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Translational Efficiency of BVDV IRES and EMCV IRES for T7 RNA Polymerase Driven Cytoplasmic Expression in Mammalian Cell Lines[1]

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Abstract—Mammalian T7 polymerase-based cytoplasmic expression systems are common tool for molecular studies. The majority of these systems include the internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV). To carry out a cap-independent translation process, this type of IRES might require the expression of an extensive array of host factors, what is a disadvantage. Other IRESes might be less dependent on the host cell factors, but their biology is characterized to a lesser degree. Here, we compare the translational efficiencies of bovine viral diarrhea virus (BVDV) IRES with that of ECMV. Both IRESes were tested in reporter vectors containing the T7 promoter, an IRES of choice and the coding sequence of the enhanced green fluorescent protein (EGFP). To provide for the expression of T7 RNA polymerase, the corresponding gene was isolated from *Escherichia coli* and inserted into pCDNA3.1-Hygro(+). After co-transfection of the T7 RNA polymerase encoding vector with either of the two IRES-containing reporter vectors into T7 baby hamster kidney (T7-BHK), human embryonic kidney (HEK) 293T, chinese hamster ovary (CHO) and HeLa cells, the translational efficiency of the reporter construct was studied by fluorescence microscopy and flow cytometry. In T7-BHK, HEK 293T and HeLa cells the translational efficiency of BVDV IRES was two to three times higher than that of EMCV IRES. In CHO cells, BVDV IRES and EMCV IRES were equally efficient. An analysis of the secondary structure of respective mRNAs showed that their ΔG values were –544.00 and –469.40 kcal/mol for EMCV IRES and BVDV IRES harboring molecules, respectively. As EMCV IRES-containing mRNA is more stable, it is evident that other, still unidentified factors should be held responsible for the enhanced translational efficiency of BDVD IRES. Taken together, our results indicate the potential of BVDV IRES as a replacement for EMCV IRES, which is now commonly used for T7 polymerase driven cytoplasmic expression of genes of interest or virus cDNA rescue experiments.

Keywords: BVDV-IRES, EMCV-IRES, T7 RNA polymerase, translational efficiency
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INTRODUCTION

Exogenous gene expression systems are common tools for basic research labs and for clinical applications including gene therapy [1]. For many of these systems the relatively low level of gene expression in transfected tissues, especially in case of non-viral gene-delivery methods, is the major limitation [2, 3]. Cytoplasmic expression techniques that directly activate the promoters while avoiding the delivery of the plasmid into the nucleus rely on the use of phage RNA polymerases [4]. In particular, T7 promoter-based vectors have higher transcription efficiencies than other phage promoters such as SP6 [5], and achieve mRNA expression levels that are folds higher than those in standard CMV-based systems which require the entry of the plasmid into the nucleus [4, 6]. These systems hold a special interest for somatic gene therapy of non-dividing cells in an adult body [7]. Indeed, T7 promoter-based systems provide a number of advantages, including high transcriptase activity, outstanding promoter specificity, no requirement for nuclear entry, higher expression of proteins, and, at last, lower probability of gene silencing after several passages, which is frequently a problem for promoters

Abbreviations: BVDV, bovine viral diarrhea virus; EMCV, encephalomyocarditis virus; HCV, hepatitis C virus; IRES, internal ribosome entry site; MFE, minimal free energy; MFI, mean fluorescence intensity.

[1] The article is published in the original.

such as cytomegalovirus (CMV) [8]. In these systems the lack of an endogenous T7 RNA polymerase is supplemented in '*trans*' by a variety of approaches such as (i) co-transfection of a T7 RNA polymerase encoding plasmid along with the T7 RNA polymerase-driven expression vector harboring the T7-responsive promoter and the target gene [9], (ii) use of cell lines that stably express the T7 RNA polymerase [10] or (iii) transducing cells with a T7 RNA polymerase containing helper virus [11].

It is important to note that T7 RNA polymerase-derived transcripts cannot be 5` capped, and, therefore, the ribosomes cannot initiate their translation. Therefore, viral vectors for T7 RNA polymerase-driven expression in mammalian cells always include internal ribosome entry sites (IRESes). IRESes are relatively long sequences found in the 5' untranslated regions (5' UTR) of some viral genomes with ribosome binding capabilities. Insertion of the IRES into the transcript upstream of the target gene makes its translation cap-independent [12, 13]. To carry out cap-independent translation, IRES structures utilize cellular RNA-binding proteins known as IRES-transacting factors (ITAFs). Different IRESes recruit different ITAFs [14]. Moreover, some IRESes, in particular, picornavirus IRESes, require eukaryotic initiation factors such as eIF4A, 4B, 4E and 4G [15, 16].

