Deadenylation of interferon-β mRNA is mediated by both the AU-rich element in the 3’-untranslated region and an instability sequence in the coding region

Muriel Paste, Georges Huez and Véronique Kruys

Laboratoire de Chimie Biologique, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, Belgium

Viral infection of fibroblastic and endothelial cells leads to the transient synthesis of interferon-β (IFN-β). The down-regulation of IFN-β synthesis after infection results both from transcriptional repression of the IFN-β gene and rapid degradation of mRNA. As with many cytokine mRNAs, IFN-β mRNA contains an AU-rich element (ARE) in its 3’-untranslated region (UTR). AREs are known to mediate mRNA deadenylation and destabilization. Depending on the class of ARE, deadenylation was shown to occur through synchronous or asynchronous mechanisms. In this study, we analysed IFN-β mRNA deadenylation in natural conditions of IFN-β synthesis, e.g., after viral infection. We show that human IFN-β mRNA follows an asynchronous deadenylation pathway typical of a mRNA containing a class II ARE. A deletion analysis of the IFN-β natural transcript demonstrates that poly(A) shortening can be mediated by the ARE but also by a 32 nucleotide-sequence located in the coding region, that was identified previously as an instability determinant. In fact, these elements are able to act independently as both of them have to be removed to abrogate mRNA deadenylation. Our data also indicate that deadenylation occurs independently of mRNA translation. Moreover, we show that deadenylation of IFN-β mRNA is not under the control of viral infection as IFN-β mRNA derived from a constitutively expressed gene cassette is deadenylated in absence of viral infection. Finally, an unidentified nuclear event appears to be a prerequisite for IFN-β mRNA deadenylation as IFN-β mRNA introduced directly into the cytoplasm does not undergo deadenylation.

In conclusion, our study demonstrates that IFN-β mRNA poly(A) shortening is under the control of two cis-acting elements recruiting a deadenylating machinery whose activity is independent of translation and viral infection but might require a nuclear event.

Keywords: mRNA stability; polyadenylation; translation.

The transient expression of human interferon-β (IFN-β) in response to double stranded RNA or viral infection is a direct consequence of transcriptional activation [1] and leads to the accumulation of mRNA. In contrast, the shutoff of IFN-β gene expression involves the induction of a transcriptional repressor as well as a rapid decay of IFN-β mRNA [2,3]. The human IFN-β mRNA contains an AU-rich element (ARE) in its 3’-untranslated region (3’UTR). AREs were first discovered as highly conserved elements present in the 3’UTR of mRNAs encoding cytokines and oncoproteins [4]. These motifs composed of the AUUUA pentamer, were shown to confer mRNA instability and to regulate mRNA translation [5]. Indeed, Shaw and Kamen first reported that the ARE located in the 3’UTR of the granulocyte macrophage colony stimulating factor (GM-CSF) mRNA was responsible for mRNA rapid degradation [6]. Later on, the destabilizing activity of several other AREs was documented (for review, ref [7]). AREs have been classified into three distinct categories based on the number and distribution of AUUUA pentamers. Class I AREs are characterized by the presence of one to three pentamers distributed into a large part of the 3’UTR coupled with a nearby U-rich region. Class II AREs have at least two overlapping copies of the nonamer UUAUUU(U/A) (U/A)U in a U-rich environment and class III do not contain any pentamers but present U-rich stretches. AREs from all three classes confer mRNA instability in cultured cells through different mechanisms that all imply mRNA deadenylation (for review, see [7]). Class II AREs (e.g., GM-CSF, TNF-α, and IL-3) induce asynchronous deadenylation resulting in the accumulation of poly(A) intermediates. In contrast, class I and class III AREs (e.g., c-fos and c-jun) direct a synchronous poly(A) shortening. Several ARE-binding proteins have been identified, among which AUFI and the tristetraprolin (TTP) were shown to participate in the destabilization of ARE-containing mRNAs. Recently, Chen et al. showed that ARE-binding proteins such as AUFI and TTP were able to interact with a multiprotein complex, called the exosome [8]. This complex first discovered in yeast [9], is composed of proteins with ribonuclease activity and is able to direct 3’–5’ mRNA degradation. Therefore, the recruitment of the exosome by ARE-binding proteins might account for the degradation of ARE-containing mRNAs.
Based on its sequence, IFN-β ARE belongs to class II. Moreover, several reports described IFN-β mRNA instability and the involvement of the ARE in this process [10,11]. In addition, another destabilizing sequence was identified within the coding region of IFN-β mRNA [12,13]. Whereas the independent removal of the ARE or the coding region instability determinant (CRID) result in a moderate stabilization of the mRNA, replacement of both elements by control sequences greatly enhances mRNA half-life [13]. It should be mentioned however, that these observations were made in heterologous cell systems using reporter DNA constructs under the control of heterologous or IFN-β modified promoters. Moreover, the role of the instability determinant in the control of IFN-β mRNA deadenylation was not addressed.

