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Activity of the human immunodeficiency virus type 1 cell cycle-dependent internal ribosomal entry site is modulated by IRES *trans*-acting factors

Maricarmen Vallejos[1], Jules Deforges[2], Terra-Dawn M. Plank[3], Alejandro Letelier[1], Pablo Ramdohr[1], Christopher G. Abraham[4], Fernando Valiente-Echeverría[1], Jeffrey S. Kieft[3,5], Bruno Sargueil[2] and Marcelo López-Lastra[1,*]

[1]Laboratorio de Virología Molecular, Instituto Milenio de Inmunología e Inmunoterapia, Centro de Investigaciones Médicas, Facultad de Medicina, Pontificia Universidad Católica de Chile, Marcoleta 391, Santiago, Chile, [2]CNRS UMR 8015, Laboratoire de cristallographie et RMN Biologique, Université Paris Descartes, 4 avenue de l'Observatoire, 75270 Paris Cedex 06, France, [3]Department of Biochemistry and Molecular Genetics, [4]Department of Microbiology and [5]Howard Hughes Medical Institute, University of Colorado Denver School of Medicine, Aurora, CO, 80045, USA

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ABSTRACT

The 5′ leader of the human immunodeficiency virus type 1 (HIV-1) genomic RNA harbors an internal ribosome entry site (IRES) that is functional during the G2/M phase of the cell cycle. Here we show that translation initiation mediated by the HIV-1 IRES requires the participation of *trans*-acting cellular factors other than the canonical translational machinery. We used 'standard' chemical and enzymatic probes and an 'RNA SHAPE' analysis to model the structure of the HIV-1 5′ leader and we show, by means of a footprinting assay, that G2/M extracts provide protections to regions previously identified as crucial for HIV-1 IRES activity. We also assessed the impact of mutations on IRES function. Strikingly, mutations did not significantly affect IRES activity suggesting that the requirement for pre-formed stable secondary or tertiary structure within the HIV-1 IRES may not be as strict as has been described for other viral IRESes. Finally, we used a proteomic approach to identify cellular proteins within the G2/M extracts that interact with the HIV-1 5′ leader. Together, data show that HIV-1 IRES-mediated translation initiation is modulated by cellular proteins.

INTRODUCTION

Initiation of protein synthesis in the eukaryotic cell leads to the assembly of the 80S ribosome at the start codon of the mRNA. At least two mechanisms for recruiting and positioning ribosomes on the mRNA have been described (1,2). The primary mechanism involves the recognition of the 5′ cap structure (m^7GpppN) by eukaryotic translation initiation factors (eIFs), followed by binding of the 40S ribosomal subunit and scanning downstream to the initiation codon (1,2). Alternatively, in some mRNAs a structural element, the internal ribosome entry site (IRES), allows assembly of the translational machinery at a position close to or directly at the initiation codon (3,4). IRES elements were first described in the uncapped poliovirus and encephalomyocarditis virus mRNAs (5,6). Functionally, the IRESes were identified by inserting the 5′-untranslated region (UTR) of the viral mRNA into the intercistronic spacer of a bicistronic construct encoding two proteins (5,6). In this context, expression of the second cistron documented the ability of the inserted sequence to promote internal ribosome binding and translation independent of the first cistron. Since the initial characterization of IRES elements in *Picornaviridae*, viruses from other families including the *Retroviridae* have been shown to initiate translation via an IRES (7–9).

The study of the mechanism of translation initiation of the full length RNA of the human immunodeficiency virus

*To whom correspondence should be addressed. Tel: +1 56 2 354 8182; Fax: +1 56 2 638 7457; Email: malopez@med.puc.cl

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

type 1 (HIV-1) revealed that this capped and polyadenylated mRNA can initiate protein synthesis through the canonical cap-dependent or by the alternative IRES-dependent mechanism (8–15). The HIV-1 full-length RNA harbors two IRESes, the first in the mRNAs 5′-UTR (here referred to as the HIV-1 IRES) (10,13), and the second within the Gag open reading frame (the HIV-1 *gag* IRES) (11,12,14). Translation initiation of the viral structural proteins, Gag and GagPol can thus be driven by three independent mechanisms, the canonical cap-dependant process (8,15), or by two internal ribosome entry events dependant on the HIV-1 IRES, or the HIV-1 *gag* IRES (8,10–12). In addition, the translation of a shorter 40K-Gag isoform of currently unknown function is directed by the HIV-1 *gag* IRES (8,9,11,12).

The observed redundancy and the conservation of the different mechanisms for the initiation of protein synthesis among primate lentiviruses suggest that translation initiation of HIV-1 mRNA is a key step during the viral life cycle (7–9,12). Alternative initiation may allow the viral mRNA to bypass the constraints of global cellular translation repression that normally target cap-dependent translation initiation, a proposal given credence by evidence that HIV-1 IRES supports translation initiation during osmotic stress (13,16). Additionally, HIV-1 gene expression is influenced by the cell cycle as evidenced by the observation that HIV-1-infected cells arrested in G2/M by the viral protein Vpr or by chemicals, exhibit enhanced levels of viral mRNA transcription and translation (17,18). Notably, the HIV-1 IRES supports translation of viral mRNA in HeLa cells that have been arrested in the G2/M phase of the cell cycle (10), when global cellular cap-dependent translation initiation is suppressed (19). IRES-mediated translation initiation may also ensure synthesis of viral structural proteins during the late stages of the replication cycle, when the eIF4G and the poly(A) binding protein (PABP), both required for cap-dependent translation initiation, are targeted by the viral protease (20–24).

To date the molecular mechanisms that determine the function of the IRESes harbored within the HIV-1 full-length mRNA are not clearly understood. However, recent reports suggest that translation initiation driven by the HIV-1 IRES can be modulated by cellular proteins (16,25,26). The heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), eIF5A, the human rev-interacting protein (hRIP) and DEAD (Asp–Glu–Ala–Asp) box polypeptide 3 (DDX3) have been identified as a cellular factor that enhance HIV-1 IRES activity (16,26), while the human embryonic lethal abnormal vision (ELAV)-like protein, HuR, has been describe as a negative modulator of HIV-1 IRES activity (25). These reports are in keeping with existing evidence that IRES-dependent translation for a number of viral and cellular mRNAs requires the presence of an additional and sometimes complex set of *trans*-acting factors for full translational activity (4,27,28). IRES *trans*-acting factors (ITAFs) are thought to bind to the mRNA, inducing conformational changes needed to structurally form the IRES, thereby facilitating ribosome recruitment (4,27,28).

In this study, we show that translation mediated by the HIV-1 IRES requires G2/M-specific cellular factors. We show that cell extracts alter the accessibility of chemical reagents to single-stranded regions present within the HIV-1 5′ leader region. Close analysis of these data reveal that cell factors protect a region of the HIV-1 5′ leader known to participate in IRES activity (10). A mutational analysis revealed that the HIV-1 IRES function is resistant to the introduction of mutations that were predicted to disrupt local RNA structures (29). This observation suggests that the requirement for a single, stable pre-formed secondary or tertiary structure may not be as rigid as has been described for other viral IRESes. Finally, using a proteomic approach we identify proteins present in cell extracts that interact with the HIV-1 5′ leader. Together our data suggest that the translational activity from the HIV-1 IRES is most probably modulated in *trans* by a group of proteins that specifically interact with the HIV-1 5′ leader during the different stages of the cell cycle.

MATERIALS AND METHODS

Plasmid

The dlΔEMCV and dl HIV-1 IRES plasmids were as previously described (10,25). The long distance interactions (LDI)/branched multiple hairpin (BMH) stabilizing mutations previously described by Abbink *et al*. (29) were introduced in the 5′ leader of the proviral clone pNL4.3 by overlapping extension PCR (30), using primers described in Table 1. In each case, the amplicon was digested with EcoRI and NcoI (both restriction sites added by PCR) and inserted into the intercistronic region of dl HIV-1 IRES plasmid as described (10), previously digested with the same enzymes (Fermentas, Vilnius, Lithuania). Upon sequencing additional mutations that were not originally included in the primers were identified in four constructs (namely Mut L5, Mut L6, Mut L7 and Mut L8); these mutants were included in the study. Mutant L9 was constructed by digesting Mut L8 with PauI and XbaI (Fermentas) and cloning the PauI–XbaI fragment into the Mut L7 digested with the same enzymes. As before, the generated mutant HIV-1 5′ leader was inserted into the intercistronic region of dl HIV-1 IRES plasmid as described (10). The authenticity of all plasmids used in this study was confirmed by sequencing (Macrogen Corp, Rockville, MD, USA).

Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) with 100 U/ml of penicillin–streptomycin (HyClone) and 10% fetal bovine serum (HyClone) at 37°C in a 5% CO_2 atmosphere. Nocodazol (400 ng/ml; Sigma-Aldrich) and L-mimosine (20 mM; Sigma-Aldrich) were used to enrich cells in the G2/M or G1 phase of the cell cycle, respectively. Cell cycle arrest was confirmed by flow cytometry as previously described (10). Cytoplasmic cell extracts were prepared following a previously described protocol (10,25). Upon preparation, extracts were tittered to determine the concentration that should

Table 1. Primers used to generate the HIV-1 Leader mutants

Mutant	Sense primer	Antisense primer
Mut L1	5′-GGTG***C***G***C***AC∆CCAAAAATTTTGACTAGCGGAGGCT-3′	5′-AAAATTTTTGG∆GT***G***C***G***CACCAGTCGCCGCCCC-3′
Mut L2	5′-GAGTACGCCA***TCCT***TTTTGACTAGCGGAGGCTAGAAGG-3′	5′-GTCAAAA***AGGA***TGGCGTACTCACCAGTCGCC3′
Mut L3	5′-ACGCCA***GGGG***TTTTGACTAGCGGAGGCTAGAAGG-3′	5′-AGTCAAAA***CCCC***TGGCGTACTCACCAGTCGCC-3′
Mut L4	5′-TTT***TT***CTAGCGGAGGCTAGAAGGAG-3′	5′-AGCCTCCGCTAG***AA***AAAATTTTTGGCGTACTCACC-3′
Mut L5–L10	5′-TTT***AG***CTAGCGGAGGCTAGAAGGAG-3′	5′-AGCCTCCGCTAG***CT***AAAATTTTTGGCGTACTCACC-3′
Mut L6	5′-G***A***CGCTGGTGAGTACGCCAAAAATTTTG-3′	5′-TTTTTGGCGTACTCACCAG∆CG***T***C***G***CCCCTCGCCTCTTG-3′
Mut L7	5′-CGACGCA***CCC***CTCGGCTTGCTGAAGCGCGC-3′	5′-CAGCAAGCCGAG***GGG***TGCGTCGAGAGATCTCCTC-3′
Mut L8–L11	5′-***CC***CGGCGACTGGTGAGTACGCC-3′	5′-ACTCACCAGTCGCCG***GGA***CTCGCCTCTTGCCGTG-3′

∆, deleted nucleotide with respect to pNL4.3 (AF 324493); bold, nucleotide changes with respect to pNL4.3.

be used in the assay. The adequate experimental concentration varied from one cell extract to another.

In vitro transcription

Capped RNAs were synthesized using the mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Applied Biosystems/Ambion, Austin, TX, USA), while capped and polyadenylated RNA transcripts were synthesized using the complete mMessage mMachine T7 Ultra Kit (Applied Biosystems/Ambion) according to the manufacturer's protocol. Uncapped RNA was synthesized by *in vitro* transcription conducted in a final volume of 200 µl using T7 RNA polymerase, 5 mM DTT, 5 mM rNTP's, 1X transcription buffer (40 mM Tris–HCl pH 8.0, 25 mM $MgCl_2$, 1 mM spermidine) and 0.04 U RNase Inhibitor (Applied Biosystems/Ambion) and incubated 2 h at 37°C. Upon synthesis, RNAs were treated with DNAse RQ1 (Promega, Madison, WI, USA) for 20 min at 37°C. RNA was precipitated with 2.5 M LiCl, centrifuged at 16 000*g*, 30 min at 4°C, washed with 70% ethanol and resuspended in 50 µl of nuclease-free water. RNA concentrations were determined spectrophotometrically (NanoDrop Technology, Wilmington, DE, USA) and RNA integrity was monitored by electrophoresis on denaturing agarose gels.

In vitro translation

In vitro transcribed dl HIV-1 IRES RNAs (8 ng/µl) were translated in 25% (v/v) nuclease-treated rabbit reticulocyte lysate (RRL; Promega), supplemented or not with cell extracts prepared as previously described (25). Final concentrations of extract used in each experiment are indicated in figure legends. Cell extracts were pre-incubated with RNA for 5 min prior to addition of the RRL mix as previously described (25). Luciferase activities were measured using the DLR™ Assay System (Promega) according to manufacturer's instructions on a Sirius Luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) as previously described (25,31).

For ^{35}S-methionine labeling, bicistronic RNA (1 pmol) was translated in 50% of RRL Flexi® (Promega) in the presence of 20 µM amino acids (lacking methionine), 0.5 mM $MgOAc_2$, 160 mM KOAc, 0.8 U/µl de RNasin® Plus RNase Inhibitor (Promega) and 0.6 mCi/ml [^{35}S]-methionine. Translation reactions, conducted in final volume of 10 µl, were incubated for 90 min at 30°C. The reaction was stopped with 90 µl of protein loading buffer as previously described (12). Ten microliter of the final mix was loaded and resolved by SDS–PAGE (12%), and bands visualized using a BAS-5000 phosphorimager (Fujifilm).

Oocyte microinjection

Oocytes were isolated from *Xenopus laevis* ovarian fragments and microinjected with glass micropipettes calibrated to deliver a final volume of 50 nl, as previously described (25). To assess the effect of G2/M HeLa extracts on IRES activity, oocytes were first microinjected with 6.25 ng of *in vitro* transcribed, capped and polyadenylated bicistronic RNA generated from either dl∆EMCV or dl HIV-1 IRES plasmids. Fifteen minutes later oocytes were microinjected with 200 ng of G2/M HeLa extracts. Oocytes were incubated for 24 h at 15°C in a standard Barth's solution supplemented with 10 UI/l penicillin–streptomicine and 2 mM pyruvate. Oocyte lysates were prepared in 1× passive lysis buffer (Promega Corporation), centrifuged at 16000*g* for 5 min and 1–5 µl of supernatant was used in the detection of luciferase as described above.

RNA probing

The secondary structure of the HIV-1 5′-UTR was probed using DiMethyl Sulfate (DMS, Across Organics), *N*-cyclohexyl-*N*-[*N*-methylmorpholino)-ethyl]-carbodiimid-4-toluolsulfonate (CMCT, Merck) and RNAse V1 (Applied Biosystems/Ambion) as described previously (12,32). RNA Selective 2′ Hydroxyl Acylation analysis by Primer Extension (SHAPE) analysis was conducted using 1-methyl-7-nitroisatoic anhydride (1M7) as a modifying agent as previously described (33,34). In brief, 10 pmol of *in vitro* transcribed RNA, which included the 5′ leader of HIV-1 (pNL4.3) and the first 58 nt of fluc (recovered from the dl HIV-1 IRES plasmid using a primer T7HIVF 5′-CCATATGTAATACGACTCACTATAG GTCTCTCTGGTTAGA-3′ and Fluc30bp 5′-CATCTTC CAGCGGATAGAATG-3′) were resuspended in 30 µl of 80 mM HEPES pH 7.5 (or 50 mM borate potassium pH 8 for CMCT), denatured for 2 min at 80°C, and then 2 µl of 3 M KCl and 2 µl of 40 mM $MgCl_2$ were added. Upon a 10 min incubation at 30°C DMS (0.2 mM final), CMCT (25 mM final), RNAse V1 (0.01 or 0.025 U) or 1M7 was added and the mixture was incubated for 5 min (10 min for CMCT). Mock controls, where the chemical was replaced by water or DMSO (for the 1M7 probing) were also

included. The modification reaction was stopped in ice by addition of 10 µg of yeast tRNA. As previously described (32,34), the time and concentration of modification agent were established to generate at the most one modification per molecule. The reaction was then immediately precipitated in dry ice with ethanol and 5 M ammonium acetate. RNA was then resuspended in 0.5 M ammonium acetate, ethanol precipitated in presence of 20 µg of glycogen, washed with 70% ethanol and resuspended in 6 µl of nuclease-free water. For DMS, CMCT and V1 probing, modifications were revealed by reverse transcription (AMV RT; Promega) using a ^{32}P-labeled primers (Fluc30bp; HIV1-336 R 5′-TTTGAAAAACACGAATT CGGTCTCTCTG-3′; 100pbHIV-1 5′-ACTTTGAGCAC TCAAGGCAAG-3′; 200pbHIV-1 5′-TTCGCTTTCAAG TCCCTGTTC-3′) according to the manufacturer's instructions (Promega). Reverse transcription products were resolved by 8% denaturing PAGE; the resulting gel was scanned on a Typhoon trio variable mode imager (Amersham Biosciences). The relative proportion of each product was determined, drawing profiles with Multi Gauge V3 software (Fujifilm). For 1M7 probing, modifications were revealed using RNAse H$^-$ M-MLV RT (Promega) and the Fluc30pb primer labeled with WellRed D2, D3, D4 (Sigma), or IR-800 (MWG Eurofins) fluorophores, cDNA fragments were resolved by capillary electrophoresis (Beckman Coulter CEQ 8000). Data were then interpreted and analyzed using the software 'shapefinder' (35) (http://bioinfo.unc.edu).

