

SHORT REPORT

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Identification of novel monocistronic HTLV-1 mRNAs encoding functional Rex isoforms

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Abstract

Background: Human T cell leukemia virus type 1 (HTLV-1) gene expression is controlled by the key regulatory proteins Tax and Rex. The concerted action of these proteins results in a two-phase kinetics of viral expression that depends on a time delay between their action. However, it is difficult to explain this delay, as Tax and Rex are produced from the same mRNA. In the present study we investigated whether HTLV-1 may produce novel mRNA species capable of expressing Rex and Tax independently.

Findings: Results revealed the expression of three alternatively spliced transcripts coding for novel Rex isoforms in infected cell lines and in primary samples from infected patients. One mRNA coded for a Tax isoform and a Rex isoform, and two mRNAs coded for Rex isoforms but not Tax. Functional assays showed that these Rex isoforms exhibit activity comparable to canonic Rex. An analysis of the temporal expression of these transcripts upon ex vivo culture of cells from infected patients and cell lines transfected with a molecular clone of HTLV-1 revealed early expression of the dicistronic *tax/rex* mRNAs followed by the monocistronic mRNAs coding for Rex isoforms.

Conclusion: The production of monocistronic HTLV-1 mRNAs encoding Rex isoforms with comparable activity to canonical Rex, but with distinct timing, would support a prolonged duration of Rex function with gradual loss of Tax, and is consistent with the two-phase expression kinetics. A thorough understanding of these regulatory circuits will shed light on the basis of viral latency and provide groundwork to develop strategies for eradicating persistent infections.

Keywords: HTLV-1, Rex, Splicing

Findings

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia-lymphoma (ATLL) and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) (reviewed in [1]). HTLV-1 expression is controlled by Tax and Rex, two key viral regulatory proteins coded by a doubly-spliced dicistronic mRNA containing exons 1, 2 and 3 (Figure 1). Tax provides a positive feedback circuit by driving transcription of the viral genome [2]; Rex enhances the nuclear export and expression of a subset of mRNAs coding for the virion-associated

proteins Gag-Pol and Env, while reducing the levels of the *tax/rex* mRNA, thus exerting a negative feedback loop on viral transcription [3, 4]. Using quantitative RT-PCR (qRT-PCR), we recently demonstrated a two-phase kinetics of HTLV-1 expression in short-term cultures of primary PBMCs from infected patients [5–7]. We also showed that this timing of viral gene expression was strictly dependent on Rex. Mathematical modelling [5, 8] indicated that a time delay between the positive (Tax) and negative (Rex) regulatory loops is necessary to explain the observed kinetics. This notion is in apparent contrast with the fact that Tax and Rex are known to be produced together from the 1-2-3 mRNA. However, we provided experimental evidence that Tax protein is more rapidly degraded than Rex, a property that might contribute, at least partially,

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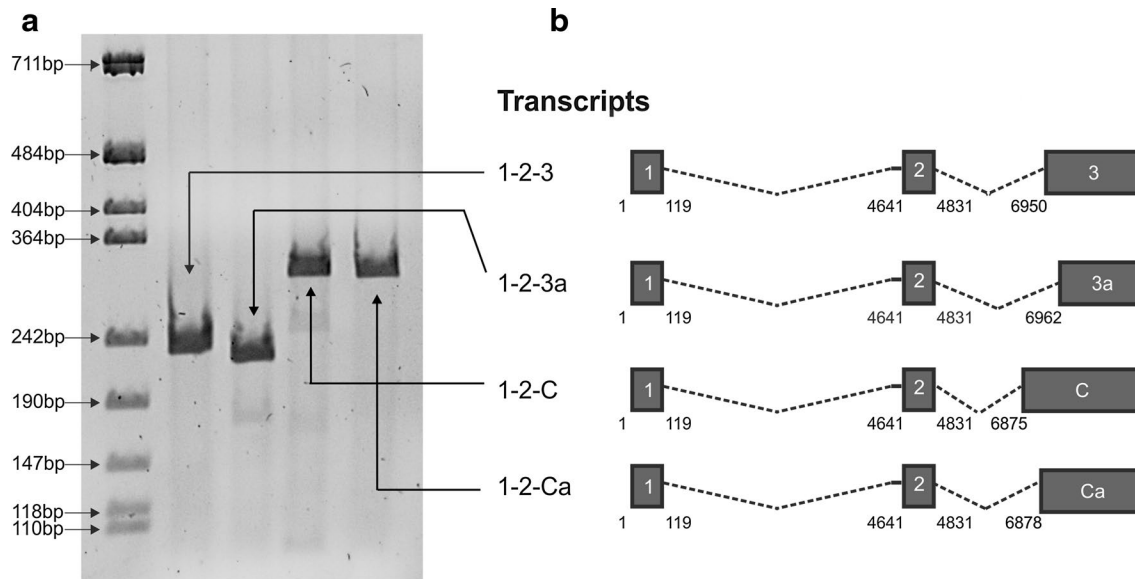


