extracts were lysed in SDS denaturation buffer and analyzed by immunoblotting. Under these conditions, a low level of hnRNP A1 protein was still observed in these HTLV-1-transformed cells (not shown). Furthermore, the analysis of nuclear proteins prepared from uninfected cord blood lymphocytes revealed that the amount of hnRNP A1 detected in these cells was quite similar to that found in Jurkat cells. Collectively, our data imply that a low level of hnRNP A1 in HTLV-1-transformed T lymphocytes correlates with an efficient activity of Rex.

hnRNP A1 Modulates the Post-transcriptional Activity of Rex—To delineate further the involvement of hnRNP A1 as an in vivo inhibitor of Rex/XRE interactions, the functional consequences of an overexpression of this protein were next evaluated. To that aim, 293T cells were transiently transfected either with a FLAG-tagged-hnRNP A1 (FLAG-A1) expression plasmid or with the pcDNA3.1 control plasmid. Overexpression of hnRNP A1 was evaluated by immunofluorescence and Western blot assays. The immunofluorescence study using the 4B10 antibody clearly showed the presence of a significant number of brighter cells among cells transfected with the FLAG-A1 vector, compared with cells transfected with the control plasmid (Fig. 6B). As expected, the tagged hnRNP A1 revealed by incu-
Modulation of Rex Function by hnRNP A1

**Fig. 2.** Identification of Jurkat nuclear proteins binding to the XRE RNA probe. A, Northwestern (NW) blot analysis of 4 μg of nuclear proteins that were separated by SDS-10% PAGE, transferred to a nitrocellulose membrane, denatured, renatured, and probed with 32P-XRE RNA. Molecular masses are indicated on the left (low molecular weight electrophoresis calibration kit, Amersham Biosciences). B, Western blot (WB) analysis of proteins eluted from biotinylated XRE RNA. Blots were incubated with the 4B10 anti-hnRNP A1 antibody (lane 1) or with the 4F4 anti-hnRNP C antibody (lane 2) and revealed by an enhanced chemiluminescence detection system. C, analysis of bound proteins from the XRE affinity column was performed by Sypro-red staining of a 12% SDS-PAGE. Molecular masses are indicated on the left.

**Fig. 3.** hnRNP A1 inhibits the in vitro formation of Rex-XRE complexes. Indicated amounts of nuclear proteins from Jurkat cells were incubated with biotinylated XRE probe in the presence of Rex protein (2 ng) and streptavidin-coated Dynabeads for 3 h at 4 °C, as indicated under “Materials and Methods.” The presence of Rex (upper panel) and hnRNP A1 (lower panel) proteins in the complexes were then assessed by Western blot analysis, by using successively the polyclonal anti-Rex and the monoclonal anti-hnRNP A1 (4B10) antibodies.

**Fig. 4.** Jurkat nuclear proteins depleted of hnRNP A1 do not inhibit the formation of the Rex-XRE complexes. A, recombinant Rex protein (2 ng) was incubated with the biotinylated XRE RNA (20 ng) and with different amounts of nuclear proteins from Jurkat cells. Binding assays performed under these conditions indicate that 15 μg of nuclear proteins are sufficient to fully inhibit the formation of Rex-XRE complexes. B, Western blot analysis of hnRNP A1-depleted nuclear extract (NE) using immunomagnetic beads. The nuclear extract of Jurkat cells (15 μg) either untreated (lane 1) or treated with the mouse 4B10 anti-hnRNP A1 antibody (lane 2) or with a control isotypic (IgG2b) monoclonal antibody (lane 3) was immunoblotted using the 4B10 antibody (Ab). C, effects of the treated nuclear extracts on the formation of the Rex-XRE complexes. Fifteen μg of each nuclear extract obtained as described in B were assayed in the in vitro binding assay performed along the experimental procedure described in A. When hnRNP A1-depleted nuclear extract was used, the formation of Rex-XRE complexes was not altered (lane 2).
mid together either with a FLAG-tagged hnRNP A1 or with a FLAG-tagged mutant hnRNP A1 referred to as FLAG A1 ΔC. This mutant, deleted of 75 amino acids in the C-terminal region, lacks part of the glycine-rich domain and the M9 sequence allowing hnRNP A1 to shuttle between the nucleus and the cytoplasm. Immunoblotting of the cell lysates performed with the anti-FLAG antibody revealed equal amounts of a 36-kDa protein in the FLAG A1-transfected cells and of a 29-kDa protein in the FLAG A1 ΔC-transfected cells (Fig. 7, lower panel). No protein was detected in cells transfected with the pcDNA vector. When overexpressed, hnRNP A1 led to an inhibition of Rex activity. Indeed, a 50% decrease in CAT activity was observed in Jurkat cells cotransfected with the FLAG-A1 plasmid together with the pCAT-XRE and the rex-expression plasmids, when compared with cells co-transfected only with the pCAT-XRE and Rex vectors (Fig. 7, compare lanes 2 and 4). Conversely, after transfection of Jurkat cells with the FLAG-A1 ΔC plasmid, no inhibition in CAT activity could be observed (compare lanes 2 and 6), indicating that this mutated protein had no effect on CAT expression. Taken together, these results underline that hnRNP A1 exerts a specific inhibitory action on Rex function.