The IRES most frequently used for driving heterologous gene expression in mammalian cells was derived from the encephalomyocarditis virus (EMCV) [17, 18]. The EMCV IRES and other similar IRESes may interfere with the translation of capped cellular mRNA [19], possibly through sequestering of cellular factors required for translation initiation [20, 21]. A few prior studies have compared abilities of various cellular and viral IRESes to enhance the expression of target genes. These studies showed that translational efficiency of the IRES depends both on its sequence and on the choice of cell line. To explain the latter, a role for cell-based ITAFs was proposed [22, 23]. Since IRESes may differ in their requirements for recruitment of particular translation initiation factors and ITAFs, some of which might be cell-specific [24, 25], substitution of EMCV IRES with other candidate sequences may improve the yield of T7 RNA polymerase driven expression.

Members of the *Flaviviridae* family, including the hepatitis C virus (HCV), bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV), are capable of initiating translation of respective mRNAs in a manner independent of eIF4s and other eukaryotic initiation factors. Moreover, to initiate the translation process IRES sequences of these viruses do not require ribosome scanning and may function without hydrolysis of ATP [16, 26]. These unique properties make these IRESes more efficient and cell type-independent. Indeed, the translational efficiency of the BVDV IRES is superior to the poliovirus IRES, another IRES commonly employed in mammalian gene expression systems [27]. More recently, translational efficiency of the BVDV IRES was substantially improved by its fusion with the first 75 bp of the BVDV N-terminal protease (*Npro*) gene [28]. All these developments prompt the incorporation of the BVDV IRES into T7 RNA polymerase-driven expression vectors and comparative studies of its efficiency.

Besides applications for cytoplasmic transgene expression in mammalian cells and high yield recombinant protein expression in *Escherichia coli* [29–32], T7 RNA polymerase-based vectors have also been developed for sophisticated reverse genetics experiments that rescue RNA viruses [33–35]. Reverse genetics techniques require precise quantification of the T7 RNA polymerase driven vector efficiency in target cells. To address this, the majority of studies relies on reporter vectors with luciferase or green fluorescent protein (GFP) genes inserted downstream of an IRES sequence that is placed under the control of the T7 promoter [8, 10, 36, 37].

To facilitate further development of the BVDV IRES containing expression vectors, we compared the translation efficiency of BVDV IRES with that of EMCV in the context of a T7 promoter-driven reporter plasmid that was transfected into a number of commonly used cell lines.

EXPERIMENTAL

Cell Lines

All cell lines employed in this study were purchased from the National Cell Bank of Iran. HEK 293T and HeLa cells were cultured in Dulbecco's Modified Eagle's Minimal Medium (DMEM) (Gibco, USA), containing 10% fetal bovine serum (FBS) (Atocel, Austria), penicillin and streptomycin, at 37°C in a 5% CO_2 atmosphere. The T7-BHK cell line, which constitutively expresses the T7 RNA polymerase gene, was maintained in the same medium, supplemented with 400 μg/mL G418 (InvivoGen, USA). For culturing CHO cells DMEM was replaced by DMEM F12 (Atocel, Austria).

Construction of the T7 RNA Polymerase-Encoding Recombinant Plasmid

To provide the '*trans*' source of the T7 RNA polymerase, the bacteriophage T7 RNA polymerase coding sequence from BL21 (DE3) strain of *E. coli* was isolated by PCR through the use of T7-F and T7-R primers (Bioneer, South Korea) (table). The amplicon (amplified T7 RNA polymerase gene) was double digested with NheI and HindIII restriction enzymes and subsequently cloned into *the same* sites of pCDNA3.1\Hygro(+) vector (Invitrogen, USA) to produce the pHygro-T7 plasmid (T7 RNA polymerase encoding plasmid).

Primers used for amplification of T7 RNA polymerase (T7-F, T7-R) and EGFP (EGFP-F, EGFP-R)* coding sequences

Primer name	Primer sequence
EGFP-F	5'-ACA**GGATCC**ATGGTGAGCAAGGGCGA-3'
EGFP-R	5'-ACC**AAGCTT**TTACTTCGTACAGCTCGTCC-3'
T7-F	5'-AA**GCTAGC**ACCATGAACACGATTAACATCGCTA-3'
T7-R	5'-AAGCGGCCGC**AAGCTT**TTACGCGAACGCGAAG-3'

*The bolded sequences indicate the inserted restriction sites used for cloning procedures. "F" and "R" denote the "Forward" and "Reverse" primers.