Figure 1 Identification of novel alternatively spliced HTLV-1 mRNAs. **a** RT-PCR analysis of doubly spliced mRNAs produced in HLTat cells transfected with a plasmid containing the ACH molecular clone [9]. RT-PCR was carried out with primers (Table 1) to detect transcripts joining exon 2 to the splice acceptor (SA) site of exon 3 (nucleotide 6950) or alternative to exon 3 (nucleotides 6875, 6878 and 6962). **b** Exon composition of the canonical (*1-2-3*) mRNA and of the three novel mRNAs (*1-2-3a*, *1-2-C* and *1-2-Ca*). The Hela-derived cell line HLTat [10] was maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Invitrogen), 100 units/mL penicillin, and 20 units/mL streptomycin. HLTat cells were seeded into 35 mm cell culture plates at 1.5×10^5 cells/plate and transfected 1 day later with 1 μ g of the ACH plasmid using GeneJuice Transfection Reagent (Novagene, Merck Millipore) and harvested 24 h after transfection for RNA extraction, DNase treatment, reverse-transcription and PCR analysis (RT-PCR) as previously described [5].

to a temporal separation between maximal Tax and Rex function [5]. To better define the molecular mechanisms determining the time delay between Tax and Rex function, in the present study we investigated whether HTLV-1 may produce novel mRNA species capable of expressing Rex and Tax independently.

Identification and coding potential of novel alternatively spliced HTLV-1 mRNAs

To determine whether HTLV-1 may produce novel Rex- and/or Tax-encoding mRNAs, we carried out a pilot in silico analysis to search for candidate splice acceptor (SA) sites in the vicinity of the canonical exon 3 SA at nt 6950. We focused our attention on potential sites defining 5' exon boundaries located at positions 6875, 6878 and 6962, and performed RT-PCR with primers (Table 1) to detect transcripts joining exon 2 (which contains the Rex and Tax initiation codons) to these potential SA. Results showed that mRNAs spliced at these sites were produced in cells transfected with the HTLV-1 molecular clone ACH (Figure 1). Position 6875 was previously described as the exon C SA in the context of a singly spliced mRNA coding for p13 [11, 12], while the other SA sites were not previously described. As these sites are located in the vicinity of the SA for exon C and exon 3, we propose to name these sites Ca (6878) and 3a (6962), respectively.

Table 1 RT-PCR and qPCR primers and probes

RT-PCR and real time PCR primers

TaxRex s: 5'-GTCCGCCGTCTAG[^]CTTCC-3' (exon 1[^]2 SA)

TaxRex as: 5'-CTGGGAAGTGGG[^]CCATGG-3' (exon 2[^]3 SA)

Tax_aRex_a s: 5'-ACCACCAACACCATGG[^]GGTTTG-3' (exon 2[^]3a SA)

Rex_b s: 5'-ACCACCAACACCATGG[^]CAGGTC-3' (exon 2[^]C SA)

Rex_c s: 5'-ACCACCAACACCATGG[^]GTCCTC-3' (exon 2[^]Ca SA)

Rex isoforms as: 5'-GAGTCGAGGGATAAGGAAC-3'

Real time probes

TaxRex: 5' (FAM)-CCCAGTGATCCCCTGGAG-3' (TAMRA)

Rex isoforms: 5' (FAM)-AAGCGACTGGTGGCCCATCTCTGGG-3' (TAMRA)

Figure 2 shows the coding potential of these new transcripts. mRNA *1-2-3a* has the potential to encode 4-amino acid-shorter isoforms of both Tax and Rex, which we propose to term Tax_a and Rex_a. The mRNAs *1-2-C* and *1-2-Ca* encode longer isoforms of Rex, which we propose to name Rex_b and Rex_c, respectively, but are not predicted to produce functional Tax, as the x-IV ORF is truncated by a premature stop codon located at position 6931 (Figure 2). The sequence differences of Rex_a, Rex_b and Rex_c (compared to Rex) are adjacent to the nuclear localization signal and upstream the multimerization domain of Rex [3, 13].

