



International Journal of **Biological Chemistry**

ISSN 1819-155X



Academic
Journals Inc.

www.academicjournals.com

Eukaryotic Release Factor 1 Affects +1 and -1 Ribosomal Frameshifting in HeLa Cells

¹M.A. Hossain and ²S.W. Peltz

¹Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh

²Department of Molecular Genetics, Microbiology and Immunology,
Robert Wood Johnson Medical School,
University of Medicine and Dentistry of New Jersey, Piscataway,
New Jersey 08854 and PTC Therapeutics, South Plainfield, New Jersey 07080, USA

Abstract: The aim of this study is to identify trans-acting element(s) that alter ribosome frameshifting event(s) result a polyprotein coded by two Open Reading Frames (ORFs) separated by a recoding signal. The hypothesis is that a depletion of eRF1 leads to inefficient recognition of stop codon or stem loop structure will force ribosomes to scan past the structure with altered efficiency resulting production of poly-protein using +1 or -1 frameshifting. The hypothesis is tested in eRF1 depleted HeLa cells using a reporter based assay system containing HIV-1 or antizyme frameshifting signaling sequence. Small interference RNA (siRNA) specific to eRF1 were transfected into cells and reporter activity was measured. The results revealed that depletion of eRF1 increased +1 frameshifting about 1.8 folds, whereas, decreased -1 frameshifting by 50%. These findings indicate that eRF1 is involved in recoding events. Particularly, alteration of -1 frameshifting in HIV-1 would be a target for drug development against the AIDS, because -1 frameshifting is the most decisive events in HIV replication that controls gag and poly-protein gag-pol ratio.

Key words: Frameshifting, HIV-1, antizyme, recoding, eRF1, siRNA

INTRODUCTION

The termination of translation occurs on ribosome's in response to one of the three stop codons, UGA, UAG or UAA. In contrast to the normal translation termination, sequences in certain mRNA i.e., recoding signal programs ribosome to undergo noncanonical translation events, such as readthrough (suppression of in-frame stop codon between two coding regions) or translational -1 or +1 frameshifting to decode two Open Reading Frame (ORF) ORF1 and ORF2 to a poly-protein separated by a stop codon (Farabaugh, 1996; Gesteland and Atkin, 1996). Most of the knowledge and understanding on recoding events came from the study of RNA viruses and retrotransposons of both eukaryotes and prokaryotes, though these events are also present in bacteria and eukaryotes. In mammalian cells, readthrough of stop codon to sense codons allow synthesis of selenocysteine-containing protein (Low and Berry, 1996), +1 frameshifting regulates Ornithine Decarboxylase Antizyme (ODC) (Hayashi *et al.*, 1996), mouse embryonic carcinoma differentiation regulated (*Edr*) gene is regulated by -1 frameshifting (Shigemoto *et al.*, 2001). The effect of recoding elements in translation recoding were studied in stop codon region of the replicase cistron from Tobacco Mosaic Virus (TMV) (a site of UAG readthrough); gag-pol junction of human immunodeficiency virus type 1 (HIV-1), transframe protein TF (Alphavirus) (a site of -1 frameshifting); Ty1 transposon and +1 mouse antizyme (a site for +1 frameshifting) (Stahl *et al.*, 1995; Grentzmann *et al.*, 1998; Bidou *et al.*, 2000; Karamysheva *et al.*, 2003; Firth *et al.*, 2008). Furthermore, very recent new

Corresponding Author: M. Anwar Hossain, Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh Tel: 880-2-9661920-73/7735 Fax: 880-2-8618355

algorithm have been developed to identify the cis-acting frameshifting signals (Belew *et al.*, 2008; Theis *et al.*, 2008). Although the effect of cis-acting recoding elements were studied in different reporter systems, identification of trans-acting factors that alter recoding efficiency are not understood and only limited work was reported on Eukaryotic Release Factor 1 (eRF1) and eRF3 (Karamysheva *et al.*, 2003; Janzen and Geballe, 2004). Furthermore, programmed -1 frameshifting allows synthesis of the required ratio of Gag to Gag-Pol, which is highly regulated in retroviruses and required for HIV-1 replication (Jacks *et al.*, 1988; Biswas *et al.*, 2004).

Small interfering RNA (siRNA) emerges as a powerful RNA interference (RNAi) tool and induces degradation and silencing of a cognate gene transcript. This technique provides a new approach for elucidation of gene function and was first employed by Elbashir *et al.* (2001) for silencing of lamin A/C in HeLa cell. This study reports recoding events +1 and -1 frameshifting under specific eRF1 gene silencing condition.