Construction of T7 Promoter-Based Reporter Plasmids

Cloning steps for construction of the pET-(EMCV IRES)-EGFP reporter plasmid (Fig. 1a) and pET-(BVDV IRES)-EGFP reporter plasmid (Fig. 1b) harboring the (EMCV IRES)-EGFP and (BVDV IRES)-EGFP sequences downstream of the T7 promoter, respectively, are shown in supplementary materials (Figs. S1 and S2, respectively, see Supplementary, http://www.molecbio.com/downloads/2017/2/supp_Ghasemi_engl.pdf).

All recombinant plasmids were constructed according to the standard procedures [38] and their accuracy was confirmed by restriction analysis followed by DNA sequencing analysis (data not shown).

Transfection

Transfections were carried out with Lipofectamine LTX and PLUS™ reagents (Invitrogen) according to the manufacturer's protocol. To reach the highest transfection efficiency in each cell line, the amounts of DNA and transfection reagent were optimized using pEGFPN1 (Clontech Laboratories Inc., USA) as a standard reporter plasmid. Briefly, 24 h before transfection, cells were seeded in 24-well plates to reach 70% of confluency at the day of transfection. Seven hundred fifty nanograms of plasmid DNA were diluted in 50 μL of_reduced serum medium Opti-MEM (Gibco) containing 0.5 μL of the PLUS™ reagent. After 5 min, the lipofectamine solution (2.5 μL lipofectamine + 47.5 μL reduced serum medium) was added to the DNA solution, the mixture was incubated for 25 min at room temperature and subsequently the DNA-lipofectamine complex was added drop-wise into the cells. Finally, 6 h after transfection, the medium was replaced with a fresh one.

To optimize the translation efficiency of the T7 promote-based reporter plasmids, different ratios of pHygro-T7 (T7 RNA polymerase-encoding plasmid) and IRES-containing reporter plasmids were used in co-transfection procedures. The best result was obtained when the pHygro-T7 plasmid was used in a 1/3 ratio to the total DNA (225 ng of 750 ng). Therefore, in co-transfection procedures, 225 ng of the pHygro-T7 plasmid and 525 ng of either IRES-containing reporter plasmids were used (for HEK 293T, HeLa and CHO cell lines). In case of the T7-BHK cell line (cells endogenously encoding the T7 RNA polymerase), instead of pHygro-T7 225 ng a mock plasmid (pCDNA3.1) was used for co-transfection.

Flow Cytometry and Fluorescence Microscopy Analyses

Twenty four hours after transfection, cells were screened for EGFP expression via inverted fluorescent microscopy INVERSO TC100 Epi Fluor (Medline Scientific, UK). For evaluation of IRESes translation efficiency, cells were washed two times with PBS, detached with trypsin and analyzed by a flow cytometer CyFlow (Partec GmbH, Germany) equipped with FloMax® software, version 2.7 (Quantom Analysis GmbH, Germany). The mean fluorescence intensity (MFI) of the FL1 channel in transfected cells indicated the translation efficiency of IRES driven plasmids [39].

Prediction of mRNA Secondary Structures

In order to predict the secondary structure and calculate the stability of the corresponding mRNA from each constructed reporter plasmid, DNA sequences were selected from the transcription initiation point (+1 nt) of the T7 promoter of pET-(EMCV IRES)-EGFP and pET-(BVDV IRES)-EGFP to the TAA stop codon of the *EGFP* gene. The secondary structure

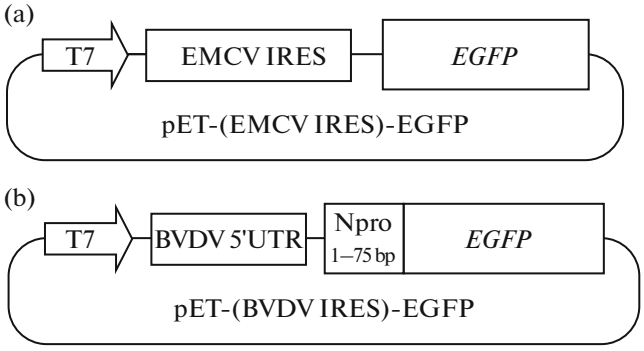

Fig. 1. Schematic diagram of the constructed IRES-driven plasmids. The *EGFP* gene is located downstream of EMCV IRES in pET-(EMCV IRES)-EGFP (a) and BVDV 5'UTR-Npro (1–75 bp) in pET-(BVDV IRES)-EGFP (b) under the control of the T7 promoter.

