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Research paper

Does HIV-1 mRNA 5'-untranslated region bear an internal ribosome entry site?

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ABSTRACT

Unspliced human immunodeficiency virus-1 (HIV-1) mRNA is capped and therefore can be translated via conventional scanning mechanism. In addition, its 5' untranslated region (5'UTR) is thought to function as an internal ribosome entry site (IRES) during G2/M-phase of cell cycle or when cap-dependent translation is inhibited. Recently, customary methods of internal initiation demonstrating have been challenged, and consequently existence of certain IRESs of cellular origin has been put under question. Since a precise knowledge of translation initiation mechanism used by HIV may be important for cure development, presence of the IRES in HIV-1 mRNA demands a careful reexamination using contemporary stringent criteria. The key point of our strategy is to compare translation efficiency of bicistronic mRNA bearing HIV-1 unspliced mRNA 5' UTR in the intercistronic position to that of the corresponding capped monocistronic mRNA. This approach allows determination of internal initiation contribution into the overall level of particular mRNA translation. We found that both in cell-free systems and in cultured cells monocistronic mRNA with HIV-1 unspliced mRNA 5'UTR is translated significantly better than bicistronic one. Importantly, it is also true for G2/M-phase stalled cells or for cells under conditions of inhibited cap-dependent translation. Thus, in our hands contribution of internal ribosome entry into the overall level of translation driven by HIV-1 unspliced mRNA 5'UTR is negligible, and 5'-dependent scanning is a primary mechanism of its translation initiation.

1. Introduction

Translation initiation in eukaryotes operates via two principle mechanisms: 5'-dependent scanning and internal ribosome entry. The former is apparently utilized by all cytoplasmic mRNAs and makes use of 5'-terminal m^7G-cap as a sticky point to attract translational machinery by virtue of cap-binding complex, eIF4F. This complex consists of eIF4E, which is a cap-binding subunit, eIF4A, which is an RNA-helicase required to melt secondary structures during ribosome movement, and eIF4G, a scaffold which enhances helicase activity of eIF4A and links cap binding with 40S ribosome through interaction with eIF3. Once a ribosome is placed onto the 5'-end of mRNA it starts scanning an mRNA until initiator codon in a suitable nucleotide context is met [1].

mRNAs of many representatives of *Picornaviridae*, *Hepaciviridae* or *Dicistroviridae* families some viruses lack m^7G-cap and therefore have to circumvent its absence. Instead, they possess specific sites which bind components of translational apparatus and attract ribosomes directly into internal positions of such mRNAs in the close proximity to initiator codon, thereby named Internal Ribosome Entry Sites (IRES) [2,3]. This mode of translation initiation is therefore cap-independent. Many cellular mRNAs have also been

List of abbreviations: 4E-BP, eIF4E-binding protein; 5'UTR, 5' untranslated region; eIF, eukaryotic translation initiation factor; EMCV, murine encephalomyocarditis virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HRV, human rhinovirus; IRES, internal ribosome entry site; RRL, rabbit reticulocyte lysate; TAR, trans-activation response element.

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reported to possess IRESs [4]. Recently, however, existence of IRESs in cellular mRNAs has been questioned [5–8].

HIV-1 encodes several mRNA species. The longest one, so called unspliced HIV-1 mRNA, encodes *gag* and *pol* genes. While this mRNA is capped and polyadenylated and there is little doubt that it can be translated via the cap-dependent mechanism [9–11], numerous reports stated that it may initiate translation in a cap-independent fashion as well (for review, see Refs. [12,13]). More specifically, HIV-1 IRES was proposed to be active in G2/M-phase of cell-cycle [14,15].

Thus the question of which mechanism prevails, if any, remains unclear. Recently, we and others proposed new criteria to examine presence of an IRES. These criteria are based on direct comparison of monocistronic and bicistronic mRNAs expression [16,17]. Such approach enables one to address contribution of both mechanisms to the overall level of capped mRNA translation. Here, we address the potential of the unspliced HIV-1 mRNA 5'UTR to function as an IRES using our stringent criteria. We show that no IRES activity can be detected in several cell lines, either not synchronized, or G2/M-phase stalled, or even under conditions when cap-dependent translation is compromised. Therefore, at least under conditions of uninfected cell HIV-1 unspliced mRNA 5'UTR can only drive translation in the cap-dependent fashion.

2. Methods

2.1. Plasmids and transcription

pGL3R-EMCV, pGL3R-EMCV^{mut}, pET28-2A^{pro} [18], pGL-HRV, pGL-LINE1 [19] and monocistronic pGL-HCV [20] have been described. Sequences corresponding to β-globin 5'UTR and adenovirus TPL were amplified from plasmids described previously [21] so that they contained T7-promoter and a part of Fluc sequence in the case of TPL. Monocistronic β-globin was obtained by inserting corresponding PCR-product between MluI and NcoI sites of pGL3R, and pGL-TPL was created by ligating corresponding sequence between MluI and NarI sites of pGL3R. HIV-1 gag-pol mRNA 5'UTR and first 30 nucleotides of gag ORF, corresponding to PV22 isolate, were amplified from pLSLP [22] and inserted between SpeI and NcoI sites of pGL3R backbone to create bicistronic pGL3R–HIV1 plasmid. Monocistronic version was then derived by amplification of the corresponding sequence with addition of T7-promoter from a primer just upstream of HIV-1 5'UTR and subsequent insertion between MluI and NcoI sites of pGL3R. pGL-HIV1ΔTAR was created by substituting nucleotides 3–57 of the 5'UTR with SpeI-site. AUG or UGA were introduced as described in Ref. [10]. m^{7}G-capped and A-capped mRNAs were prepared as described using ARCA or ApppG (NEB) [18].