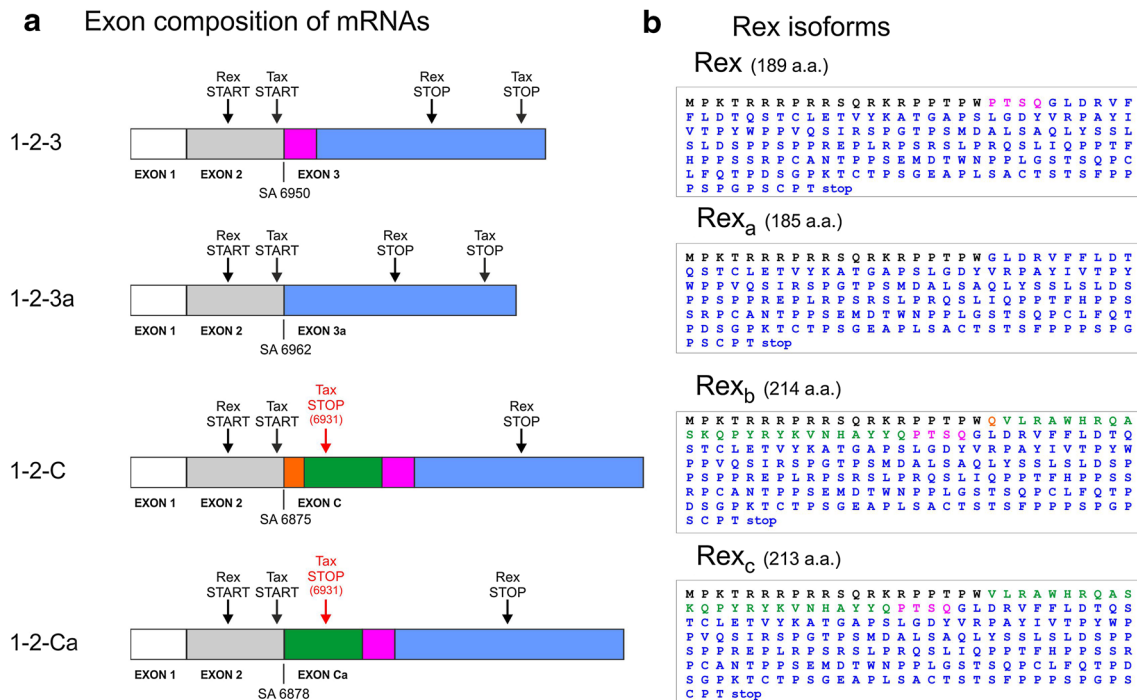


Figure 2 Coding potential of the novel mRNA species encoding Rex-protein isoforms. **a** Schematic representation of 1-2-3, 1-2-3a, 1-2-C and 1-2-Ca mRNAs; start and stop codons are indicated by arrows. Coloured boxes (not in scale) indicate the amino acid sequences differing in the alternatively spliced mRNAs. **b** Predicted amino acid sequences of proteins coded by the x-III ORF in the different mRNAs. Amino acid sequences are indicated in colours matching the boxes indicated in **a**.

Quantitation of novel alternatively spliced HTLV-1 mRNAs

We next employed qRT-PCR to measure the levels of expression of these transcripts in the HTLV-1-infected

cell line C91PL (Figure 3a), in the HeLa-derived cell line HLtat transfected with the HTLV-1 molecular clone ACH (Figure 3b), and in PBMCs from HTLV-1-infected ATLL