**DISCUSSION**

The expression of HTLV-1 and of HIV-1 structural and enzymatic proteins is regulated by viral regulatory proteins, which operate at the transcriptional and post-transcriptional levels. In particular, both viruses encode trans-acting proteins, Rex and Rev, respectively, which allow the nucleo-cytoplasmic export of unspliced and singly spliced mRNAs (1, 3). The functionality of these proteins depends on binding to viral RNAs and on interacting with CRM1 (a cellular protein belonging to the importin/karyopherin β family transport receptors), via their activation domains, which act as nuclear export signals (22–24). The leucine-rich activation domains of Rex and Rev are functionally interchangeable, underlining that the specificity of these RNA-binding proteins is dependent on the recognition of highly structured sequences defining their response elements, XRE and RRE, respectively (5–7).

In the present study, we have addressed the molecular and cellular mechanisms underlying the decreased functionality of Rex in Jurkat lymphoblastoid T cells, in which incidentally Rev was found to be fully efficient (9). We therefore investigated whether Rex interaction with the XRE might be impaired in these cells. We first showed that Jurkat nuclear proteins are able to interfere with Rex binding to the XRE. By means of Northwestern assays, we characterized one protein with a molecular mass of 36 kDa able to recognize and to bind XRE sequences. Immunoblot of Jurkat nuclear proteins bound to the XRE RNA probe identified this 36-kDa protein as hnRNP A1.

We confirmed by *in vitro* competition assays that hnRNP A1 has the ability to disrupt the Rex-XRE complexes. These data therefore provide evidence that hnRNP A1 competes with Rex in binding XRE in a concentration-dependent manner, suggesting that the amount of these RNA-binding proteins is a critical parameter in controlling Rex activity. Indeed, when compared with that detected in Jurkat cells, a lower amount of hnRNP A1 was found to characterize HTLV-1-producing T cell lines, in which Rex is functionally efficient. Furthermore, overexpression of hnRNP A1 led to a decrease in the Rex/XRE-dependent expression of a reporter gene. As expected, the Rev/RE-dependent expression of the same reporter gene was not affected by the overexpression of hnRNP A1. Consequently, the present study supports the premise that among cellular proteins implicated in the life cycle of HTLV-1, hnRNP A1 is endowed with the ability to restrict the production of structural and enzymatic viral proteins.

One intriguing finding of the present study concerns the different levels of hnRNP A1 detected in uninfected cord blood lymphocytes and in *in vitro* HTLV-1-transformed cells. In the former, the amount of hnRNP A1 was quite similar to that found in Jurkat cells, whereas, for example, C91PL cells that were obtained after co-cultivating cord blood cells with X-irradiated producing T cells were expressing a lower amount of hnRNP A1.

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We confirmed by *in vitro* competition assays that hnRNP A1 has the ability to disrupt the Rex-XRE complexes. These data therefore provide evidence that hnRNP A1 competes with Rex in binding XRE in a concentration-dependent manner, suggesting that the amount of these RNA-binding proteins is a critical parameter in controlling Rex activity. Indeed, when compared with that detected in Jurkat cells, a lower amount of hnRNP A1 was found to characterize HTLV-1-producing T cell lines, in which Rex is functionally efficient. Furthermore, overexpression of hnRNP A1 led to a decrease in the Rex/XRE-dependent expression of a reporter gene. As expected, the Rev/RE-dependent expression of the same reporter gene was not affected by the overexpression of hnRNP A1. Consequently, the present study supports the premise that among cellular proteins implicated in the life cycle of HTLV-1, hnRNP A1 is endowed with the ability to restrict the production of structural and enzymatic viral proteins.