In the present study, we analysed the expression of endogenous IFN-β in human cells upon viral infection. We also investigated the influence of the ARE and the CRID on the poly(A) status of the human IFN-β mRNA in natural conditions of IFN-β synthesis.

Materials and methods

Reagents

All the reagents and enzymes used in this study were purchased from Roche Molecular Biochemicals and Life Technologies Inc., unless specified. The Sendai virus (Can
tell strain, ATCC VR-907 Parainfluenza 1) was obtained from Charles River Laboratories. [α-32P]UTP, [α-35S]UTP and [35S]-Met were purchased from Amersham-Pharmacia Biotech. The anti-IFN-β ELISA kit was purchased from Biosource. Rat monoclonal anti-HA Ig (clone 3F10) was purchased from Roche Molecular Biochemicals.

Plasmid Construction

The complete sequence of the human IFN-β gene including the IFN-β promoter (EcoRI/EcoRI fragment described in reference [14]) was inserted in the pcDNA3 plasmid (Invitrogen) from which the cytomegalovirus (CMV) promoter was deleted previously. In the pIFNHA construct, the IFN-β gene was tagged by PCR using an oligonucleotide containing three repetitions of the sequence corresponding to the human influenza A virus hemaglutinin (HA) epitope. The plIFNHAUU construct was generated by deleting the 75-nucleotide region corresponding to the ARE (from nucleotides 740–815 of the mRNA). The plIFNCRIDHA and plIFNCRIDHAU constructs were obtained by deletion of a 32-nucleotide region (from 513–545) in the PIFNHA and PIFNHAU constructs, respectively.

A stable hairpin (hp) structure obtained by oligomerization of a Sall linker was inserted in the Hin cII site of the plIFNHA construct located at the beginning of IFN-β mRNA 5’ UTR. To place the IFN-β gene under the transcriptional control of a constitutive promoter, the HA-tagged IFN-β cDNA was inserted between the EcoRI and BamHI sites of the pSG5 plasmid (Stratagene) (pSG5IFNHA) downstream of the simian virus 40 (SV40) promoter.

For in vitro transcription, the pBSIFNpA vector was generated as follows. The poly(A) tail was obtained by hybridization of a 15-A and a 15-T oligonucleotide. The single stranded extremities were filled with the Klenow polymerase before oligomerization. The DNA fragments were cloned in the T4 DNA polymerase blunted PstI site of the pSP65 vector. The length of the inserted fragments was estimated on agarose gel and the vector containing a 100–150 nucleotides insert was selected. The poly(A)100–150 tail was then cloned in the HindIII/SalI sites of the pBluescript SK (Stratagene). The restriction sites between SacI and PstI were deleted in this pBluescript SK poly(A) and the IFN-β gene without its promoter (EcoRI/BamHI fragment from the pSP65IFNc plasmid described elsewhere [5]) was then cloned between the EcoRI and HindIII sites. The deletion of the ARE was performed by inserting a EcoRI/NdeI fragment of the IFN-β gene [5].

Cell culture and treatments

The human endometrial adenocarcinoma cells (Hec-1B, ATCC number, HTB-113) were maintained in DMEM containing 10% of fetal bovine serum (FBS; Myoclone Super Plus, Life Technologies) and 1% of penicillin/streptomycin. The cells were infected by addition of 80 U·mL−1 of Sendai virus during 2 h. Actinomycin D and cycloheximide were used at final concentrations of 5 μg·mL−1 and 10 μg·mL−1, respectively.