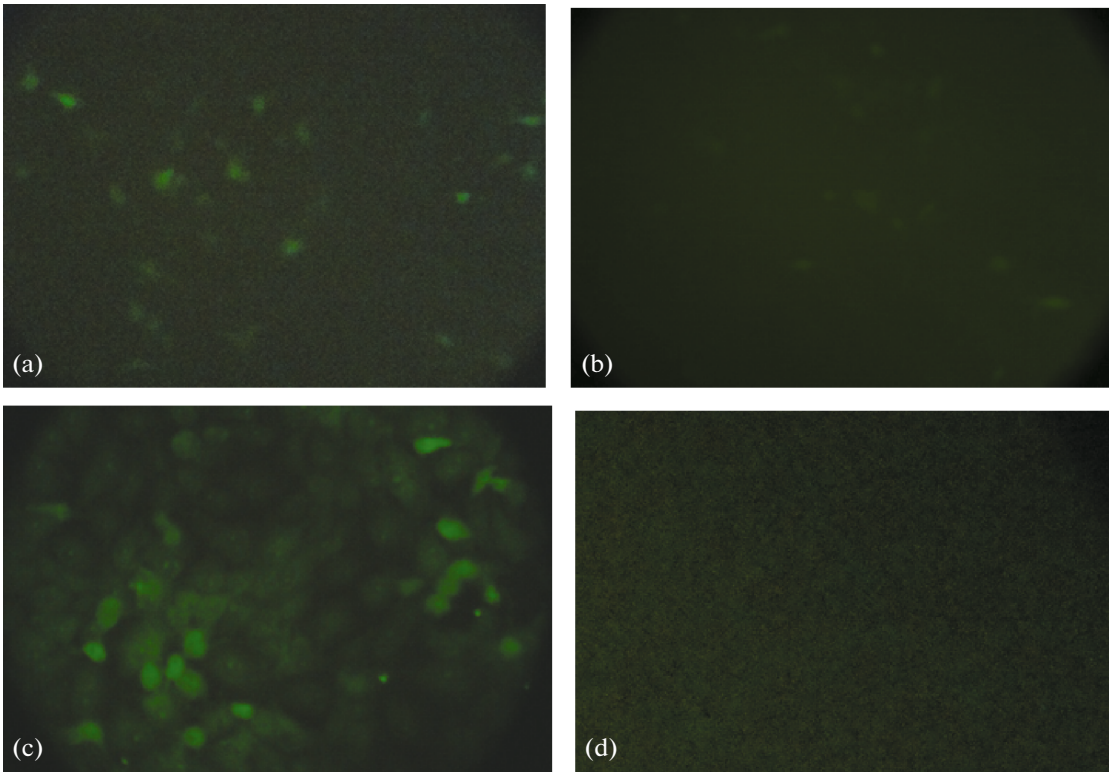

Fig. 2. Fluorescence microscopy analysis of T7-BHK cells 6 h after transfection with pET-(BVDV IRES)-EGFP (a), pET-(EMCV IRES)-EGFP (b), pEGFPN1 as a positive control (c), and mock plasmid pCDNA3.1\Hygro(+) (d).

of RNA was predicted using the RNA Fold Web server (http://rna.tbi.univie:ac.at/cgi-bin/RNAfold.cgi) based on minimum free energy (MFE).

Statistical Analysis

Data were analyzed with the SPSS statistical package (16.0 Version). All data are expressed as mean value ± SD unless otherwise mentioned. Factorial analysis of variances was applied for statistical analysis when appropriate. P values < 0.05 were considered statistically significant.

RESULTS

Construction of Recombinant Plasmids

As mentioned in the Experimental, the T7 RNA polymerase gene was isolated from the *E. coli* BL-21(DE3) genome by PCR. Agarose gel electrophoresis results of the amplicon showed a 2654 bp band (Fig. S3a, see Supplementary, http://www.molecbio.com/downloads/2017/2/supp_Ghasemi_engl.pdf) what is in accordance to the expected size of the T7 RNA polymerase gene [8, 10]. The PCR product was further cloned into NheI-HindIII sites of the pCDNA3.1\Hygro(+) vector to provide the pHygro-T7. Details on the restriction analysis carried out to confirm the accuracy of pHygro-T7 and the final reporter vectors, pET-(EMCV IRES)-EGFP and pET-(BVDV IRES)-EGFP, are illustrated in the supplementary materials (Fig. S3). All constructs were confirmed by sequencing analysis (data not shown).