1M7 footprinting experiments were carried as described above except that non-synchronized, G1 or G2/M synchronized, Hela extracts (6 µg of total proteins) were added after RNA renaturation, the mixture was then incubated for 10 min at 30°C before 1M7 addition. Profiles were compared to the profile obtained with a mocked control containing the equivalent amount of buffer instead of extracts. Increases or decreases of 2-fold of the reactivity, with the higher reactivity being at least 0.3, was considered significant and reported.

Synthesis of 1-methyl-7-nitroisatoic anhydride was as described by Mortimer and Weeks (34).

DNA transfection

Cells were seeded at 1×10^5 cell/well in 12-well plates and DNA transfection (200 ng/well) was performed at 60% confluence using the JetPEI transfection system (PolyPlus transfection, France) according to the manufacturer's protocol. After 24 h, the culture medium was removed and cells lysed with 1× passive lysis buffer (Promega) as described in the DLR™ Assay System manual (Promega) (10,25,31). Protein concentration was determined by a Bradford assay using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). FLuc and RLuc activities were measured as described above.

RNA constructs and cellular extracts preparation for pull-down experiments

HeLa cells were grown to confluency in standard media or media supplemented with 400 ng/ml nocodazole. Cells were detached with trypsin/EDTA, and pelleted. Following a cold phosphate buffered saline (PBS) wash, the cell pellet was resuspended in cold buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, supplemented with protease inhibitors) and incubated on ice for 5 min. Cells were lysed with a pre-chilled Dounce homogenizer for 20 strokes using a tight fitting pestle. Dounced cells were centrifuged at 228*g* for 5 min at 4°C to pellet nuclei and other fragments. The supernatant was retained as the cytoplasmic fraction and the pellet was retained as the nuclear fraction. The cytoplasmic fraction was supplemented with 5× RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, supplemented with protease inhibitors) to a 1× final concentration and centrifuged at 2800*g* for 10 min at 4°C to pellet solids. The nuclear pellet was resuspended in buffer S1 (0.25 M Sucrose, 10 mM $MgCl_2$), layered over a sucrose cushion (0.8 M Sucrose, 0.5 mM $MgCl_2$) and centrifuged at 2800 × g for 10 min at 4°C. The pellet was resuspended in 10 pellet volumes 1× RIPA buffer and sonicated on ice. The lysate was centrifuged at 2800 × g for 10 min at 4°C to pellet solids. Protein concentration was determined using a Bradford assay (BioRad).

Templates for *in vitro* transcription of strep-aptamer tagged HIV-1 5′ leader (nucleotides 1–384) and the reverse complement HIV-1 5′ leader (RC) were generated by PCR amplification from plasmid HIV 1–384 described by Brasey *et al.* (10). A T7 promoter was added to the 5′-end, and the sequence for a streptomycin binding RNA aptamer (36) was added on the 3′ end using the primer pair 5′TAATACGACTCACTATAGggtctctctggttagaccag3′ (forward) and 5′GGATCCGACCGTGGTGCCCTT GCGGGGCAGAAGTCCAAATGCGATCCcccatttat ctaattctccc3′ (reverse) for the HIV-1 5′ leader and primers 5′TAATACGACTCACTATAGGcccatttatctaattctccc3′ (forward) and 5′GGATCCGACCGTGGTGCCCTTGC GGGGCAGAAGTCCAAATGCGATCCggtctctctggttag accag3′ (reverse) for the HIV-1 RC 5′ leader. RNA was *in vitro* transcribed from PCR templates using T7 RNA polymerase as previously described (37). RNA was purified on a denaturing polyacrylamide gel, passively eluted in diethylpyrocarbonate treated water, and concentrated using spin concentrator columns (Amicon). RNA integrity and purity was verified by denaturing polyacrylamide gel electrophoresis, and concentration was determined by ultraviolet (UV) spectroscopy.

Pull-down experiments

Pull-down experiments were performed using aptamer tagged RNA and a streptomycin-conjugated sepharose column (36). Aptamer tagged RNAs were folded over a series of incubations (65°C for 5 min, 37°C for 5 min, room temperature for 5 min) and added to 1 ml of column buffer (50 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$, 250 mM NaCl). Folded RNA was loaded onto a column packed with 1 ml bed volume of streptomycin conjugated sepharose that had been equilibrated three times with column buffer and blocked with 20 µg tRNA. The RNA was incubated on the column for 10 min, and washed

twice with 1 ml column buffer. 150 μg of protein extract (supplemented with RNASE inhibitor) was loaded onto the column and incubated for 10 min. The column was washed 10× with column buffer, and RNA–protein complexes were eluted three times with 1 ml column buffer supplemented with streptomycin to 10 μM. Elutions were concentrated using spin concentrator columns (Amicon) according to the manufacturer's protocol.

Mass spectrometry analysis

Eluted protein samples were reduced with 5 mM DTT (30 min at 70°C) and alkylated with 15 mM iodoacetamide (30 min at room temperature in the dark). Samples were digested with 1.2 μg trypsin overnight at room temperature and peptides desalted using C18 tips (Omix) according to the manufacturer's protocol. Acetonitrile was evaporated and the samples were brought up to a final volume of 15 μl with 1% formic acid. Tryptic peptides were analyzed by the UC-Denver proteomics core on an Agilent 1200 nanoLC system directly infused for MS/MS analysis on a LTQ-FT Ultra hybrid mass spectrometer (ThermoFisher). Peptides were separated using a 90 min gradient of increasing acetonitrile (8–35%) with 0.1% formic acid as a pairing agent. Electrospray ionization was performed at 200 V on the column eluent. Parent scans (MS) were acquired in the ICR cell at 50 000 resolution. Collision induced dissociation was performed in the ion trap and product ions recorded (MS/MS). Ion peak lists were created using PAVA (UCSF) and searched against the human Swiss Prot database using the Mascot server (Version 2.2, Matrix Science). Mass tolerances of ±10 ppm were used for MS peaks, and ±0.8 Da for MS/MS fragment ions. The modifications of cysteine carbamidomethylation, methionine oxidation, N-terminal acetylation of protein, N-terminal pyroglutamic acid formation and phosphorylation of serine, threonine, and tyrosine residues were allowed for. Protein identifications were considered significant if two or more peptides matched with an expect value of below 0.01.

RESULTS

Cap-independent translation initiation from the HIV-1 IRES in RRL requires cytoplasmic cellular factors

The HIV-1 IRES was identified by cloning the 5′ leader region of the laboratory adapted HIV-1 infectious recombinant proviral clone pNL4.3 or the *CXCR4 (*X4*)*-tropic HIV-1 *primary isolate* HIV-LAI in the intercistronic region of a dual luciferase (dl) reporter construct (10,13). In the context of this bicistronic RNA the HIV-1 IRES was shown to be cell-cycle regulated and preferentially active during G2/M (10), and to be functional during osmotic stress (13,16). In addition, the HIV-1 IRES is functional in HeLa cell translational extracts (10), in transfected HeLa or Jurkat T cells (10,13,16,25), and in *X. laevis* oocytes (25); however, it is poorly active in RRL (8,10).