MATERIALS AND METHODS

Dual reporter system is used in the present investigation to study recoding events in HeLa cells. For frameshifting assay -1/or +1 in HeLa cells, the dual luciferase reporter system were used (Grentzmann *et al.*, 1998). All the frameshifting reporters -1 or +1 and their products are presented in Fig. 1A and B.

siRNA Synthesis

Most of the siRNA used in this study (Table 1) synthesized using Silencer™ siRNA construction kit (Ambion) according to the instruction of manufacturer. The procedure based on *in vitro* siRNA synthesis using T7 RNA polymerase. Design of sense and antisense DNA template and blast search

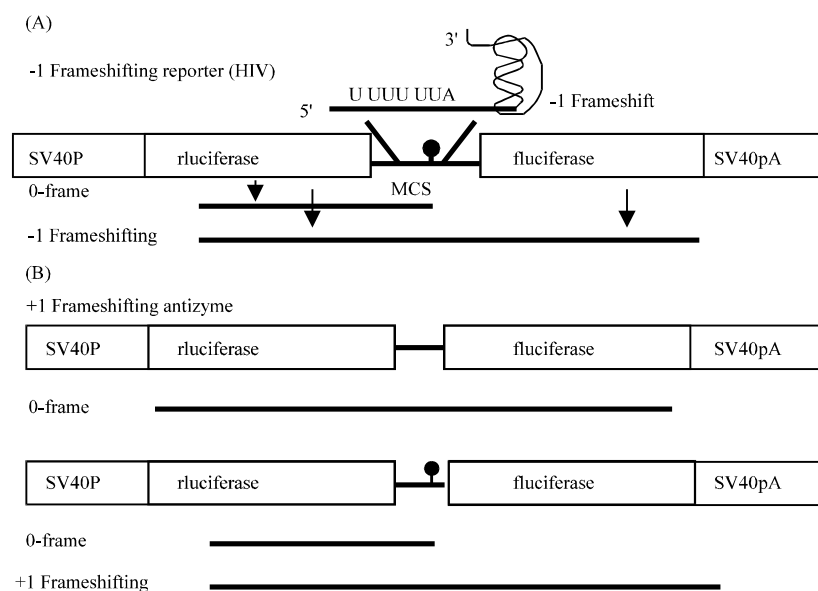


Fig. 1: Schematic diagrams of the dual reporters used in the study. (A) HIV-1 -1 frameshifting signal inserted in between two luciferase gene (*rLuc* and *fluc*) and (B) contain human ornithine decarboxylase +1 antizyme signal

Table 1: List of siRNA used in this study. The name of each siRNAs, target sequence and percentage of gene silencing effect of each siRNAs are shown

Name	Target sequence	Silence (%)
eRF1.160	eRF1.160-181	40 $\leq$
eRF1.759	eRF1.759-780	70 $\leq$
eRF1.1084	eRF1.1084-1105	75 $\leq$
eRF1.1179	eRF1.1179-1200	85 $\leq$
eRF1.com	eRF1.1187-1208	65 $\leq$
Scrambled Duplex (SD)	No target	
LuciferaseGL2 (Luc-GL2)	firefly luciferase	70 $\leq$

were carried out using the web site of Ambion. Chemically synthesized eRF1, luciferase siRNA (*fluc*-siRNA) and scramble duplex siRNA were purchased from Dharmacon.

Cell Culture and Transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U of penicillin G mL⁻¹ and 100 μ g of streptomycin mL⁻¹. HeLa cells grown over night at 60-80% confluent were transiently transfected either with reporter alone or reporter plus specific siRNA or SD oligos or *fluc*-siRNA with LipofectamineTM 2000 or Lipofectamine plus plus reagent (Invitrogen) according to the instruction of the manufacturer.

Reporter Assay

Cells grown for 50-52 h after transfection were lysed using one X passive lysis buffer provided by the manufacturer and the activity of the dual luciferase renilla and firefly were determined using the assay kits (Promega) using instrument Turner 20/20 luminometer (Palo Alto, Calif).

RNA Extraction and cDNA Synthesis

Total RNA was isolated from cultured HeLa cells using RNeasy Mini Kit (Qiagen). An on-column RNase-Free DNase treatment was carried out to remove the trace of DNA contamination according to the instruction of manufacturer. Before preparing cDNA a second step RNase-Free DNase treatment was done, this treatment improved DNA free RNA sample. One half of 1 μ g RNA sample was reverse transcribed into cDNA and other half was also treated same way without enzyme and used as RNA control during qPCR.