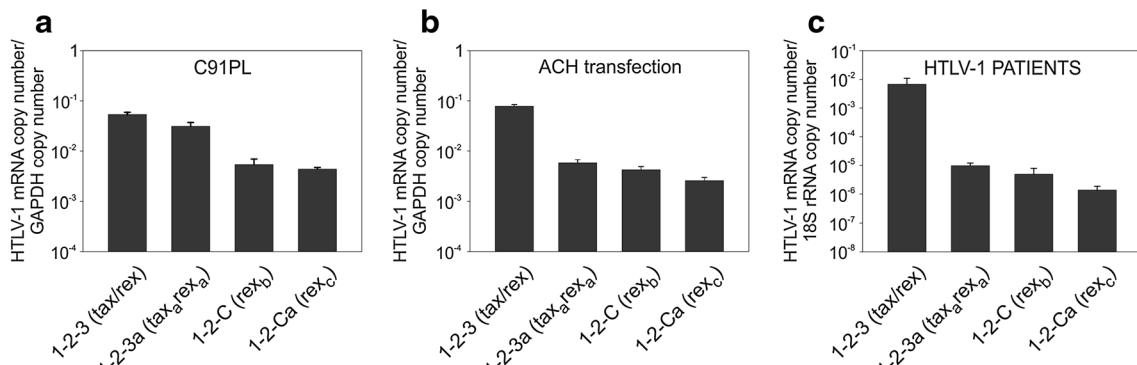


Figure 3 Quantitation of novel alternatively spliced HTLV-1 mRNAs. The *tax/rax* (1-2-3), *tax_a/rax_a* (1-2-3a), *rex_b* (1-2-C) and *rex_c* (1-2-Ca) mRNAs were quantified by qRT-PCR in **a** the C91PL T-cell line, which is chronically infected with HTLV-1, **b** HLtat cells 24 h after transfection with the ACH molecular clone, and **c** Peripheral blood mononuclear cells (PBMC) from three HTLV-1-infected patients (ATLL-2, TSP-3 and TSP-4), after 48 h of culture in vitro. **a, b** Mean values and standard error bars from three independent experiments. C91PL cells [14] were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS, 2 mM L-glutamine (GIBCO) and penicillin/streptomycin. PBMCs were isolated from peripheral blood samples donated by patients with a clinical diagnosis of ATLL or TSP/HAM attending the clinic at the National Centre for Human Retrovirology, Imperial College Healthcare NHS Trust, St. Mary's Hospital or King's College Hospital, London, UK, and treated as previously described [5, 15]. RNA extraction, DNAase treatment, reverse-transcription and qRT-PCR for the detection of HTLV-1 transcripts were performed as previously described [5] by using the primers and probes listed in Table 1. The absolute copy number of each transcript was determined and normalized (normalized copy number, NCN) for the copy number of 18S rRNA (patients' samples) or GAPDH (C91PL and ACH transfections).

and TSP-HAM patients (Figure 3c). The novel transcripts were detected in all of these samples. In C91PL cells the *I-2-3a* transcript was detected at levels similar to that of *I-2-3*, while the *I-2-C* and *I-2-Ca* transcripts were less abundant. In ACH-transfected HLTat and PBMC samples, all three novel transcripts were less abundant than the canonical *I-2-3 tax/rex* mRNA.

Intracellular localization of Rex protein isoforms

Plasmids encoding Rex, Rex_a, Rex_b and Rex_c were constructed and transfected into HLTat cells. Immunofluorescence with anti-Rex antibodies followed by laser scanner microscopy (IF-LSM) showed that Rex and Rex_a accumulated in the nucleus (visualized by propidium iodide, PI), while Rex_b and Rex_c were mainly localized to the cytoplasm