Isolation of total RNA and RNase H treatment

Total RNA was prepared by the Trizol method (Life technologies, Inc.). RNase H treatment was performed according to the method described by McGrew et al. [15].

Northern blot analysis

Northern blot analysis was performed as described by Kruys et al. [16]. Total RNA (10 μg per lane) was separated by electrophoresis in a 2.2% agarose gel, electrotransferred to nylon membrane and cross-linked by UV-irradiation. Blots were hybridized with antisense [α-32P]UTP or [α-35S]UTP labelled riboprobes.

In vitro Transcription and translation

DNAs were linearized at unique restriction sites and capped mRNA were generated by in vitro transcription with T3 or Sp6 polymerases. RNA was quantified by absorbance at 260 nm and its integrity was verified by agarose gel electrophoresis followed by ethidium bromide staining. Translation was carried out in rabbit reticulocyte lysate (Promega) in the presence of 35S-labelled Met (Amersham Pharmacia Biotech).

DNA and RNA transfection

Hec-1B cells were transfected with DNA using the Fugene reagent (Life technologies) following the procedure provided by the supplier. RNA transfections were carried out using the lipofectamine reagent (Life technologies) as described by the supplier. In brief, cells were grown to 50% confluency in six-well plates before transfection. The culture medium was then replaced by serum-free medium and the transfection mix was
added. The transfection mix contained 10 μg of RNA (between 10–100 ng of the in vitro transcribed mRNA supplemented by a carrier tRNA) and 10 μL of lipofectin in a total volume of 200 μL of serum-free medium.

In both cases, the culture media were harvested to measure IFN-β concentration by ELISA and the cells were harvested for total RNA extraction.

Metabolic protein labeling and immunoprecipitation
Hec-1B cells were plated in six-well plates at 200 000 cells per well and were incubated for 6 h before transfection. After transfection, the cells were incubated for another 24 h and then infected by Sendai virus for 2 h. Cells were washed and preincubated in a Met and Cys-depleted medium for 1 h. Metabolic labeling was performed by adding 500 μCi/mL of 35S-labelled Met and Cys in the cell culture for 5 h. The cell culture medium was harvested for immunoprecipitation. Immunoprecipitation was performed in RIPA buffer (25 mM Tris pH 8.2, 50 mM NaCl, 0.5% Nonidet P40, 0.5% deoxycholate, 0.1% SDS) using an anti-\textit{HA} Ig and protein A-Sepharose. Proteins were analysed by SDS/PAGE followed by autoradiography.

Results
Deadenylation of the human IFN-β mRNA in virus-infected cells
So far, all the studies aimed at understanding the post-transcriptional regulation of human IFN-β mRNA have been performed in heterologous cell systems. Therefore, we chose to analyse the regulation of human IFN-β mRNA in human cells (Hec-1B) that naturally produce IFN-β upon viral infection [17]. We first performed a kinetic analysis of IFN-β production by Hec-1B cells after infection by the Sendai virus for 2 h. IFN-β appeared in the cell culture 2–5 h after the infection, reaching a maximum between 8–11 h and subsequently levels dropped at later times (Fig. 1A). We then analysed, by Northern blot, the induction and decay of IFN-β transcript in the same conditions. As shown in Fig. 1B, IFN-β mRNA was detectable 4 h after the beginning of the infection, reached a maximum after 6–7 h and then rapidly disappeared thereafter. Interestingly, two IFN-β mRNA species were observed, the shorter form appearing later in the infection process. As class II AREs are known to mediate mRNA degradation by