Real-Time Quantitative-Polymerase Chain Reaction Analysis

Real-time PCR was performed on DNA Engine Opticon2 (MJ Research) using iQTM SYBR Green supermix (Bio-Rad). The relative amount of PCR product was determined by the comparative Ct value method, in which each sample was normalized to human glyceraldehyde 3-phosphate dehydrogenase (*hGapdh*) and expressed as fold-increase versus controls. The following HPLC pure primer pairs were used:

hGapdh, 5'-AATCCCATCACCATCTTCCAG-3' (forward)
 5'-CCTTCTCCATGGTGGTGAAGAC-3' (reverse);
 eRF1, 5'-AGAGGCTCTTACAGCACTAC-3' (forward)
 5'-CACAGTGAATTTGTGCAGGAC-3' (reverse);
 fluciferase, 5'-TTCTATCCTCTAGAGGATGG-3' (forward)
 5'-ATAGCTTCTGCCAACC GAAC-3' (reverse)

Western Blot Analysis

Fifty to one hundred microgram of protein lysate were separated using 5-15% gradient SDS-PAGE gel, electroblotted to PVDF membrane, probed with antibodies to eRF1 (gift of LE Goff, CNRS, France) and hGraph (abcam) and detected using HRP kits (Pierce) according to the manufacturer's specifications. In case of 2nd probing, the membrane were stripped with stripping buffer (Pierce), blocked and re-probed with desired antibody and detected as earlier.

RESULTS

eRF1 Affects -1 or +1 Frameshifting

The alternate reading of two open reading frames ORF1 and ORF2 requires frameshifting in the expression of some genes. Such events depend on specific cis- and trans-acting elements. The cis-acting elements are present in specific mRNA sequences. It has been reported that exogenous increase of eRF1 decreased +1 antizyme frameshifting in rabbit reticulocyte lysates (Karamysheva *et al.*, 2003). Furthermore, -1 frameshifting signal functioned as a cis-acting mRNA destabilizing element i.e., increased NMD in yeast (Plant *et al.*, 2004). It is therefore evaluated the effect of NMD trans-acting elements (hUPF1, hUPF2, hUPF3a/b, hSMG1) and eRF1 on recoding for which a cell-based dual luciferase reporter system was employed (Grentzmann *et al.*, 1998). The reporter contained the recoding site (ODC antizyme +1 frameshifting and HIV1 -1 frameshifting) and placed between *Renilla luciferase (rluc)* and Firefly luciferase (*fluc*) genes (Fig. 1A, B). The *fluc* protein can only be produced as a consequence of ribosomal either -1 or +1 frameshifting. We transiently transfected the HeLa cell together with specific siRNA or control SD siRNA and either -1 or +1 frameshifting reporter and the ribosomal frameshifting efficiency were measured by measuring both luciferase activity. The depletion of eRF1 had been measured both by measuring the total mRNA and eRF1 protein using western blotting. The results clearly demonstrated that present synthesized eRF1 siRNA was found more effective in depleting eRF1 than that of eRF1 siRNA purchased from Dharmacon (Table 1, Fig. 2). It is noted that *in vitro* synthesized siRNA work at 5 nM concentration, whereas, to get same level depletion of eRF1 mRNA required 50 nM (data not shown). Measurement of Upfs, Smg1 and luciferase mRNA and protein were also done by the same method (data not shown). In the system -1 ribosomal frameshifting in control experiment using SD siRNA was obtained 6% similar to that of reported value of Biswas *et al.* (2004). We consider

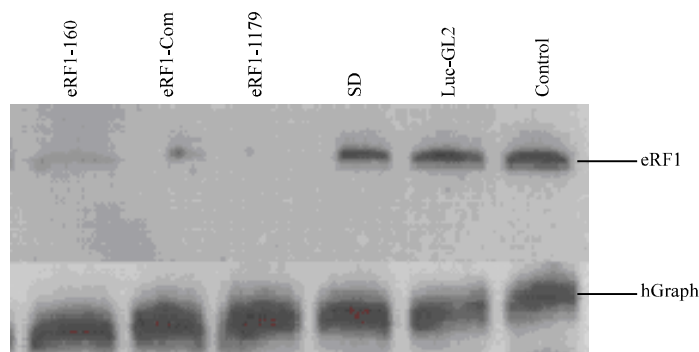


Fig. 2: Synthetic and commercial eRF1 siRNA deplete eRF1 protein in the transfected HeLa cell. Synthetic eRF1 siRNA (eRF1-160, eRF1-1179); Commercial eRF1 siRNA (eRF1-Com); SD, Scramble duplex; Luc-GL2, siRNA against luciferase and control, no siRNA used in this experiment