Poor activity of the HIV-1 IRES in RRL suggests that, similar to poliovirus (PV) and human rhinovirus (hRV) IRES elements (38–40), additional factors entirely absent or present only at low concentration in the RRL may be required for HIV-1 IRES activity. To assess this possibility, RRL programmed with the dl HIV-1 IRES RNA (depicted in Figure 1A) was supplemented with 0.5 or 1 μg of cytoplasmic HeLa extracts generated from non-synchronized, mimosine-G1 or nocodazole-G2/M arrested cells. Cell arrest in G1 or G2/M was confirmed by flow cytometry (10). Translation reactions were conducted in the presence of ^{35}S-methionine and resolved and visualized as indicated in 'Materials and Methods' section. One representative experiment is shown in Figure 1B. It should be noted that optimal translation of the upstream reporter occurred at a narrow range of cell extract concentrations, since translation of the first cistron was consistently abolished with high concentrations of cell extract (Figure 1B compare lanes 2, 4, 6 with lanes 3, 5, 7). In contrast, the addition of 0.5 μg of cell extracts to the RRL had no significant impact on RLuc synthesis, thus no effect on the translation of the upstream message of the bicistronic reporter (Figrue 1B lanes 2, 4 and 6). RNA did not significantly vary among the different assays suggesting that extracts did not affect RNA integrity (data not shown). Based on this observation, in this particular experiment, data generated with 1 μg of HeLa cell extracts were not taken into account in further analysis. In agreement with previous reports (8,10), HIV-1 IRES activity was negligible in non-supplemented or G1-supplemented RRL (Figure 1B, lanes 1 and 2). When RRL was supplemented with 0.5 μg of cytoplasmic extracts generated from non-synchronized (NS; Figure 1B, lane 6) or G2/M (Figure 1B, lane 4) cells, HIV-1 IRES activity increased markedly as evidenced by the appearance of FLuc protein. IRES activity was always greatest when RRL was supplemented with G2/M extract (Figure 1B). These observations echoed previous studies conducted in HeLa cells or HeLa cell based translational extracts (10).

To further extend these observations, we next studied the kinetics of protein synthesis using luciferase activity as the experimental readout. The RLuc/FLuc bicistronic RNA dlΔEMCV, that harbors a defective encephalomyocarditis virus (ΔEMCV) IRES known to inhibit ribosome reinitiation and read-through, inserted upstream of the FLuc reporter, was used as a negative control (10,31,41). In Figure 1C, RLuc and FLuc activities are independently displayed. The maximal RLuc and FLuc activity obtained in non-supplemented translation reactions in RRL was arbitrarily set to 100%. In agreement with data presented in Figure 1B, the activity of the HIV-1 IRES increases when RRL is supplemented with G2/M extracts (Figure 1C). As before (Figure 1B), the addition of G2/M extracts to the RRL had little or no impact on translation initiation from the first cistron (see RLuc, Figure 1C). Together, the observed enhancement by G2/M cell extracts which only impacts translation from the second cistron implies that one or more cellular factor(s) present in G2/M HeLa extracts may act in *trans* to specifically overcome the translational inefficiency of the HIV-1 IRES in RRL.

Next, we evaluated the effect of G2/M extracts on HIV-1 IRES activity in *X. laevis* oocytes, a system

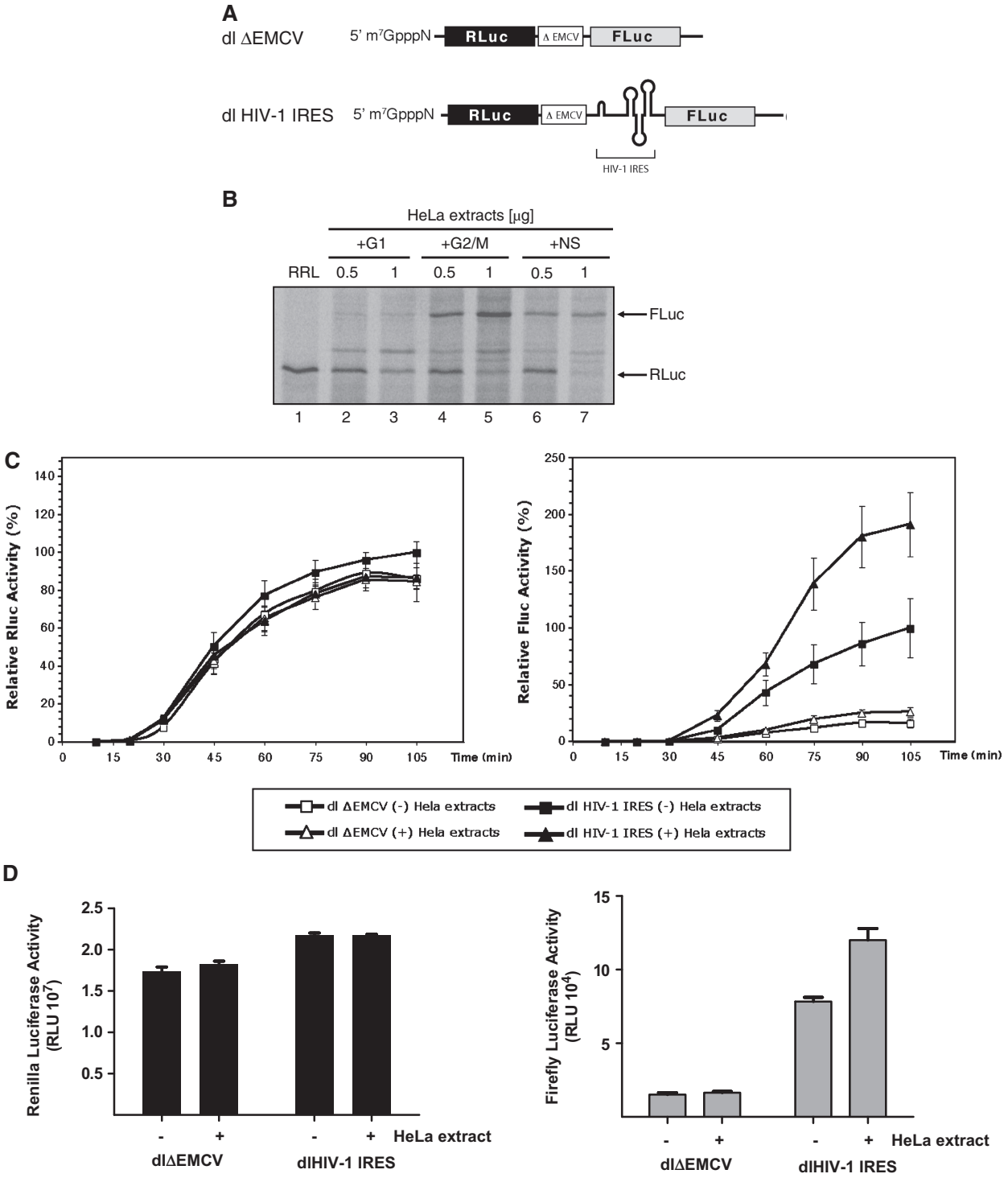

Figure 1. HeLa cell cytoplasmic factors are required for HIV-1 IRES activity. (**A**) Schematic representation of the dl HIV-1 IRES and dlΔEMCV RNAs used in this study. (**B**) RRL alone or supplemented with cytoplasmic extracts (0.5 or 1 μg of total protein) generated from NS HeLa cells or cells arrested in G1 or in G2/M were programmed with the dl HIV-1 IRES RNA. [^{35}S]-methionine-labeled proteins were resolved by SDS–PAGE and visualized as indicated in 'Materials and Methods' section. **(C)** Kinetics of HIV-1 IRES translation in the presence of G2/M HeLa extracts. The capped dlΔEMCV (open shapes) or dl HIV-1 IRES (filled shapes) RNAs (8 ng/μl) were used to program RRL. *In vitro* translation reactions were supplemented with 160 ng/μl of G2/M cytoplasmic extracts (Δ). Renilla luciferase (RLuc) and Firefly luciferase (FLuc) activities were measured at the indicated times. The RLuc and FLuc activities of the dl HIV-1 IRES measured at 105 min of *in vitro* translation in non-suplemented RRL (filled square) were arbitrary set to 100%. Relative RLuc activity (left panel) and relative FLuc activity (right panel) are shown. Values are the means ± SEM (error bars) of five independent experiments. (**D**) Capped and polyadenylated RNA corresponding to the dlΔEMCV or dl HIV-1 IRES vectors (6.25 ng) were microinjected into *X. laevis* oocytes with (+) or without (−) cytoplasmic extracts generated from G2/M arrested HeLa cells (200 ng) as described in 'Materials and Methods' section. Oocytes were harvested 24 h after the microinjection and processed and RLuc and FLuc activities were determined RLU. The RLuc (left panel) and FLuc (right panel) activities for each RNA are shown. Each value is the mean ± SEM from at least three oocytes obtained from different animals.

known to support its function (25). To this end the control dlΔEMCV or the dl HIV-1 IRES RNA were microinjected into *X. Laevis* oocytes either alone or with G2/M extract as previously described by others (42). Once more, no effect was evident on RLuc translation as indicated by a constant luciferase activity (Figure 1D). In contrast FLuc activity increased >50% in the presence of cell extracts (Figure 1D). Just as in RRL, factors present in G2/M cell extracts seem to be capable of specifically stimulating HIV-1 IRES activity.