Fluorescence Microscopy and Flow Cytometry

To evaluate the effect of BVDV and EMCV IRESes on the intensity of EGFP expression and to compare the MFI induced by each IRES, we co-transfected pHygro-T7 with either of the IRES containing reporter plasmids into different cell types including T7-BHK cells which constitutively express the T7 RNA polymerase gene (transfected with IRES containing reporter plasmids only).

Expression of EGFP was detectable 6 h after transfection. Fluorescence microscopy images showed that the intensity of EGFP was higher in all cells (T7-BHK, HEK 293T and HeLa) transfected with pET-(BVDV IRES)-EGFP rather than pET-(EMCV IRES)-EGFP reporter vector, except CHO cells that showed no recognizable difference for either reporter vector (data not shown). In Fig. 2, EGFP fluorescent intensity of reporter plasmid expression in T7-BHK cells are presented.

Analysis of detached cells for expression of EGFP by flow cytometry the day after transfection indicated that MFI values corresponding to the transfected T7-BHK, HEK 293T and HeLa cells was 2, 2.5 and 3 fold higher

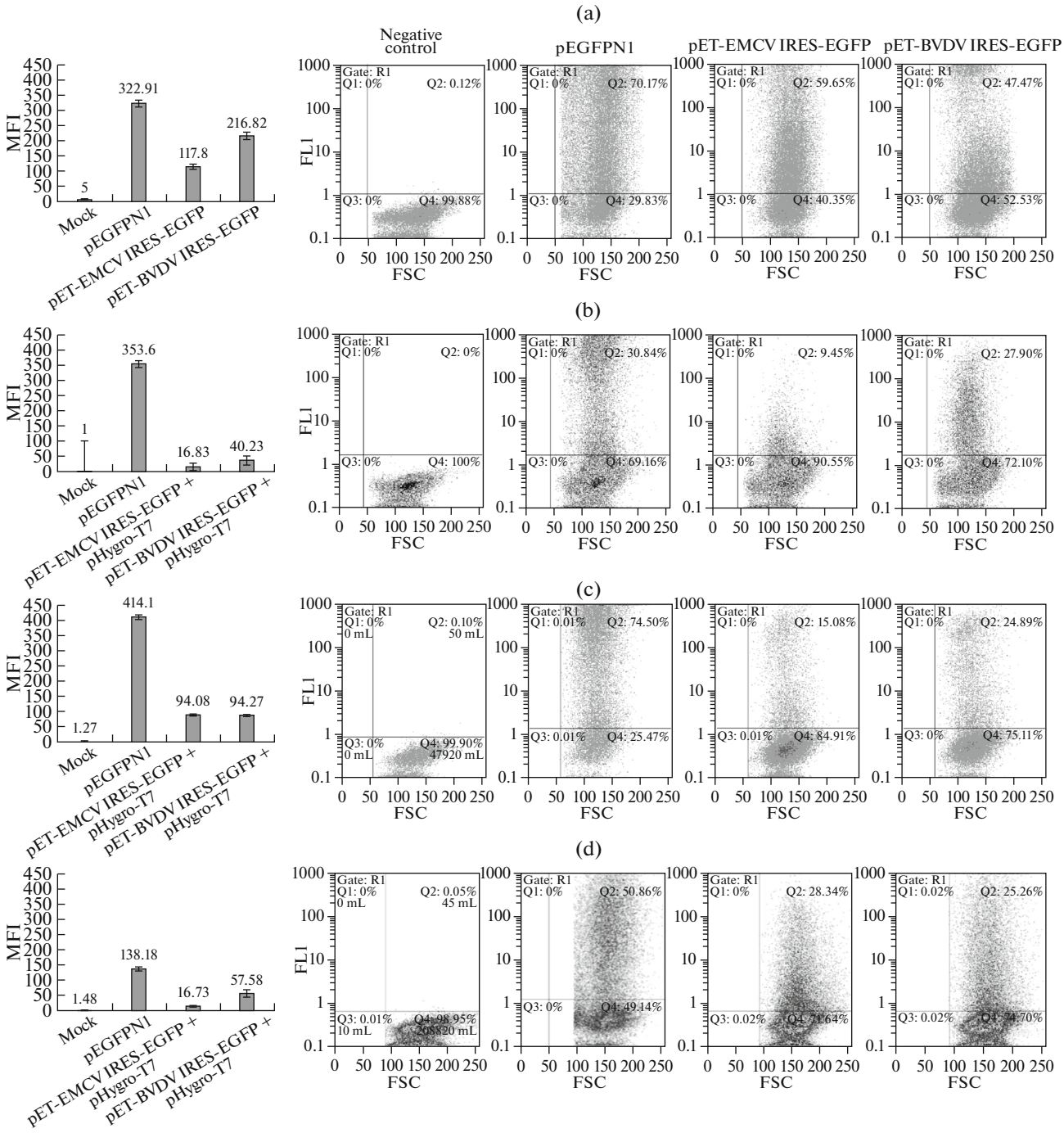

Fig. 3. Measured MFI values for EGFP expressed by pET-(EMCV IRES)-EGFP and pET-(BVDV IRES)-EGFP constructs in T7-BHK (a), HEK 293T (b), CHO (c) and HeLa (d) cell lines. Cells were assayed for MFI by flow cytometry 24 h post-transfection (mean ± SD, N = 3). Representative samples of flow cytometry graphs corresponding to 24 h post-transfection in each cell line are demonstrated in the right panel. pEGFPN1 was used as the control plasmid to optimize transfection rates in all four cell lines.

for BVDV IRES than EMCV IRES, respectively (P values < 0.05) (Figs. 3a, 3b, 3d) However, as shown in Fig. 3c, there was no significant difference in MFI values for CHO cells transfected with either reporter plasmid (EMCV IRES or BVDV IRES harboring vectors).

mRNA Secondary Structure Prediction and Calculation of ΔGs

Thermodynamic analysis based on MFE calculations [40] for secondary structure of RNAs indicated ΔG values of −544.00 and −469.40 kcal/mol for the

mRNA transcribed from pET-(EMCV IRES)-EGFP and pET-(BVDV IRES)-EGFP, respectively. The lower ΔG values for mRNA represents a generally more stable and strongly folded secondary structure and thus suggests a more stable mRNA for pET-(EMCV IRES)-EGFP.

DISCUSSION