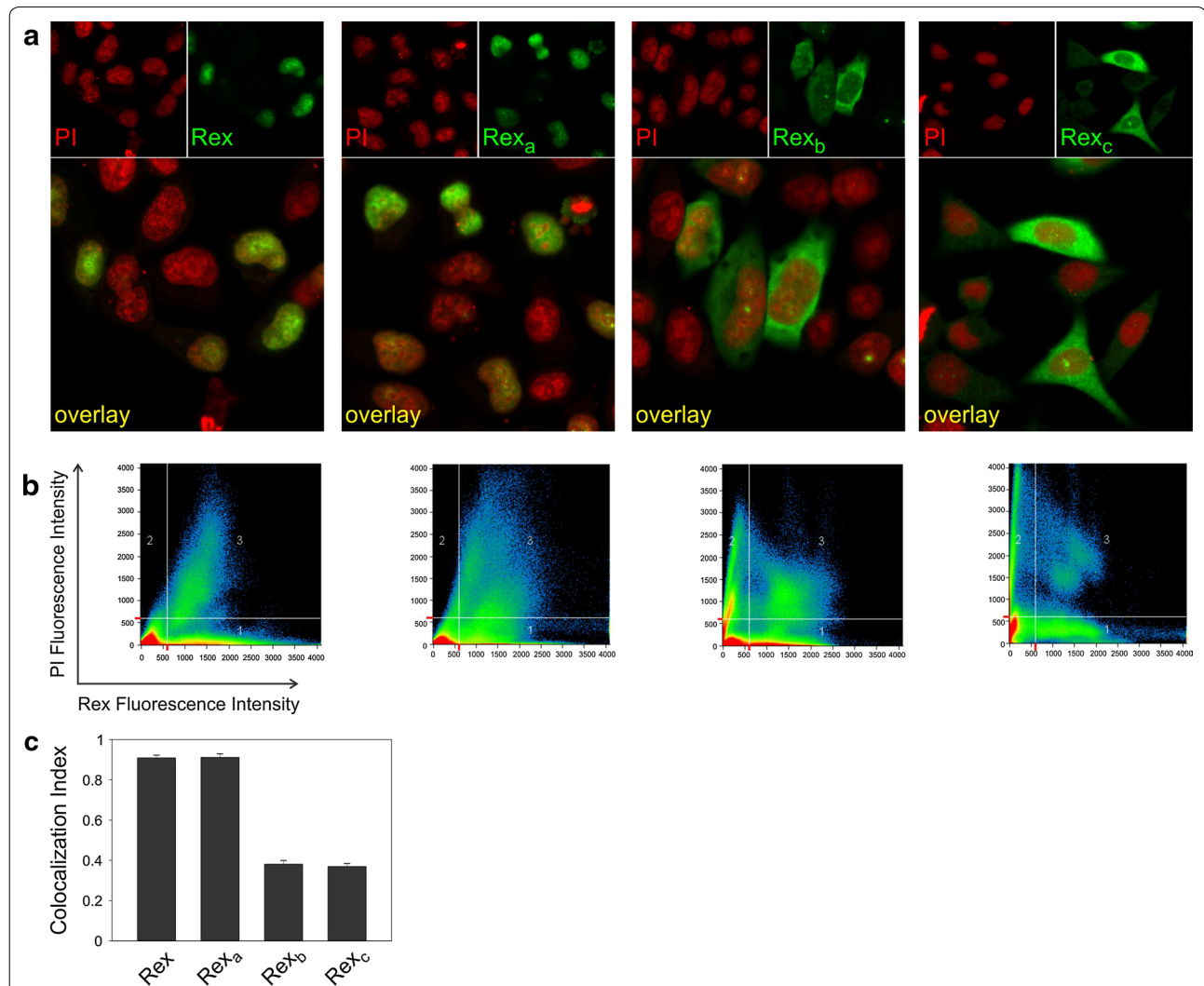


Figure 4 Intracellular localization of Rex protein isoforms. **a** IF/LSM of Rex isoforms in HLTat cells transfected with pMH-Rex, pMH-Rex_a, pMH-Rex_b and pMH-Rex_c plasmids. The Rex signal is visualized in *green*, and propidium iodide (PI), used as a nuclear marker, is visualized in *red*. **b** Quantitative analysis of the Rex (X axis) and PI (Y axis) fluorescence per pixel (represented by *dots* in the scatter plot). **c** The “Histogram” software tool was used to measure the Colocalization Index (CI), indicating the fraction of Rex-positive pixels that were also PI-positive; *bars* represent mean CI values and standard error bars for at least 50 cells. Plasmids encoding Rex, Rex_a, Rex_b or Rex_c (termed pMH-Rex, pMH-Rex_a, pMH-Rex_b and pMH-Rex_c, respectively) were generated by recombinant PCR to join exon 2 to exons 3, 3a, C and Ca, respectively. The resulting products were cloned in the expression vector pMHneo (Stratagene). HLTat cells were transfected with 1 μg of pMH-Rex, pMH-Rex_a, pMH-Rex_b or pMH-Rex_c. At 40 h after transfection, the cells were fixed in 4% formaldehyde (added to the culture medium) for 20 min, permeabilized with phosphate-buffered 0.1% NP40 for 10 min and treated with 100 μg/ml RNase for 1 h at room temperature. Cells were then incubated with a rabbit antibody (1:500) raised against amino acids 98–111 of Rex [16] for 1.5 h at 37°C, followed by incubation with an Alexa 488-conjugated anti-rabbit antibody (1:1,000, Molecular Probes) for 1 h at 37°C. Cells were then stained with 500 nM PI for 15 min at room temperature. Images were acquired with a Zeiss LSM 510 microscope using the Argon and Helium–Neon lasers (×63 optical magnification, ×4 scanning magnification). All parameters were standardized to allow comparison of signals obtained in different samples. Fluorescence signals were analyzed using a 505- to 530-nm band-pass filter for Alexa 488 and a long-pass 560-nm filter for PI.

(Figure 4a). Figure 4b shows a quantitative colocalization analysis of the Rex (X axis) and PI (Y axis) fluorescence per pixel (represented by dots in the scatter plot) that permitted calculation of the Colocalization Index (CI) indicating the fraction of Rex-positive pixels that are also PI-positive; CI values can range between 0 (no colocalization) and 1 (100% colocalization). A plot of the mean CI measured in 50 cells for each sample (Figure 4c) revealed that approximately 90% of Rex and Rex_a were localized in the nucleus, while less than 40% of Rex_b and Rex_c were in the nucleus.

Functional analysis of Rex protein isoforms

We next tested the ability of the Rex isoforms to rescue the expression of Gag (a Rex-dependent protein) from a Rex knock-out HTLV-1 molecular clone (ACH Rex-KO, Figure 5a) [5]. Results showed that Rex_a, Rex_b and Rex_c were all capable of rescuing Gag expression (Figure 5b) with comparable relative activity (normalized activity, calculated as the ratio between the Gag and Rex signals).

