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Effects of the Adenovirus Type 5 Tripartite Leader Sequence with Partial and Complete Exons on mRNA Transport, Stability and Translation in Chinese Hamster Ovary Cells

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Abstract

Adenoviruses have been used as a model system for understanding gene expression, oncogenic transformation, DNA replication, gene delivery and other molecular biological phenomenon. The adenovirus major late promoter (MLP) is the predominant promoter during late viral events, and together with the tripartite leader sequence (TPL) is responsible for the abundant late viral protein production. In this study, we have constructed a series of plasmids containing the MLP, with partial and complete TPL exons (exon 1, exon 1 & 2 and exon 1,2 & 3). Gene expression was evaluated in Chinese hamster ovary (CHO) cells using green fluorescence protein (GFP) as a reporter gene. Upon transfection, GFP mRNA transcription levels were measured over time via qRT-PCR. Furthermore, quantification of nuclear and cytoplasmic mRNA was monitored to determine the transport rates and mRNA stability over time. Translation levels were determined by western blotting using anti-GFP antibodies. No significant changes in mRNA transcription were measured with the incorporation of the different TPL exons. On the other hand, mRNA export and stability, in addition to GFP levels, increased with the incorporation of the TPL exons. Maximum levels were reached with the complete TPL form. The results show the importance of the entire TPL structure in mRNA stability and function.

Introduction

- The late phase of adenovirus infection is characterized by the production of an abundant amount of late proteins required to form and assemble the new viral capsids. Active translation in this phase is attributed to the activity of the major late promoter (MLP) and the presence of the tripartite leader sequence (TPL).
- TPL is a 5' untranslated sequence present in all of the late, but none of the early, viral mRNA. The adenovirus serotype 5 (Ad5) leader sequence is 201 bp formed by the splicing of three exons during the post-translational modifications. TPL facilitates mRNA transport and accumulation in the cytoplasm and is responsible for the selective translation of the late viral proteins in preference to the cellular proteins (2, 12, 17).
- Adenovirus E1B-55K and E4-orf6 play the main role in active transport of TPL-containing mRNA from the nucleus to the cytoplasm (1, 3, 9, 11, 14-16). The viral transcription sites in the nucleus contain a complex of E1B-55K and E4-orf6 (8, 13). Evidence suggests that viral mRNA interacts with this complex through the ability of E1B-55K to bind RNA (10), and facilitates its transport to the cytoplasm using the E4-orf6 proteins nuclear localization and transport signals (4). Cellular mRNA transport is blocked by the same complex (7).
- Translation of any TPL-attached mRNA is eIF-4F-independent (6). The relaxed secondary structure of TPL facilitates its function in translation initiation even when eIF-4F is inhibited (5). It is not known if the complete TPL, or just a partial sequence, is needed for such activity.
- In order to understand the effect of the TPL on transcription from MLP and mRNA transport and translation, independent from other viral proteins, four plasmids were constructed. This allows us to determine whether the complete sequence of TPL, or just partial requirements, is responsible for its function.The four plasmids contain MLP as a common promoter and TPL with all, partial or no exons. We investigated mRNA transcription, transport, stability and expression levels of green florescence protein (GFP) gene from each construct, after transfection into Chinese hamster ovary (CHO) cells.

Methods

- Four plasmids (pMGA, pMT1GA, pMT1,2GA and pMT1,2,3GA) were constructed with partial or complete Ad5 TPL exons and GFP as a reporter gene under the control of Ad5 MLP (Figure 1). Plasmids were prepared by CsCl gradients.
- Equal amounts from each plasmid were transfected into CHO cells using the calcium phosphate method. Transfection was done in triplicate wells in 6-well plates and the medium was replaced 6 h post-transfection.
- Transfected cells in each of the triplicate wells were divided into two isolation procedures. The first was used to isolate cytoplasmic and nuclear RNA using Norgen's Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek. Corp., Thorold, ON, Canada), and the second was used to isolate total RNA, DNA and proteins using Norgen's RNA/DNA/Protein Purification Kit (Norgen Biotek. Corp., Thorold, ON, Canada). Samples were collected after 0, 12 and 24 h post-transfection.
- All isolated RNAs were treated with Ambion`s turbo DNase (Ambion, Austin, TX, USA) to digest any residual DNA background and were then cleaned using Norgen`s RNA Clean-Up and Concentration Kit (Norgen Biotek. Corp., Thorold, ON, Canada). Specific PCR for GFP fragment was used to check the success of the digestion step.
- qPCR and qRT-PCR were performed on the isolated DNA and RNA samples using 1x SYBR GREEN master mix (Bio-Rad, Hercules, CA, USA) and specific primers for GFP fragment (Forward: 5' ATCCTGATCGAGCTGAATGG 3' and Reverse: 5' TGCCATCCTCGAT-GTTGTG 3'). Reactions were performed using the Bio-Rad iCycler thermal cycler.