In the present study, we designed two T7 RNA polymerase (T7 promoter)-based reporter plasmids, pET-(EMCV IRES)-EGFP and pET-(BVDV IRES)-EGFP (Fig. 1), which only differ in their IRES sequence (EMCV IRES versus BVDV IRES). We compared the translation efficiency of BVDV IRES with that of EMCV in a number of selected cell lines to gain information on cell-based potential applications of the BVDV IRES for cytoplasmic expression in mammalian cells and virus rescue studies. The main reasons that prompted us to conduct such a comparative study were both advantages of the T7 RNA polymerase-based system for cytoplasmic expression of exogenous genes (i.e.: high transcriptase activity, promoter specificity, rapid and large scale expression of proteins [7, 41], less gene expression silencing [8] as well as their independent functionality from nuclear entry) and potential efficiency of BVDV IRES for incorporation into T7 RNA polymerase-based vectors on translational efficiency of mRNAs of the target proteins as compared to other traditionally used IRESes like EMCV IRES [25, 42]. The procedures employed in this study for constructing the T7 promoter-based reporter plasmids may be used as a model for constructing other cytoplasmic gene expression systems or evaluation of T7 RNA polymerase activity in eukaryotic cells prior to rescue of T7 promoter-driven viral cDNA.

Selection of a suitable reporter gene is one of the major aspects in construction of reporter plasmids. Chloramphenicol acetyl transferase (CAT), lusiferase and EGFP are among the most recognized reporter genes [43, 44]. Despite the higher sensitivity of luciferase, it needs to normalize for transfection efficiency and cannot be quantified in each cell. EGFP due to both the possibility of direct analysis (visual observation) of functionality via fluorescent microscopy (qualitative analysis) [8] and quantitative analysis via flow cytometry [10] has gained more popularity. Accordingly, in the present study and in accordance with prior similar studies [8, 10, 36], EGFP was used as the reporter gene that enabled us to evaluate and compare EMCV IRES and BVDV IRES activities by employing both fluorescent microscopy (Fig. 2), like in a previous work wherein EGFP was used as a reporter for T7 RNA polymerase activity and without quantification of EGFP gene expression [8], and flow cytometry (Fig. 3), similar to a prior study that used the EGFP gene linked with FMDV IRES [10] followed by EGFP expression quantification by flow cytometry analysis. As shown in Fig. S2, for construction of pET-(BVDV IRES)-EGFP we amplified the EGFP gene using pEGFPN1 as a template for PCR. Agarose gel electrophoresis revealed the expected 715 bp band of the size of the EGFP gene which was in agreement with prior reports on size of the gene (Fig. S3c) [45]. Accordingly, the size of the isolated T7 RNA polymerase gene from *E. coli* BL-21 in this study (2654 bp) was in accordance with reports of previous studies (Fig. S3a) [8, 10] and the sequencing results confirmed the authenticity of the final construct (pHygro-T7).