Fig. 1. Interferon-β production by Hec-1B cells infected by Sendai virus. Hec-1B cells were infected for 2 h by the Sendai virus, the cells were then washed with NaCl/Pi and fresh medium was added. (A) Every 3 h, the supernatant was sampled and replaced by fresh culture medium. The IFN-β was quantified in the supernatants by ELISA. (B) Cells were harvested for total RNA extraction 0, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 h after infection. The amount and the length of IFN-β mRNA was analysed by Northern blot, using a 32P-labelled antisense IFN-β riboprobe. As a control, the membrane was hybridized with a GAPDH antisense riboprobe. (C) Total RNA of cells infected with the Sendai for various lengths of time was digested (or not) by RNase H in the presence of oligo(dT). Treated and untreated RNAs were analysed by Northern blotting. (D) Hec-1B cells were infected by the Sendai virus and further cultured with cycloheximide (10 μg/mL). At the indicated times after infection, total RNA was extracted and analysed by Northern blot. (E) Hec-1B cells were infected by the Sendai virus and further cultured with cycloheximide (10 μg/mL) and actinomycin D (5 μg/mL). At the indicated times after induction, total RNA was extracted and analysed by Northern blot with a 32P-labelled antisense IFN-β riboprobe. The results presented in A, B and C are representative of more than five independent experiments. Data in D and E are representative of two independent experiments.
promoting deadenylation, we investigated whether the two transcripts differed by the length of their poly(A) tail. Therefore, total RNA of Hec-1B cells infected by the Sendai virus was treated (or not) by RNase H in the presence of oligo(dT) before the Northern blot analysis with an IFN-β probe. This treatment led to the detection of a single band comigrating with the short IFN-β transcript in untreated samples (Fig. 1C), indicating that the large and short transcripts observed in untreated samples corresponded to polyadenylated and deadenylated mRNAs, respectively. The poly(A) tail of IFN-β mRNA was estimated to be about 200 nucleotides long based on the difference of electrophoretic migration between the adenylated and deadenylated IFN-β mRNA (Fig. 1B). We then analysed the effect of cycloheximide on the accumulation of the two IFN-β transcripts to determine whether IFN-β mRNA deadenylation process required ongoing translation. As shown in Fig. 1D, the addition of the translation inhibitor after the infection of the Hec-1B cells did not prevent the appearance of the IFN-β short transcript. Moreover, as reported previously, cycloheximide led to a marked increase of IFN-β mRNA accumulation at later times of infection resulting from mRNA stabilization and/or absence of transcriptional repression [10,12]. Treatment by both actinomycin D and cycloheximide did not prevent IFN-β mRNA deadenylation either as the polyadenylated IFN-β transcript accumulated in response to the viral infection was also shortened before being degraded. Moreover, the transcriptional blockade by actinomycin D abrogated the increase of mRNA accumulation due to the cycloheximide (Fig. 1E). This latter observation indicates that increased accumulation of IFN-β mRNA in cycloheximide-treated cells is due to the absence of transcriptional repression of the IFN-β promoter.

Altogether, these results indicate that viral infection triggers the synthesis of a polyadenylated IFN-β mRNA that is deadenylated rapidly before degradation. Moreover, this deadenylation process does not require IFN-β mRNA translation and/or protein synthesis as it is effective in the presence of a translational inhibitor.

Deadenylation of IFN-β mRNA occurs when IFN-β synthesis is induced by other agents such as synthetic double-stranded polyriboinosinic polyribocytidylic acid (poly rLrC) and was observed in other cell types such as Namalwa B cells (data not shown). These observations indicate that deadenylation is a general mechanism controlling the length of IFN-β mRNA poly(A) tail.

**IFN-β mRNA deadenylation is mediated by both the ARE and the CRID**

The IFN-β mRNA contains in its 3′UTR an AU-rich element (ARE) which is very similar to AREs present in other unstable mRNAs [4]. As AREs present in other cytokine mRNAs were demonstrated to induce mRNA degradation by triggering poly(A) shortening, we first analyzed the role of such an element in the deadenylation process of IFN-β mRNA. To this end, two DNA constructs were generated in which the IFN-β gene contained or not the ARE (pIFNHA and pIFNHAUU). In addition, the sequence encoding the HA epitope was inserted at the end of the IFN-β coding sequence to distinguish the products resulting from the expression of the DNA constructs and the endogenous gene (Fig. 2). These constructs were transfected in Hec-1B cells and the cells were subsequently infected with the Sendai virus for 2 h. Cells were lysed 3 or 8 h after infection to extract the RNA which was treated (or not) by RNase H in the presence of oligo(dT) before the Northern blot analysis with a HA antisense riboprobe. As shown in Fig. 3A (lanes 3 and 7), the HA-tagged IFN-β transcript underwent significant deadenylation 8 h after infection independently of the presence or the absence of the ARE. Another RNA instability determinant was identified in the 3′-end of the IFN-β coding region [13]. This element named CRID (coding region instability determinant), has been mapped between nucleotides 513–545, the first nucleotide corresponding to the adenosine of the initiation codon. Deletion of this element by itself from the IFN-β gene (pIFNCRIDHA) did not abolish mRNA deadenylation (Fig. 3B, compare lanes 2 and 3). However, deletion of both the ARE and the CRID (pIFNCRIDHAUU) led to a blockade of the deadenylation process (Fig. 3B, compare lanes 6 and 7). These results demonstrate that deadenylation is controlled by both the ARE and the CRID.