Results

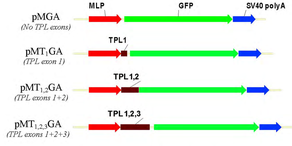

Figure 1: Schematic diagrams of the constructs used. Each of the four constructed plasmids (pMGA, pMT1GA, pMT1,2GA and pMT1,2,3GA) contains a common promoter (MLP), reporter gene (GFP) and poly A signal (SV40 poly A). Tripartite leader sequence exons were cloned downstream of the MLP and upstream from the reporter gene. Exon 1, exons 1,2 and the full TPL sequence were cloned to construct pMT1GA, pMT1,2GA and pMT1,2,3GA, respectively.

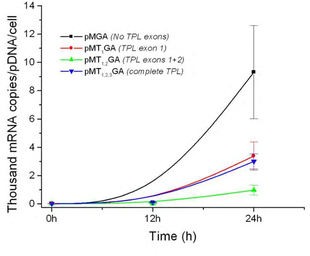

Figure 2: Transcription efficiency of GFP over 24 hours post-transfection of the different plasmids into CHO cells. Using a standard curve of known concentration, qPCR on 4 ng DNA and qRT-PCR on 25 ng RNA were used to determine plasmids and GFP mRNA copy numbers, respectively. Copy numbers of both plasmid DNA and GFP mRNA were calculated per cell and used in determining transcription efficiency over time (Transcription efficiency = mRNA copy number/pDNA copy number/cell).

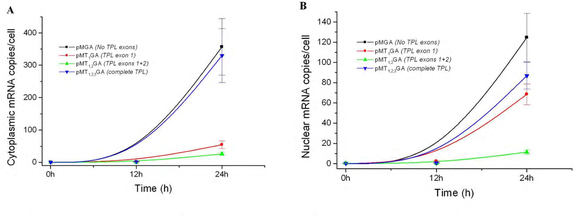

Figure 3: Cytoplasmic (A) and nuclear (B) GFP mRNA copy number per cell over 24 hours post-transfection of the different plasmids into CHO cells. mRNA copy numbers were measured by qRT-PCR, using a standard curve of known concentrations.

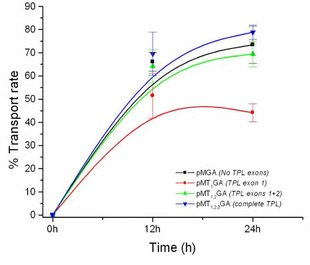

Figure 4: GFP mRNA transport rate over 24 hours post-transfection of the different plasmids into CHO cells. mRNA copy numbers were measured by qRT-PCR, using a standard curve of known concentrations. Transport rate was calculated as the percentage of cytoplasmic mRNA over the sum of cytoplasmic and nuclear mRNA.

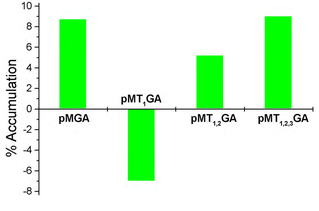

Figure 5: GFP mRNA accumulation rate after transfection of the different plasmids into CHO cells. Percentage accumulation rate = [(cytoplasmic/(cytoplasmic + nuclear mRNA)]24 h – [cytoplasmic/ (cytoplasmic + nuclear mRNA)]12 h x 100.

Conclusions

- Incorporation of TPL exons reduced the transcription efficiency from the MLP; minimum transcription rates were obtained with incorporating TPL exons 1+2.
- Transport rate was insignificantly increased with incorporating the complete TPL form; however significant reduction in the transport rate was shown when using just the first TPL exon.
- No significant changes in accumulation rate were observed between the TPL-free, exons 1+2 and the complete TPL form. Incorporating only Exon 1 resulted in mRNA instability.
- In general, the complete TPL increased mRNA transport and accumulation when compared to the incomplete forms. However, it does not have any significant effects when compared to the TPL-free construct.
- The data suggest that the lack of adenoviral proteins that interacts with TPL (E1B 55K and E4 orf6) severely affects its function and activity.

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