To validate these findings, we employed a second assay based on the pcGagRXRE reporter plasmid [11, 17], which contains the HIV-1 LTR and Gag ORF, linked to the RXRE (Figure 6a). Upon transfection of the HLTat cell line, the gag-RXRE mRNA is transcribed, but its expression is greatly impaired by cis-acting inhibitory sequences located in the *gag* gene, unless Rex is provided [18]. Cotransfection of pcGagRXRE with increasing amounts of expression plasmids coding for the Rex isoforms resulted in a dose-dependent increase in Gag expression (Figure 6b), thus confirming their functional activity.

The finding that Rex_b and Rex_c show a functional activity comparable to that of Rex and Rex_a suggests that their prevalent cytoplasmic localization is likely to result from increased nuclear export or decreased nuclear retention rather than an intrinsic defect in nuclear import, which would be expected to seriously impinge on their function.

The amino-acid changes in Rex_b and Rex_c do not affect the sequence of the nuclear localization signal (NLS) or the leucine-rich nuclear export signal/activation domain (NES/AD). However, the presence of 25 or 24 extra amino-acids in Rex_b and Rex_c respectively, immediately downstream of the NLS and 55 amino-acids upstream of the NES/AD (Figure 2) might induce conformational changes affecting the function of the NLS and/or the NES/AD. It is also possible that the extra residues affect the protein phosphorylation status, which is known to be important for Rex function [19], as well as subcellular localization in the case of Rex of HTLV-2 [20].

Distinct temporal regulation of mRNAs encoding Rex isoforms

As described above, simultaneous action of Tax and Rex at their maximal levels (as suggested by the fact that

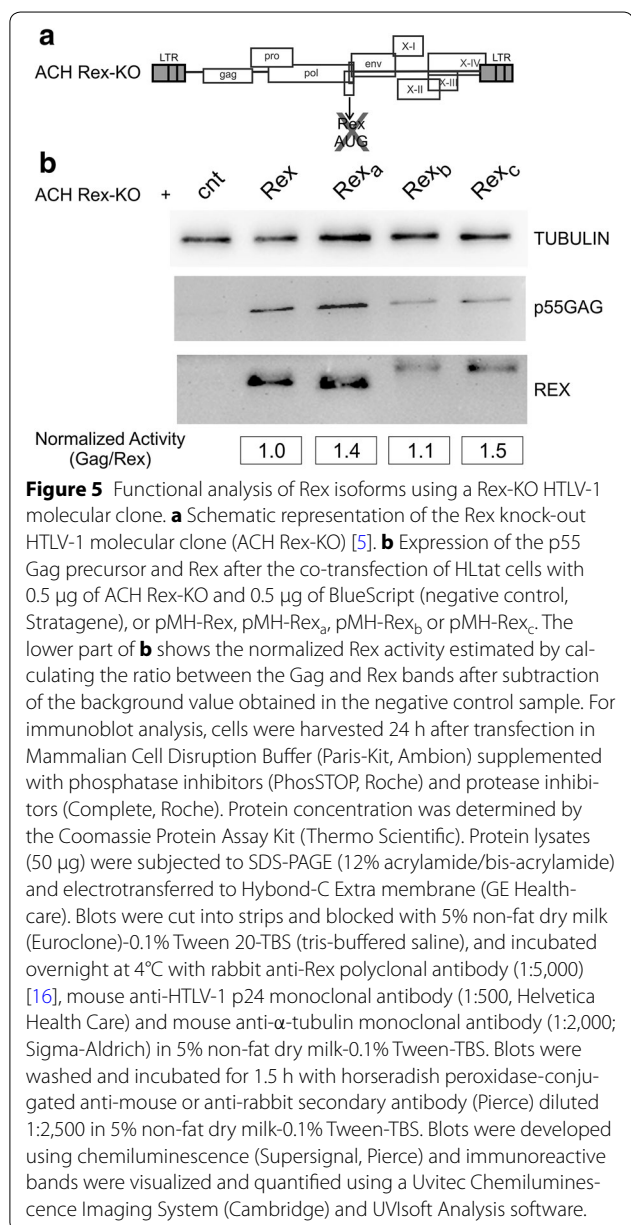
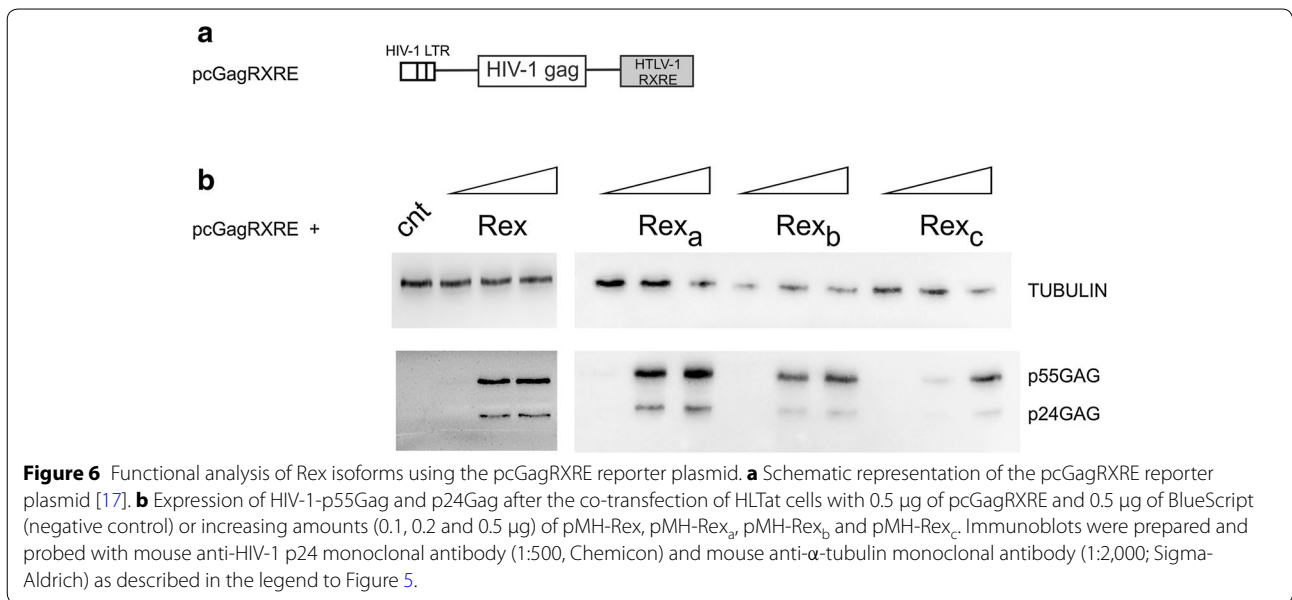