2.2. Cell culture

293T or RKO cells were maintained in DMEM supplemented with 10% FBS. The day before transfection growing cells were plated at density about 1:3. Next day cells were transfected with total 100 ng of *in* vitro transcribed RNA (either bicistronic or equimolar mixture of monocistronic ones) and Lipofectamine 2000 (Invitrogen) or Unifectin 56 (RusBioLink) as described [16]. Generally, 3 h after transfection cells were washed with PBS, lysed with Passive Lysis Buffer (Promega), and assayed for luciferase expression with Dual Luciferase Assay Kit (Promega). In the case of kinetic experiments cells were harvested at indicated time after transfection.

For synchronisation experiments 293T cells of ~50% confluence were treated with 0.05 mM nocodazole (Sigma) for 11 h. An hour before transfection, the cells were washed with PBS and nocodazole-free medium was added. Transfection was performed essentially as described above. Torin 1 (Tocris Bioscience) treatment (250 nM, as suggested in Ref. [23]) was performed 1 h before transfection.

Jurkat cells were grown in RPMI-1460 supplemented with 10% FBS. Transfection of Jurkat cells was performed with 3 μg of *in vitro* transcribed RNA and Unifectine-56 (RusBioLink) as a transfection reagent. Attempts to use other commercially available reagents (Lipofectamine 2000 (Invitrogen), RNotion (5 Prime), Turbofect (Thermo Scientific), Metafectene Pro (Biontex); listed in the order of transfection efficiency decline) were less successful. Typically, ~10^6 cells per well were used. Torin 1 treatment was performed essentially as described for 293T cells.

2.3. Preparation of Jurkat cells extract

The extract was performed according to a protocol described previously [18]. Typically, five 150 mm culture dishes were grown to ~$5*10^5$ cells/ml (culture must be in a log phase). Cells were harvested by centrifugation, washed by 30 ml of ice-cold PBS, harvested again and resuspended with 1 ml of ice-cold PBS and centrifuged once more (3000 rpm, 5 min). After that, cells were suspended in lysolecithin buffer (1 ml per $8x10^7$ cells; 20 mM HEPES-KOH pH 7.4, 100 mM KOAc, 2.2 mM $Mg(OAc)_2$, 2 mM DTT, 0.1 mg/ml lysolecithin (Sigma)), stored for 1 min on ice, and rapidly centrifuged for 10 s at 10000 g. Then rapidly but carefully supernatant was discarded. Cells were then suspended in equal volume of ice-cold hypotonic extraction buffer (20 mM HEPES pH 7.5, 10 mM KOAc, 1 mM $Mg(OAc)_2$, 4 mM DTT, Complete Protease Inhibitor Cocktail (EDTA-free; Roche)), incubated for 5 min on ice, and disrupted in a tiny Dounce homogenizer (pestle « B») by 20–25 strokes. The lysate was clarified by centrifugation for 10 min at 10000 g. Aliquots were frozen with liquid nitrogen and stored at −80 °C.

2.4. In vitro translation

Preparation of cytoplasmic extract from mouse Krebs-2 ascites cells was described previously [24]. Translation reactions were performed in a total volume of 10 μl which contained 50% v/v Krebs-2 or Jurkat extract, translation buffer, and 15 nM mRNA at 30 °C for 1 h. When indicated, translation reaction were pre-incubated with 4E-BP1 or 2A^{pro} as described previously [18]. 4E-BP1 and 2A^{pro} were expressed and purified as described [18]. Translation in combined RRL + HeLa system has also been described [25]. The luciferases' activities were assayed using the Dual Luciferase Assay or the Luciferase Assay System kit (both Promega), when appropriate.

3. Results

3.1. Stringent criteria to address IRES activity

Conventional way of studying internal translation initiation relies on expression of bicistronic reporters, when the second cistron is only expressed if its translation is driven by an IRES. Most frequently, transfected plasmids are used as a source of corresponding bicistronic mRNA (Fig. 1A). However, DNA-transfection cannot be regarded as a reliable method to address IRES activity in a bicistronic assay. This is due to cryptic promoter activity or artifactual splicing [16,19,26,27]. The easiest way to find out that transfection of a bicistronic plasmid leads to unanticipated mRNA species has been proposed by R. Lloyd and colleagues [26]. Their approach is based on RNA-interference against first cistron sequence, Renilla luciferase in our case. In the case of the authentic