In our study, we compared two types of IRESes, EMCV IRES and BVDV IRES, in a T7-based reporter system. Insertion of the IRES sequence between the T7 promoter and the reporter gene in order to enhance the translational efficiency of the reporter gene in a cap-independent manner was first reported in 1989 [17]. Subsequently, several other studies employed this system [8, 46] and the use of multiple IRES-reporter sequences in a single plasmid as an IRES-mediated polycistronic reporter system in order to improve the translational efficiency was also demonstrated [19]. However, to our knowledge, up to date the BVDV IRES has not been used in construction of such reporter systems. In fact, there are only a few prior comparative studies based on bicistronic mRNA systems which were mostly concerned with the activity of IRESes of *Picornaviridae* family and HCV IRES [15, 18, 22]. Results of some of these studies showed different activities of the FMDV and EMCV IRESes, although they both are type II IRESes of the *Picornaviridae* family [18, 22]. Thus, IRESes from even the same types and families may not necessarily show equal efficiency.

In the present study, different concentrations of pHygro T7 and IRES-containing plasmids were co-transfected into the cells to gain the best reporter output. The most efficient translation was registered when the pHygro T7 and the IRES-driven plasmids were used at a ratio of 3/7 which is consistent with the report by Gao et al. [46]. Subsequently, to evaluate their translational efficiencies, we co-transfected the pHygro-T7 plasmid with IRES-containing reporter plasmids in four commonly used for reverse genetics rescue system and recombinant protein expression studies mammalian cell lines (HEK 293T, CHO, HeLa and BHK) [47, 48]. It has been previously shown that an EMCV IRES-containing vector is able to translate the genes of interest efficiently in HEK 293T, CHO and HeLa cell lines [49] but not BHK cells [18, 22]. Our results however, indicated that, although translation efficiency of BVDV IRES in T7-BHK cells was 2 fold higher than that of EMCV RES (Fig. 3a), contrary to results of Borman et al. [22], who found the introduced EMCV IRES to be inefficient IRES in BHK cells, we found this IRES to be relatively efficient in T7-BHK cells (Fig. 3a). Our results also indicated that replacement of EMCV IRES with

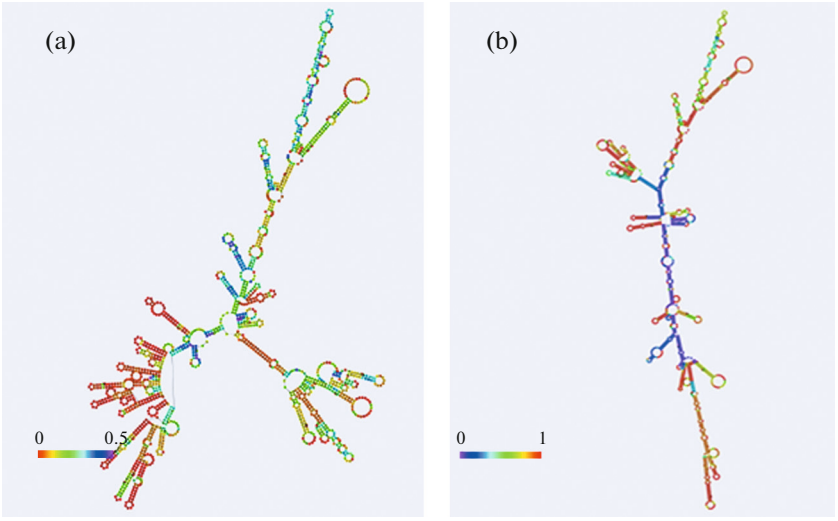

Fig. 4. RNA secondary structure prediction based on MFE. mRNA sequences for both (EMCV IRES)-EGFP (a) and (BVDV IRES)-EGFP (b) constructs were subjected to secondary structure prediction using the RNA fold Web Server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Predicted MFE ΔG values of −544.00 kcal/mol for (EMCV IRES)-EGFP mRNA and −469.40 kcal/mol for (BVDV IRES)-EGFP mRNA suggest a more stable secondary structure for the mRNA corresponding to the first construct.