Cytoplasmic cellular factors alter the accessibility of chemical reagents to single-stranded regions present within the HIV-1 5′ leader

We next sought to establish whether cytoplasmic cell extracts altered the chemical modification profile of the HIV-1 5′ leader. Before addressing this specific question, the secondary structure of the HIV-1 5′ leader recovered from the dl HIV IRES construct (nucleotides 1–336 from clone pNL4.3 followed by 58 nt of fluc gene) was probed using DiMethyl sulfate (DMS), *N*-Cyclohexyl-*N*-[*N*-Methylmorpholino)-ethyl]-Carbodiimide-4-Toluolsulfonate (CMCT) and RNAse V1 as described (12,32). CMCT and DMS were used to detect accessible RNA functional groups consistent with single-stranded regions, while RNAse V1 revealed stacked or paired nucleotides. Modifications were mapped by reverse transcription using a ^{32}P-labeled primer. For each run the reverse transcription pattern of the modified RNA was compared to the profile obtained with a non-modified RNA. Modifications were classified into two categories according to the relative intensity of the induced stop by comparison with the control. Modifications were classified as 'weak' when inducing a 2- to 3-fold increase in intensity of the RT stop, and as 'highly reactive' for higher intensities. A typical example of our results using DMS and covering the full HIV-1 5′ leader is shown (Figure 2A). The strong hits for DMS and CMCT were considered in the initial secondary-structure modeling using the Mfold algorithm as previously described (12,32). The structure obtained was then fitted onto a model structure of the HIV-1 leader (43), taking into account the V1 data and the weak DMS and CMCT hits. To further validate our probing data and to gain information on the intrinsic nucleotide flexibilities that characterize the secondary structure we also performed a detailed RNA Selective 2′Hydroxyl Acylation analysis by Primer Extension (SHAPE) analysis using the same RNA and 1-methyl-7-nitroisatoic anhydride (1M7) as a modifying agent (33,34). 1M7 reacts with flexible ribose groups of nucleotides that are not in a strong Watson–Crick pair or any other rigid tertiary interaction (33). In these experiments, modifications were mapped by reverse transcription using a fluorescent primer and the raw data were processed using 'Shapefinder' (35). The secondary structure of the HIV-1 5′ leader was modeled using 'RNA structure 5.03′ and the pseudo-energy constraints derived from the probing analysis (44). The integrated data, DMS/CMCT/RNAse V1 probing and RNA SHAPE analysis, were fitted onto a model structure of the HIV-1 leader (Figure 2B). Both methods, DMS/CMCT/RNAse V1 probing and RNA SHAPE analysis (Figure 3A), showed high consistency and yield essentially the same structure model. Most of the discrepancies observed when comparing both models consist in nucleotides reactive with DMS or CMCT but unreactive to 1M7 (for example A_{66}–A_{67} and G_{92}). As DMS and CMCT probe the availability of 'Watson–Crick' position and 1M7 the flexibility of the ribose, such results could indicate bases involved in non-canonical base pairs. This is particularly interesting in the case of A_{66}–A_{67} and G_{92} which are located within an asymmetrical bulge (Figure 2B). The observed probing signatures and the established model are similar to those obtained by others, *in vitro* or *ex vivo* (43,45–47). As notable differences, the regions involved in the LDI with the *gag* open reading frame (ORF) (U5 G_{108}–C_{114} and part of the polyA loop G_{79}–C_{85}) were accessible to single-strand probes. This observation is expected because the construct used in the study lacks the *gag* coding sequence. Interestingly, 3 nt within the palindrome in the DIS loop are reactive to 1M7, suggesting that the kissing-loop interaction that initiates non-covalent dimerization of the genomic RNA (48), was not formed under the experimental conditions used for probing.

Once we had a model secondary structure for the HIV-1 5′ leader we probed the RNA using 1M7 in the presence of cytoplasmic cell extract (see 'Materials and Methods' section) and compared the data with those generated in absence of extracts (Figures 2B and 3A). A position was considered as protected (or exposed) when the reactivity value was at least 2-fold lower (or higher) than in the mock control. We tested RNA with or without cell extracts generated from non-synchronized cells (NS) (red bars in Figure 3A), cells arrested in G1 (green bars in Figure 3A), or cells arrested in G2/M (yellow bars in Figure 3A). The modification of the pattern upon addition of NS cell extracts or extracts generated from cells blocked in G1 or G2/M is summarized on Figure 3B. Interestingly, distinct 1M7 accessibility RNA patterns are observed when NS, G1 or G2/M extract is used (Figure 3A and B). However, significant protections are seen upon addition of cell extracts independently of the stage where the cells were blocked. Specifically, we observe a strong protection of the TAR apical loop (C_{29}–A_{34}), in the PBS (G_{167}–A_{170}) and in three occurrences in the DIS element (G_{240}–A_{242}, A_{255}–A_{256} and A_{271}–G_{273}). As depicted in Figure 3, some positions are protected in the presence of G2/M extracts while few others are highly reactive. These reactivity modifications map to four specific regions: the poly(A) loop (increased reactivity of C_{60}–U_{61} and U_{64}–A_{66}; protection of A_{76}–A_{78} and C_{95}–A_{97}), the PBS structure (protection of G_{130}–A_{133}, G_{190} and G_{223}–G_{226}), the SD loop (increased reactivity of G_{289} and the Psi stem–loop immediately upstream from the initiation codon (protection of G_{318}–G_{320} andA_{324}–A_{327}). It is interesting to highlight that the PBS structure, the SD loop, and the Psi stem–loop are regions previously identified as crucial for HIV-1 IRES activity (10). As reported in Figure 3, G1-specific alterations of the modification pattern exclusively consist of positions that become highly reactive. Most interestingly, sequences