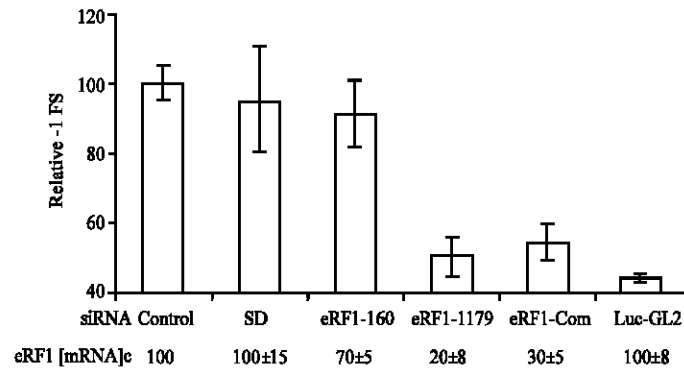


Fig. 3: Depletion of eRF1 decreases -1 frameshifting in HeLa cells. X-axis, denotes siRNA used as mentioned in Fig. 2; bottom, showed the concentration eRF1 mRNA measured by RT-PCR. Proteins levels were measured by western blotting (Fig. 2)

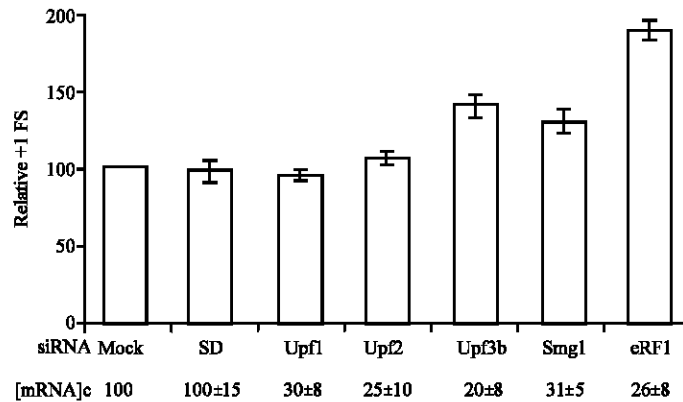


Fig. 4: Depletion of eRF1 increases +1 frameshifting in HeLa cells. X-axis, denotes siRNA used in the experiment; bottom, showed the concentration mRNA of respective nonsense mediated (NMD) mRNA decay pathway gene products and eRF1 measured by RT-PCR. Proteins levels were measured by western blotting (Fig. 2)

this value as 100%. In contrast to control, depletion of eRF1 decreased -1 frameshifting by 50% at 50 nM of eRF1.ch siRNA (Fig. 3). On the other hand depletion of hUPFs and hSMG1 did not change -1 frameshifting (data not shown) which corroborate the report of Bidou *et al.* (2000). Interestingly, depletion of eRF1 had opposite effect in +1 frameshifting and two-folds increase of +1 frameshifting was observed over control (Fig. 4), whereas, hUPFs and hSMG1 had little or no effect (data not shown).

DISCUSSION

We observed that depletion of eRF1 increases +1 frameshifting, but decreases -1 frameshifting. It has been reported that efficiency of antizyme frameshifting in rabbit reticulocyte lysates was reduced by the addition of eRF1 (Karamysheva *et al.*, 2003). The opposite effect of the depletion of eRF1 in case of +1 and -1 frameshifting may be due to the organizational differences between the

frameshifting signals in both the case. For +1 frameshifting to occur, the UGA (stop) must occupy the ribosomal A-site. Therefore, it is expected that depletion of eRF1 will decrease termination efficiency at initial open reading frame stop codon and thus increase +1 frameshifting. But for -1 HIV-1 gag-pol frameshifting such stop codon occupancy rule at ribosome A-site is not applicable, because the initial stop codon for gag open reading frame resides further down to slippery site. We propose ribosome stalling occurs at the HIV-1 slippery site due to the presence of adjacent stem-loop, which allow eRF1 to assemble through the interaction of 18S rRNA at A-rich site (Manuvakhova *et al.*, 2000) and the failure of recognition of proper stop codon abort this association and ribosome scanning through mRNA continue until it reaches the first stop codon and producing gag -protein. But a reduced fraction of pausing ribosome still could cross-talk with stem-loop via eRF1 by protein-RNA or protein-protein (trans-acting protein binds at stem-loop) interaction and will slip or push back by -1 frame and producing gag-pol poly-protein. According to our proposed model, it is obvious that depletion of eRF1 will decrease -1 frameshifting in HIV-1. This model is currently testing in the present laboratory.