Figure 5 Functional analysis of Rex isoforms using a Rex-KO HTLV-1 molecular clone. **a** Schematic representation of the Rex knock-out HTLV-1 molecular clone (ACH Rex-KO) [5]. **b** Expression of the p55 Gag precursor and Rex after the co-transfection of HLTat cells with 0.5 μ g of ACH Rex-KO and 0.5 μ g of BlueScript (negative control, Stratagene), or pMH-Rex, pMH-Rex_a, pMH-Rex_b, or pMH-Rex_c. The lower part of **b** shows the normalized Rex activity estimated by calculating the ratio between the Gag and Rex bands after subtraction of the background value obtained in the negative control sample. For immunoblot analysis, cells were harvested 24 h after transfection in Mammalian Cell Disruption Buffer (Paris-Kit, Ambion) supplemented with phosphatase inhibitors (PhosSTOP, Roche) and protease inhibitors (Complete, Roche). Protein concentration was determined by the Coomassie Protein Assay Kit (Thermo Scientific). Protein lysates (50 μ g) were subjected to SDS-PAGE (12% acrylamide/bis-acrylamide) and electrotransferred to Hybond-C Extra membrane (GE Healthcare). Blots were cut into strips and blocked with 5% non-fat dry milk (Euroclone)-0.1% Tween 20-TBS (tris-buffered saline), and incubated overnight at 4°C with rabbit anti-Rex polyclonal antibody (1:5,000) [16], mouse anti-HTLV-1 p24 monoclonal antibody (1:500, Helvetica Health Care) and mouse anti- α -tubulin monoclonal antibody (1:2,000; Sigma-Aldrich) in 5% non-fat dry milk-0.1% Tween-TBS. Blots were washed and incubated for 1.5 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Pierce) diluted 1:2,500 in 5% non-fat dry milk-0.1% Tween-TBS. Blots were developed using chemiluminescence (Supersignal, Pierce) and immunoreactive bands were visualized and quantified using a Uvitec Chemiluminescence Imaging System (Cambridge) and UVIssoft Analysis software.