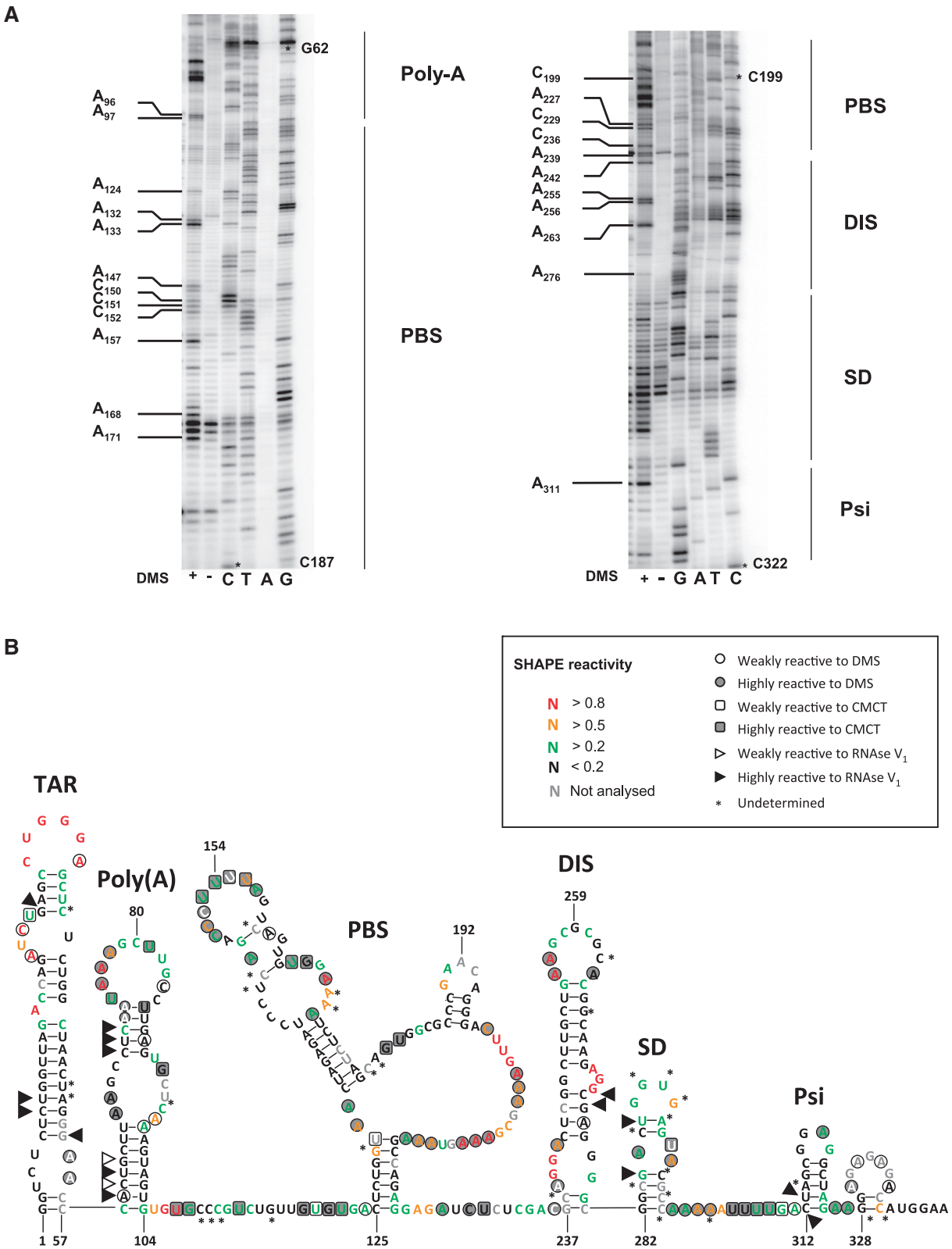

Figure 2. Secondary structure model of the HIV-1 leader. The HIV-1 5′ leader recovered from the dl HIV IRES construct (nucleotide 1–336 from clone pNL4.3 followed by 58 nts of fLuc gene) was probed using DiMethyl Sulfate (DMS), *N*-Cyclohexyl-*N*- [*N*-Methylmorpholino)-ethyl]-Carbodiimide-4-Toluolsulfonate (CMCT) and RNAse V1 as previously described (12,32) or using 1-methyl-7-nitroisatoic anhydride (1M7) as a modifying agent. (**A**) Typical examples of probing DMS probing. The HIV-1 5′ leader was probed using (+) DMS. Reverse transcription (RT) products were separated on a 8% gel as indicated in the 'Materials and Methods' section. Sequencing lanes were also included. Note that DMS induces a premature RT stop 1 nt before the hit. Therefore the DMS induced stops migrate faster than the corresponding sequence product (12,32). The RT pattern of the modified RNA was compared to the profile obtained with an unmodified RNA. Some hits are indicated in the figure. The asterisks on the gel denote the nucleotide position. (**B**) Results were fitted in a model of the HIV-1 5′ leader (43), the respective reactivity of the different probes is indicated as motioned in the box. The main HIV-1 structural elements present in the 5′ leader the TAR and poly(A) loops, PBS, DIS, SD and Psi are indicated (43).

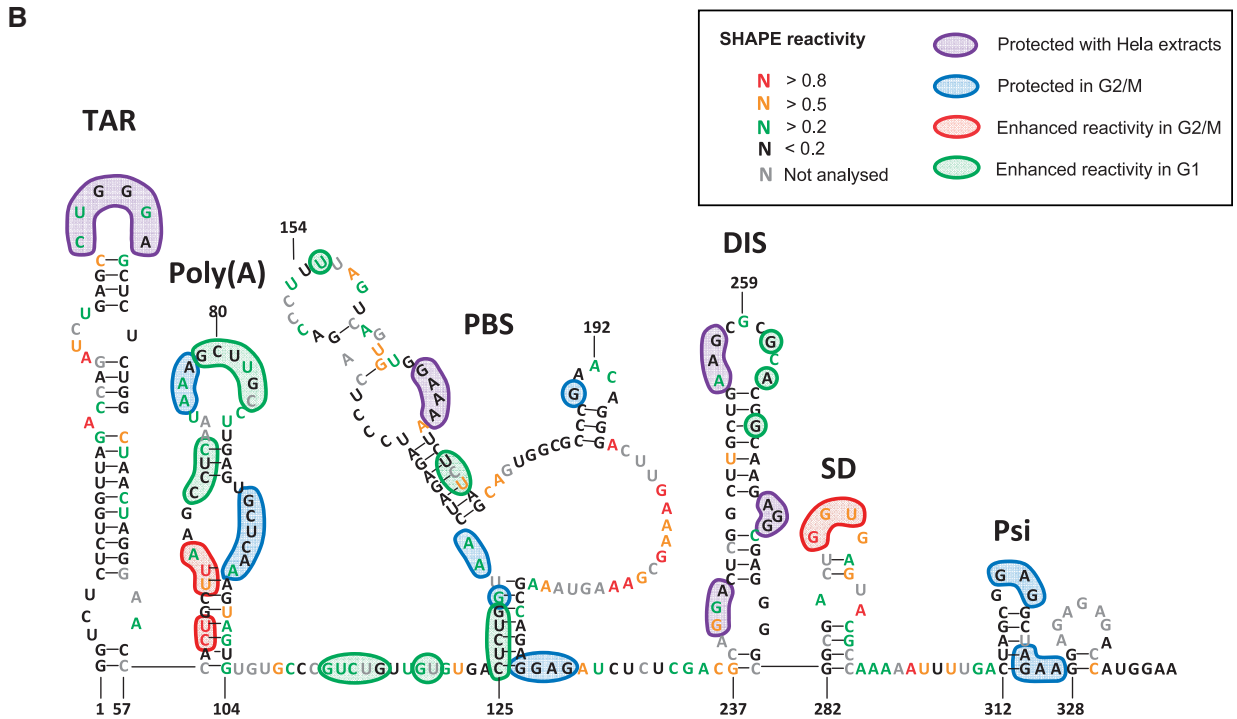

Figure 3. HeLa cell extracts alter the accessibility of 1M7 to single-stranded regions present within the HIV-1 5′ leader. The HIV-1 5′ leader RNA was probed using 1M7 in the presence or absence of HeLa cell cytoplasmic extracts generated from NS, or cells arrested in the G1, or in the G2/M phase of the cell cycle. (**A**) Histogram representing the 'SHAPE' reactivity for each nucleotides of the 5′-UTR in absence of extracts [(-) blue bars] in presence of NS (red bars), G1 arrested [(G1) green bars] or G2/M arrested [(G2M) yellow bars] HeLa extracts. Nucleotides which reactivity is undetermined in at least one of the tested conditions are boxed in grey. Local RNA structures are indicated as hallmark. (**B**) Data presented in A, are incorporated in the model depicted in Figure 2, the nucleotides protected by NS, G1 and G2/M extracts are boxed in purple, those specifically protected by G2/M extracts are boxed in blue, while those which reactivity is enhanced in presence of G2/M cytoplasmic extracts are boxed in red, nucleotides which reactivity is enhanced in presence of G1 extracts are boxed in green. The reactivity of each nucleotide in presence of G2/M extract is encoded by a specific colour, the reactivity are thus also valid for the area protected by all extracts (boxed in purple), but not for the nucleotides which reactivity is enhanced in presence of G1 extracts.

modeled as double stranded in all conditions (C_{70}–C_{72} in the poly(A) stem–loop, C_{125}–G_{129} and U_{174}–U_{176} in the PBS structure) clearly react as unpaired nucleotides upon G1 extract addition.

In summary, we find that the addition of HeLa cell extracts alters the accessibility of 1M7 to discrete regions of the HIV-1 5′ leader. Most interestingly, we observed a G2/M-specific pattern most probably confirming that one or more proteins delivered with those cell extracts interacts with the HIV-1 5′ leader. It should be stated, however, that at this point we cannot tell if our observation results from the footprint of proteins, or from a structural rearrangement, or from both.