BVDV IRES enhanced EGFP intensity in HEK 293T and HeLa cell lines by 2.5 and 3.0 folds, respectively (Figs. 3b, 3d). However, MFI corresponding to both EMCV IRES- with BVDV IRES-driven plasmids indicated almost the same values (no significant difference) in CHO cells (Fig. 3c). With regards to our fluorescence microscopy and flow cytometry results (Figs. 2 and 3), it might be concluded that the BVDV-IRES works efficiently in plenty of different cell lines. These results are in accordance with the data of a prior study which showed the superior activity of the BVDV IRES as compared to a poliovirus IRES, an IRES that is functionality strongly dependent on cellular translation initiation factors (similarly to the EMCV IRES) [27]. Indeed, in agreement with our observations and contrary to BVDV IRES-mediated translation-resistance to inhibition by an R362Q mutant form of eIF4A, IRES-mediated translation of those viruses was shown to be prohibited by this inhibitor (R362Q mutant form of eIF4A) [50, 51], what may further imply the smaller dependency of BVDV IRES functionality on cellular translation initiation factors as compared to that of the EMCV IRES. From another aspect, despite of the more relaxed structure of the BVDV IRES, the secondary structure of EMCV transcript is too complex, so it needs very specific cellular factors to interact with it and start translation. For this reason the inefficiency of the EMCV IRES in some cells can be the result of a failure to bind one or more translation initiation factors or RNA binding proteins.

Finally, we analyzed and compared the mRNA secondary structures and stability of IRESes in our reporter vectors to see their potential role in the higher translational efficiency of the BVDV IRES as compared to the EMCV IRES. The secondary structure of an IRES is usually complex and consists of several domains. These domains play a pivotal role in interactions with respective RNA and cellular factors such as RNA-binding proteins [52] and microRNAs [53]. These interactions and the secondary structure itself contribute to the stability of the RNA molecule and thereby determine the expression levels of the RNA at a posttranscriptional level [39]. Interestingly, prediction of the secondary structure of the respective mRNAs suggested a more stable secondary structure for (EMCV IRES)-EGFP mRNA as compared to that of (BVDV IRES)-EGFP mRNA (Fig. 4). This final software based prediction may further emphasize the particular role of interacting/binding cellular components for the efficient expression of BVDV IRES-containing constructs as compared to those containing the EMCV IRES, rather than the sole structural stability of their transcripts. In agreement to this proposition, very recently, the minor impact of the liver specific miR-122 on the translational efficiency of HCV IRES due to sole transcript stabilization was suggested and thus other miR-122-dependent mechanisms for sustained translation of HCV IRES are currently being investigated [54]. Although, in the present study we did not aim to investigate cell-specific factors that might contribute to increased expression levels of the BVDV IRES, previous studies have suggested some potential roles for viral elements and their interactions with cell-specific factors while concerning mRNA stability and its function at expressional levels [39, 55]. Indeed, to our knowledge there are no prior studies on interactions of cellular proteins and BVDV IRES with the corresponding mRNAs that might enhance trans-

lational efficiencies. Sanderbrand and coworkers [56] reported that 3 proteins with molecular masses of 50, 65 and 72 kDa interact with BVDV IRES RNA without any suggestion on the potential influence of these proteins on the IRES's translation activity. Furthermore, since the human LA antigen (p52 or SS-B) is capable of stimulating translation directed by the IRES of HCV (an IRES closely related to BVDV IRES) [57, 58], Myers et al. [59] suggested a possibility of interaction of the LA antigen with the BVDV IRES with a role in translation initiation. Accordingly, a major topic of future investigations might be the exploration of binding proteins for a potential stimulatory action on BVDV IRES-mediated translation.

In summary, we designed and constructed EGFP-based reporter plasmids to compare the translation efficiency of the BVDV and EMCV IRESes in the context of T7 promoter-based vectors and tested them in several cell lines. Our protocols could be used as a model for constructing other cytoplasmic gene expression/reporter systems or for evaluating T7 RNA polymerase activity in eukaryotic cells prior to rescue of T7 promoter-driven viral cDNA. To our best of knowledge, no prior study has compared BVDV and EMCV IRES-driven translation efficiencies in any system. We showed that in a variety of cell lines the BVDV IRES enhances translation more efficiently than the EMCV IRES, thus indicating its potential as an alternative to commonly used IRESes, such as EMCV in T7 promoter-dependent expression designed for either gene therapy or virus rescue experiments.

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