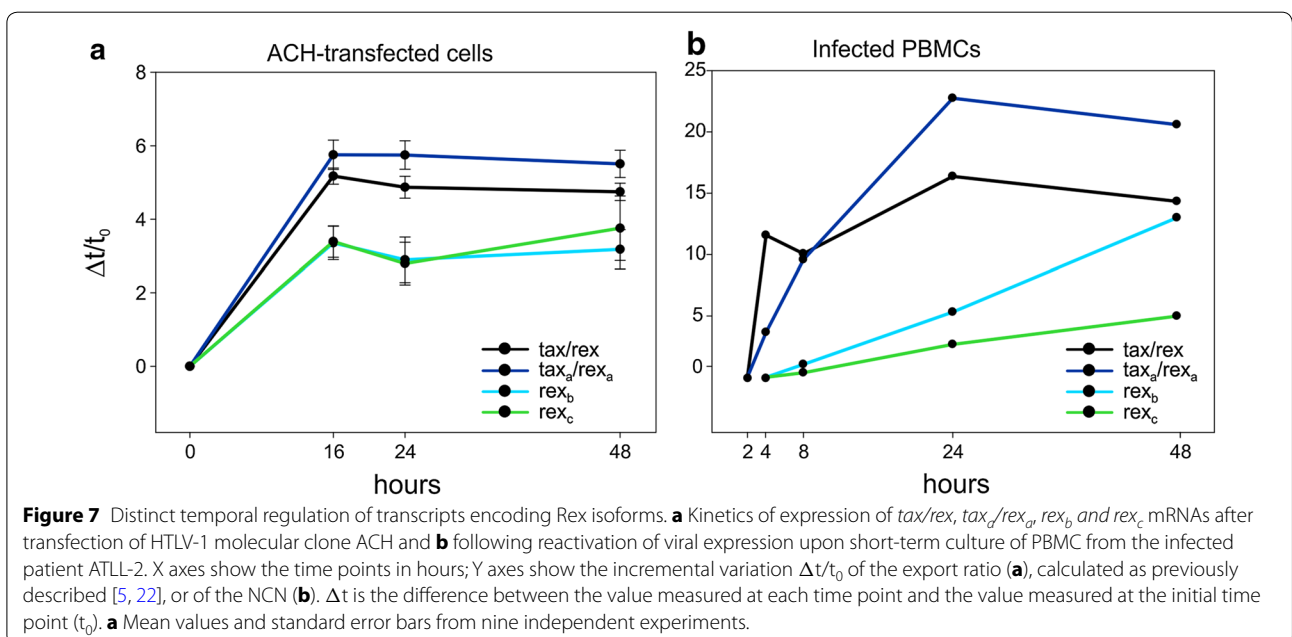
they coded by the same mRNA) is predicted to quickly shut down viral transcription due to loss of the *tax/rex* mRNA [7, 8]. It is thus necessary to assume a temporal separation between early transactivation of HTLV-1 expression by Tax and later post-transcriptional negative feedback loop mediated by Rex. In a previous study we showed that this delay could, at least in part, be attributed to an increased stability of Rex compared to Tax [5]. To test whether the delay in Rex function might also result from a distinct temporal regulation of the additional Rex-coding mRNAs, we investigated the kinetics of viral expression both following transfection of the ACH molecular clone (Figure 7a) and following reactivation of



viral expression upon short-term culture of PBMC from an infected patient (Figure 7b). Both assays revealed an early selection of splice acceptors (3 and 3a) that give rise to dicistronic mRNAs encoding functional Tax and Rex isoforms. However, as the levels of the *tax/rex* and *tax_a/rex_a* mRNAs declined, the expression of the monocistronic *rex_b* and *rex_c* mRNAs followed a steady rise, thus contributing to a prolonged duration of Rex function. These data are somewhat reminiscent of the expression strategy of HIV-1, where the two essential regulatory

proteins Tat and Rev are coded by distinct mRNAs [9, 10, 21].

These findings provide insight into the mechanisms controlling HTLV-1 expression and suggest that the production of alternatively spliced monocistronic mRNAs coding for Rex isoforms contributes to the two-phase kinetics of HTLV-1 expression. The resulting pattern of viral gene expression might be important to temporally restrain the expression of highly immunogenic viral epitopes (e.g. Tax, Gag, Env), thus favouring escape from



the immune response and establishment of long term persistence in the host, a key feature of HTLV-1 infection. A better understanding of how these regulatory circuits are established and maintained will shed light on the basis of viral latency and may provide groundwork for the development of new therapies for eradicating persistent infections.

Authors' contribution

FR carried out the molecular cloning, immunofluorescence, immunoblot and functional assays, analyzed and interpreted the data, IC carried out the qRT-PCR assays, analyzed and interpreted the data. VA and VVV helped with in initial pilot experiments. VC and GF conceived the study, analyzed and interpreted the data, and DMD participated in its design and coordination and helped to interpret the results. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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