Cap-independent translation initiation driven from HIV-1 IRES is resistant to point mutations

The HIV-1 5′ leader is capable of forming a complex secondary structure with multiple junctions, internal loops and stem–loop elements that is involved in many steps of the viral life cycle including translation (10,43). The most probable secondary structure model of the HIV-1 5′ leader, known as the Branched Multiple Hairpin (BMH, see Figure 2), comprises the PBS, dimer-initiation site (DIS), splice donor (SD) and hairpin loops, all structural elements involved in genomic RNA dimerization, reverse transcription, splicing and packaging (46,47,49). An alternative secondary structure model of the 5′ leader known as the LDI would engender alternative base pairing that disrupts the DIS hairpin loop (49–51). When comparing the LDI and the BMH models the region embedding the Gag start codon is contained in different secondary structure elements, but the initiation codon itself is mostly occluded in both structures (49–51). Abbink *et al.* (2005) reported a series of mutations designed to alter the LDI-BMH equilibrium (29). However, one important caveat of the study was that the specific secondary structure of the described mutants was not probed and thus the predicted changes to the structure of the HIV-1 leader remain largely speculative (29). Nonetheless, we decided to evaluate the effect of the mutations described by Abbink *et al.* (2005) on translation initiation driven by the HIV-1 IRES. The rational for this experiment was based on the observation that the introduction of point mutations within the sequence of a viral IRES can have a profound impact on its function (4,52–55). Therefore, the mutations reported by Abbink *et al.* (2005) were introduced into the 5′ leader of the HIV-1 clone pNL4.3 and examined in the context of bicistronic mRNAs (similar to those depicted in Figure 1A). Based on what has been previously reported (29), mutants leaders (Mut L) Mut L7, Mut L8 and Mut L11 are expected to favor the BMH conformation, Mut L1, Mut L2, Mut L4, Mut L5, Mut L6 and Mut L10 favor the LDI conformer while with the wild-type and mutants Mut L3 and Mut L9 the two conformers are expected to be in equilibrium.

Vectors were transfected into HeLa cells and the RLuc and FLuc activities were monitored as described in previous studies (10,16,25). Vector dl HIV-1 IRES, harboring the leader (nucleotide 1–336) of the HIV-1 infectious recombinant proviral clone pNL4.3 (10), was used as a positive control and sets the 100% of activity, while construct dlΔEMCV, used as a negative control (10,31,41), showed only 0.8% of the activity of the HIV-1 IRES. As in previous studies (10,16,25,31), the FLuc/RLuc ratio was used as an index of IRES activity and the activity of the mutants are expressed as relative translation (%) with respect to the wild-type construct. As shown in Figure 4, the most affected mutant was Mut L6 which exhibits *an increase (1.3-fold) in IRES activity* with respect to the control HIV-1 IRES (pNL4.3). However, none of the relative translation efficiencies were significantly different from the control IRES, as determined by a *one*-way ANOVA ($P > 0.05$). Therefore, echoing previous reports *(13,29)*, no correlation between the IRES activity and the putative BMH/LDI switch of conformation is evidenced. To this respect, it is important to note that the existence of the LDI conformation is not supported *in vivo* (46,47). Somewhat surprisingly, data do show that the activity of the HIV-1 IRES is resistant to mutations spread all along the 5′ leader sequence, a feature that directly contrast what has been described for most viral IRES elements (4,52–55).

Specific cellular factors from G2/M extracts bind the HIV-1 5′ leader

Results presented in Figures 3 and 4 suggest that IRES-mediated translation initiation is most probably modulated by a distinct group of proteins that specifically interact with the HIV-1 5′ leader during the G2/M phase of the cell cycle. If indeed the case, different sets of proteins would be expected to bind the HIV-1 5′ leader in different stages of the cell cycle. To evaluate this possibility we used a proteomic approach to identify the proteins present in HeLa cell extracts generated from NS or G2/M arrested cells that interact with the HIV-1 5′ leader. In these assays, an uncapped streptomycin binding RNA aptamer (strep-aptamer)-tagged HIV-1 5′ leader was used as bait (36), while the reverse complement HIV-1 5′ leader was used as a control RNA to discard non-specific RNA-binding proteins from the subsequent analysis. Pull-down experiments were performed using extracts from either NS or G2/M arrested cells. Mass Spectrometry analysis, conducted as indicated in the 'Materials and Methods' section, allowed the identification of 54 proteins that pulled-down with the HIV-1 5′ leader when cytoplasmic extracts from non-synchronized cells were used as the protein source (data not shown). Interestingly, only 18 proteins were identified as pulling-down with the viral 5′ leader RNA when G2/M extracts were used (Table 2). From these, only two proteins where common between NS and G2/M extracts (Table 2) indicating that a distinct set of proteins interact with the leader during G2/M. This result correlates with biochemical assays demonstrating that the accessibility of 1M7 to discrete regions of the HIV-1 5′ leader changes when extracts from NS or G2/M cells are used.

Taken together, the results of the pull-down experiments suggest that the assembly of protein factors on

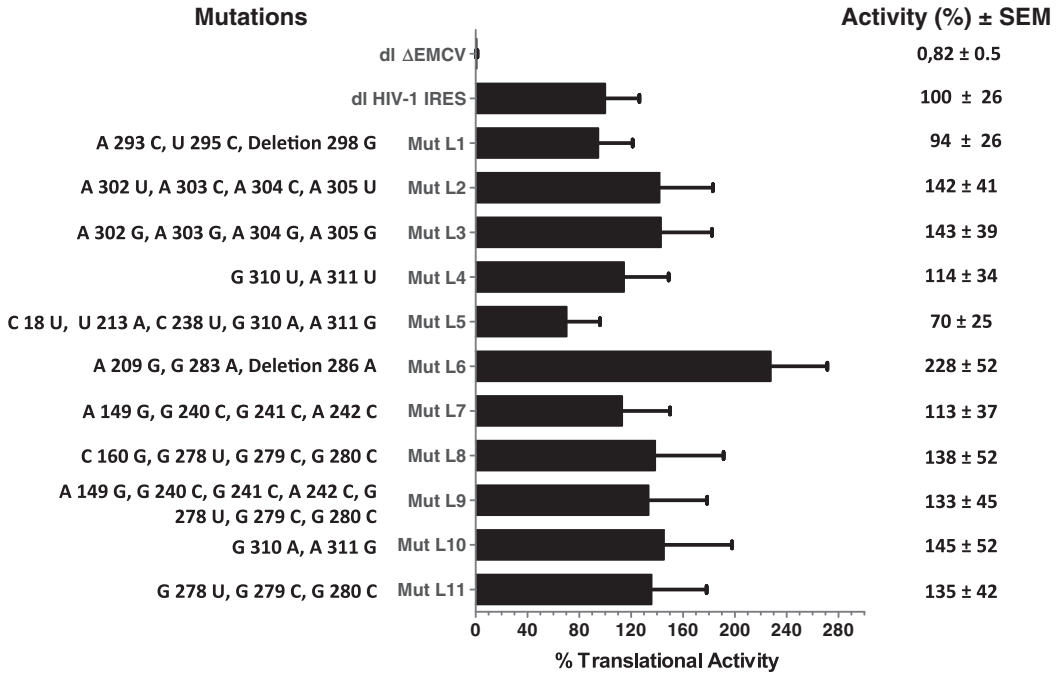

Figure 4. Translational activity of the mutant HIV-1 IRES elements. HIV-1 mutant leaders generated as described in 'Materials and Methods' section and arbitrarily named Mut L1–L11 were cloned into the dual luciferase bicistronic vector (dl-vector). The dl HIV-1 mutant vectors (Mut L1–L11) were transfected into HeLa cells and IRES-mediated translational activity was evaluated in comparison with the dl HIV-1 IRES vector and the dlΔEMCV control (10,41). The nucleotide changes with respect to the pNL4.3 (AF 324493) leader included in the dl HIV-1 IRES vector, are shown (left). RLuc and FLuc activities were measured and the [(FLuc/RLuc)] ratio was used as an index of IRES activity. The [(FLuc/RLuc)] ratio of the dl HIV-1 IRES vector (10), was arbitrarily set to 100%. Values are the means ± SEM (error bars) of three independent experiments.