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Fig. 6. Overexpression of hnRNP A1 in 293T cells inhibits the XRE-dependent mRNA export pathway. A, schematic view of the reporter plasmid pCAT-XRE and the FLAG-tagged hnRNP A1 protein used in this assay. The sequence corresponding to this tagged protein was inserted into the pcDNA 3.1 plasmid. B, paraformaldehyde-fixed 293T cells transfected with either the FLAG-A1 vector or with the pcDNA 3.1 plasmid were incubated with either the 4B10 anti-hnRNP A1 antibody or an anti-FLAG antibody, followed by a fluorescein isothiocyanate-conjugated anti-mouse antibody. C, Western blot analysis of the expression of the FLAG-hnRNP A1 and of the endogenous hnRNP A1 in 293T cells transfected as described in B. Proteins were detected by using an enhanced chemiluminescence detection system. D, effect of the overexpression of hnRNP A1 on CAT expression dependent on Rex/XRE or Rev/RRE interactions. Cells were transfected with 150 ng of the pCAT-XRE or pCAT-RRE or pDM138 reporter plasmids with or without the rex- or rev-expression vectors (100 ng) in combination with either the FLAG-A1 vector (300 ng) or the pcDNA3.1 control plasmid. CAT activity was assessed in cell lysates prepared 48 h after transfection. The results shown are expressed as picomoles of [3H]acetylchloramphenicol produced per min. The values of CAT activity are the means of one representative experiment (out of four experiments) performed in triplicate with the S.D. of the means. The lower panels represent the expression of the FLAG-A1 in these experiments, evaluated by Western blotting using a chemiluminescent detection system. E, effect of the concentration-dependent expression of hnRNP A1 on CAT expression mediated by Rex/XRE interactions. Cells were transfected with 150 ng of the pCAT-XRE reporter plasmid with or without the rex-expression vector (100 ng) in combination with indicated amounts of the FLAG-A1 vector. The results shown are expressed as the percent inhibition of Rex transactivation evaluated as follows: 100 – (CAT activity in Rex and FLAG-A1-transfected cells/CAT activity in Rex transfected cells). The values represent the averages of triplicates from two independent experiments.
cells with a low expression of hnRNPA1 in the population of cord blood lymphocytes may also be considered.

hnRNPA1 proteins are the major pre-mRNA binding proteins in eukaryotic cell nuclei and play important roles in the biogenesis of mRNAs (25). Indeed, they remained associated with these molecules during the processing of pre-mRNAs into mRNAs, which are then transported from the nucleus into the cytoplasm, where they are translated (26). Among these RNA-binding proteins, the 36-kDa hnRNPA1 protein plays essential functions in RNA metabolic processes. It binds to most RNA polymerase II transcripts (21), and because of its abundance and of its intracellular trafficking, it is influencing gene expression both in the nucleus and cytoplasmic compartments (27). In the nucleus, hnRNPA1 is implicated in pre-mRNA processing by binding intronic sequences and regulating alternative splicing (35). Furthermore, hnRNPA1, which shuttles between the nucleus and the cytoplasm, has been implicated in the nucleo-cytoplasmic transport, signaled by a 38-amino acid C-terminal sequence, termed M9 (between amino acids 268 and 305) (30, 31). Few observations have been devoted so far to the intervention of hnRNPA1 in the replication of viruses. Thus, hnRNPA1 has been shown to bind HTLV-1 RNA regulatory elements, suggesting that it may alter viral RNA processing (32, 33). More recently, this RNA-binding protein was found to repress HIV-1 splicing (34). Interestingly, hnRNPA1 has been shown to stimulate synergistically the HIV-1 Lrev-dependent export of unspliced mRNA (35). Finally, the participation of hnRNPA1 in the transcription and replication of a cytoplasmic RNA virus, mouse hepatitis virus, has been delineated (36).

Further characterization of hnRNPA1 binding sites to the HTLV-1 XRE-RNA will help to elucidate the precise mechanism by which hnRNPA1 is able to interfere in the RNA-mediated export of mRNAs from the nucleus. The XRE-RNA consists of two stems and four stem-loops. Alterations of these stem-loops and the stem structures strongly reduced Rex function and have indicated that the central part of the stem-loop D is sufficient to mediate Rex function (37). This stem-loop, which functions upon duplication as efficiently as the full-length XRE, contains a high affinity binding site for Rex, as revealed by a quantitative gel retardation assay (37–39). The search for binding sites for hnRNPA1 in the XRE identifies two putative high affinity-binding sequences. Thus, a 6-nt UAGGAUA sequence (nt 8768–8773) is similar to the winner UAGGG(AU) binding sequence. Furthermore, a 9-nt CUACCAGA sequence (nt 8825–8833) is similar to the hnRNPA1 binding sites CUAGACUAGA in the HIV-1 tat exon (40) and the UUAAGAUAGA in the trans-regulatory region of mouse hepatitis virus RNA (41). Interestingly, the two sequences identified in the XRE are found in the stem of the stem-loop D. Therefore, it could be speculated that hnRNPA1 binds these sequences, thus hindering the binding of Rex to its XRE-binding site.

In conclusion, our data provide direct experimental evidence that hnRNPA1, by interfering with the formation of Rex-XRE complexes, may be involved in the post-transcriptional activity of the Rex protein. Based on our results, we propose that the competition between hnRNPA1 and Rex is modulating the post-transcriptional regulation of HTLV-1 expression. Finally, the dependence of Rex functionality on hnRNPA1 by revealing another level in the control of this retrovirus life cycle might provide novel insights on HTLV-1 pathogenesis.

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Heterogeneous Nuclear Ribonucleoprotein A1 Interferes with the Binding of the Human T Cell Leukemia Virus Type 1 Rex Regulatory Protein to Its Response Element

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