In conclusion, this report demonstrated that eRF1 altered general recoding event like readthrough, -1 and +1 ribosomal frameshifting.

ACKNOWLEDGMENTS

The author thanks PTC, therapeutics for providing all single firefly luciferase reporter and Jason Carnes for dual lacZ-fluc reporters, Jon Dinman for *rluc-fluc* (pHIVlucwt, p2luc and p2luci) reporters and John F. Atkins *rluc-fluc* (antizyme) reporter. We thank Catherine Le Goff, France for eRF1 antibodies.

REFERENCES

- Belew, A.T., N.L. Hepler, J.L. Jacobs and J.D. Dinman, 2008. PRFdb: A database of computationally predicted eukaryotic-programmed -1 ribosomal frameshift signals. *BMC Genomics*, 9: 339-339.
- Bidou, L., G. Stahl, N. Hatin, O. Namy and J.P. Rousset, 2000. Nonsense-mediated decay mutants do not affect programmed -1 frameshifting. *RNA*, 6: 952-961.
- Biswas, P., X. Jiang, A.L. Pacchia, J.P. Dougherty and S.W. Peltz, 2004. The human immunodeficiency virus type 1 ribosomal frameshifting site is an invariant sequence determinant and an important target for antiviral therapy. *J. Virol.*, 78: 2082-2087.
- Elbashir, M.S., J. Harborth, W. Lendeckel, A. Yalcin and K. Weber, 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in mammalian cell culture. *Nature*, 411: 494-498.
- Farabaugh, P.J., 1996. Programmed translational frameshifting. *Microbiol. Rev.*, 60: 103-134.
- Firth, A.E., B.Y.W. Chung, M.N. Fleeton and J.F. Atkin, 2008. Discovery of frameshifting in alphavirus 6K resolves a 20-year enigma. *Virol. J.*, 5: 108-108.
- Gesteland, R. and J. Atkins, 1996. Recoding: Dynamic reprogramming of translation. *Ann. Rev. Biochem.*, 65: 741-768.
- Greentzmann, G., J.A. Ingram, P.J. Kelly, R.F. Gesteland and J.F. Atkins, 1998. A dual-luciferase reporter system for studying recoding signals. *RNA*, 4: 479-486.
- Hayashi, S., Y. Murakami and S. Matsufuji, 1996. Ornithine decarboxylase antizyme: A novel type of regulatory protein. *Trends Biochem. Sci.*, 21: 27-30.
- Jacks, T., M.D. Power, F.R. Masiarz, P.A. Luciw and P.J. Barr, 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature*, 331: 280-283.
- Janzen, D.M. and A.P. Geballe, 2004. The effect of eukaryotic release factor depletion on translation termination in human cell lines. *Nucleic Acids Res.*, 32: 4491-4502.

- Karamysheva, Z.N., A.L. Karamyshev, K. Ito, T. Yokogawa and K. Nishikawa, 2003. Antizyme frameshifting as a functional probe of eukaryotic translational termination. *Nucleic Acids Res.*, 31: 5949-5956.
- Low, S.C. and M.J. Berry, 1996. Knowing when not to stop: Selenocysteine incorporation in eukaryotes. *Trends Biochem. Sci.*, 21: 203-208.
- Manuvakhova, M., K. Keeling and D.M. Dedwell, 2000. Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. *RNA*, 6: 1044-1055.
- Plant, E.P., P. Wang, J.L. Jacobs and J.D. Dinman, 2004. A programmed -1 ribosomal frameshifting signal can function as a cis-acting mRNA destabilizing element. *Nucleic Acids Res.*, 32: 784-790.
- Shigemoto, K., J. Brennam, E. Walls, C.J. Watson and D. Stott, 2001. Identification and characterization of a developmentally regulated mammalian gene that utilises -1 programmed ribosomal frameshifting. *Nucleic Acids Res.*, 29: 4079-4088.
- Stahl, G., L. Bidou, J.P. Rousset and M. Cassan, 1995. Versatile vectors to study recoding: Conservation of rules between yeast and mammalian cells. *Nucleic Acids Res.*, 23: 1557-1560.
- Theis, C., J. Reeder and R. Giegerich, 2008. KnotInFrame: prediction of -1 ribosomal frameshift events. *Nucleic Acids Res.*, 36: 6013-6020.