Table 2. Cellular factors from G2/M extracts identified to that were pulldown bind the HIV-1 5′ leader

Accession No.	Protein Name	Score	Mass (Da)	Matches	Coverage (%)	emPAI	References
P53999	Activated RNA polymerase II transcriptional coactivator p15[a]	157	14 386	5	26	1.05	(62)
Q07021	Complement component 1 Q subcomponent-binding protein[a]	174	31 742	4	5	0.25	(63)
P11387	DNA topoisomerase 1[a]	391	91 125	25	17.3	0.43	(64–69)
P14866	Heterogeneous nuclear ribonucleoprotein L (hnRNP L)	127	60 719	6	12.9	0.06	
P07196	Neurofilament triplet L protein	122	61 536	4	4.1	0.12	
P19338	Nucleolin (Protein C23)[a]	105	76 625	4	3.4	0.1	(70,71)
P06454	Prothymosin α[a]	113	12 196	2	12.6	0.32	(72)
P37108	Signal recognition particle 14 kDa protein (SRP14)[b]	146	14 675	4	28.7	0.61	
P55072	Transitional endoplasmic reticulum ATPase (TER ATPase)	444	89 950	11	8.4	0.33	
P09234	U1 small nuclear ribonucleoprotein C (U1 snRNP protein C)	191	17 552	4	18.9	0.49	(73)
P62861	40 S ribosomal protein S30	124	6644	13	20.3	6.08	
P16989	DNA-binding protein A (Cold shock domain-containing protein A)	225	40 066	10	14.8	0.31	
P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β 1	85	38 151	3	12.1	0.1	
Q9BUJ2	Heterogeneous nuclear ribonucleoprotein U	79	91 164	33	25.5	0.89	
P17096	High mobility group protein HMG-I/HMG-Y (HMG-I(Y))[a]	232	11 669	6	15	1.41	(74,75)
Q04837	Single-stranded DNA binding protein, mitochondrial precursor (Mt-SSB)[a]	71	17 249	2	20.3	0.23	(76)
P07951	Tropomyosin β-chain (β-tropomyosin)[a]	61	32 945	5	10.6	0.24	(77)
Q13748	Tubulin α-2 chain (α-tubulin 2)[a,b]	143	50 612	6	16.9	0.24	(78–82)

Score = protein identification score, reflects amount of peptide matches and percent coverage obtained for the protein; Mass = protein mass; Matches = number of peptides corresponding to the protein; percent coverage = sequence coverage of identified peptides over the protein; emPAI = exponentially modified protein abundance index.
[a]Proteins known to play a role in the HIV-1 replication.
[b]Proteins also found in non-synchronized cytoplasmic cell extracts.

the HIV-1 leader varies when non-synchronized or G2/M arrested extracts are used. Combined with the results from Figures 1 and 4, these data support the notion that G2/M-specific proteins bind the HIV-1 leader to support IRES-mediated translation initiation.

Overall, the biological significance of the pull-down analysis is still to be determined, work is being performed to determine the role of these and other RNA binding proteins in the control of HIV-1 IRES-mediated translation initiation.

DISCUSSION

In this study, we report that in common with most cellular IRESes and some viral IRESes translation initiation from the HIV-1 IRES in RRL requires the addition of exogenous proteins (Figure 1). Echoing previous reports suggesting that activity of the HIV-1 IRES is enhanced in the G2/M phase of the cell cycle (10), G2/M extracts were more effective at stimulation of IRES-mediated translation initiation than G1 or extracts generated from no-synchronous cells (NS in Figure 1). This could be due to the presence of specific G2/M factors, or simply reflect the reduced competition for the translation machinery due to the impairment of cap-dependent translation during the G2/M phase of the cell cycle. The latter possibility was disregarded as the addition of G2/M extracts did not significantly impact translation initiation from the first cistron in two independent translation systems, the RRL and the *X. laevis* oocytes (Figure 1). In an attempt to resolve this issue and to further characterize the molecular events involved in this process, we show that the addition of cell extracts induces an alteration of the 1M7 modification profile of the HIV-1 leader (Figure 3). The sequences directly upstream the initiation codon appear to be protected with the extracts generated from G2/M arrested cells, possibly reflecting bound G2/M-specific proteins or perhaps even the presence of the initiation complex on the start codon. In good agreement with the functional data, we observe a specific G2/M footprint on the sequences spanning from the poly(A) stem–loop to the SD stem–loop. It should be noted that Brasey *et al.* (10) previously reported that the poly(A) loop (nucleotide 58–104) is not part of the minimal IRES (nucleotide 104–336), but the PBS structure, the SD loop and the Psi stem–loop are regions previously identified as crucial for HIV-1 IRES activity (10). Our data do not formally allow us to conclude if the protection of specific position within the HIV-1 leader is due to protein or ribosome binding or if we observe some local RNA structural rearrangement. However, beside the case of the poly(A) loop, protections in loop or bulges suggest the presence of proteins on those nucleotides rather than a structural modification. In contrast, the modification pattern obtained with G1 cell extracts suggests a significant alteration of the global structure of the HIV-1 5′ leader. This observation raises the possibility that IRES activity is inhibited in G1 due to a disruption of the active structure. In conclusion, our observations suggest that the G2/M cellular extracts contain proteins that stimulate HIV-1 translation, and that one or several G2/M-specific ITAFs bind within regions known to be critical for the IRES function.

To gain information on the set of proteins from G2/M cell extracts that interact with the HIV-1 5′ leader a proteomic approach was used. Surprisingly, a discrete set of proteins from G2/M arrested extracts pull-down with the HIV-1 leader (Table 2). Interestingly, many of the proteins identified have been previously implicated in HIV-1 replication (Table 2), although their direct role in HIV-1 IRES-mediated translation initiation is still unknown. Together, our data (Figures 1, 3 and 4; Table 2) warrants the notion that a protein complex that forms on the viral RNA somehow imprints the information required to determine IRES activity.

An additional and unexpected finding reported in this study is that the HIV-1 IRES activity is resistant to a number of mutations designed to disrupt RNA structure (29). Mutations introduced in the TAR stem–loop or within the region spanning between the major 5′ splice site and the initiation codon (ML1-ML6) had no significant effect on translation [Figure 4 and references (13,29)]. This confirms the results of a precedent study that shows the TAR element, and the nucleotides beyond the splice site (U_{291}) could be deleted without significantly affecting the IRES-mediated translational activity (10). More surprisingly, mutations within the PBS or the DIS stem–loop which are known to be essential for HIV-1 IRES do not significantly alter the translation efficiency in our bicistronic assay (Figure 4). Even though unexpected, these results are consistent with a recently published report that conducted a similar set of experiments in the context of a bicistronic mRNA (13). Together, these observations are in direct contrast to what is observed with other viral IRESes such as those present within picornavirus, HCV and CrPV RNAs, where simple point mutations that alter their secondary/tertiary RNA structure can totally abolish IRES activity (4,52–55). However, it remains possible that point mutations to as-of-yet undiscovered critical elements in the HIV-1 IRES may have a stronger effect than those explored here.

Interestingly, point mutations or deletions within many cellular IRESes have little impact on translation initiation, suggesting that the structure–function relationship in cellular IRESes is not as rigid as that observed for viral IRESes (4,28,56). In this respect, it is tempting to speculate that the HIV-1 IRES is an atypical viral IRES as it seems to shares certain properties normally ascribed to cellular IRESes (4,28,57,58), but that are absent from their viral counterparts. Moreover, a striking difference between the HIV-1 full-length mRNA and most viral mRNAs that harbor an IRES, is that the former possesses both a 5′ cap and the poly(A) tail. Incorporation of a cap structure onto a picornavirus mRNA inhibits IRES-mediated translation, suggesting that in the context of viral mRNAs the two mechanisms are mutually exclusive (59). Yet, as cellular mRNAs that harbor IRES elements, the HIV-1 mRNA is also capped. However, at this point, we cannot tell if the two mechanisms are used simultaneously or at two different stages of the virus life cycle (8). Additionally, and in sharp contrast to most (+) RNA viruses that harbor IRES elements that are synthesized by a viral RNA-dependent RNA polymerase in the cell cytoplasm where it is translated (27), transcription of the HIV-1 mRNA takes place in the host cell nucleus. As nascent cellular mRNAs, the HIV-1 mRNA would be expected to first encounter RNA binding proteins in the nucleus structuring a distinct ribonucleoprotein (RNP) complex with nuclear RNA binding factors (60,61). These RNA binding proteins of nuclear origin might be part of an 'IRES RNP'-specific signal that is further modified by interaction with cytoplasmic proteins prior to its associating with the translation apparatus; as

would be the case of cellular mRNA that harbor IRES elements (4,27,28).

In summary, in this study, we describe that G2/M extracts harbor factors capable of enhancing HIV-1 IRES activity. Furthermore, we describe that a discrete set of proteins present within G2/M extracts bind the HIV-1 5′ leader. Even though the role on IRES function of the identified proteins is presently unknown, our data are consistent with the notion that cellular proteins are directly involved in the regulation of HIV-1 IRES activity. In fact, current evidence indicate that cellular proteins cannot only stimulate HIV-1 IRES-mediated translation initiation [Figure 1, Table 2 and reference (16)], but they can also inhibit it (25). Thus, we propose a mechanism by which G2/M-specific proteins bind to local structures within the 5′ leader, and subsequently recruit, or stimulate the internal recruitment of the initiation complex. Together, findings presented herein give new insights into the RNA structure/function relationship and provide a valuable framework for further dissection of the molecular mechanism involved in HIV-1 IRES activity.

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Conflict of interest statement. None declared.

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