**Fig. 2. Schematic representation of the DNA constructs.**
Deadenylation of IFN-β mRNA is uncoupled from its translation

The role of translation in ARE-mediated mRNA deadenylation and subsequent decay is still a subject of controversy. Indeed, while several reports support a translation-dependent mechanism [18–20], other observations deny any coupling between the recruitment of the mRNA into polysomes and its deadenylation/degradation [7].

Here, we analysed whether ongoing translation is a prerequisite for IFN-β mRNA deadenylation. Therefore, we generated a IFN-β gene construct containing a stable hairpin in the 5'UTR (pIFNHAhp, Fig. 2) and the deadenylation of the derived mRNA was compared to that of the mRNA lacking such a secondary structure (pIFNHA). As shown in Fig. 4A, the presence of the hairpin in the 5'UTR does not influence the deadenylation process. To verify that the hairpin effectively prevented the translation of the mRNA, the secretion of HA-tagged IFN-β was monitored in the culture medium of cells transfected with these constructs. Whereas cells transfected with the construct lacking the hairpin produced detectable amounts of HA-tagged IFN-β, no translation product was detectable with the construct containing the hairpin in the 5'UTR (Fig. 4B).

Deadenylation of IFN-β mRNA occurs independently of viral infection

We then analysed whether IFN-β mRNA deadenylation resulted from the infection of the cells by the Sendai virus. In order to ensure the production of IFN-β transcripts in absence of infection, the HA-tagged IFN-β gene was placed downstream of the SV40 early promoter (pSG5IFNHA, Fig. 2). Hec-1B cells were transfected with the pSG5IFNHA construct and were subsequently infected (or not) with the Sendai virus. Deadenylation of the HA-tagged IFN-β mRNA was monitored in the presence of actinomycin D to block further accumulation of HA-tagged IFN-β mRNA. As shown in Fig. 5, deadenylation of the HA-IFN-β transcript occurs even in absence of viral infection.

Deadenylation of IFN-β mRNA requires a nuclear event

We next determined whether IFN-β mRNA deadenylation requires a nuclear event. To approach this question, a synthetic IFN-β transcript containing a poly(A) tail of ≈100–150 residues was generated by in vitro transcription in the presence of 32P-labelled UTP (see Materials and
The Hec-1B cell was transfected with this synthetic transcript for 2 h, and total RNA was extracted at various times after transfection to be analysed by agarose electrophoresis and autoradiography. As shown in Fig. 6B, the IFN-β transcript is rapidly degraded without prior deadenylation, suggesting that IFN-β mRNA must originate from the nucleus to be a substrate of the deadenylation process. To verify the poly(A) status of the IFN-β transcript, we compared its migration in agarose gel to poly(A)+ IFN-β transcripts, containing (or not) the ARE after transfection into Hec-1B cells. The migration of the different transcripts confirmed that the poly(A)+ IFN-β mRNA bore a 100–150 nucleotides long poly(A) tail (Fig. 6C). Moreover, in order to verify the effective introduction of the synthetic mRNA into cells, IFN-β production was assayed in the culture medium after transfection. As shown in Fig. 6D (lane 1), transfection of polyadenylated mRNA led to IFN-β synthesis (Fig. 6D, lane 1) in contrast to the poly(A)− transcripts which were poorly translated (Fig. 6D lanes 2 and 3). Similar results were obtained when cells were infected by the Sendai virus before RNA transfection (data not shown).

Discussion

In the present study, we analysed the expression and the poly(A) status of human IFN-β mRNA in human endothelial Hec-1B cells in response to infection by the Sendai virus. As observed in other cell types, IFN-β synthesis is transiently induced and results from a strong accumulation of IFN-β mRNA that rapidly disappears at later times of infection [12,21]. We showed that the disappearance of IFN-β mRNA is accompanied by the shortening of its poly(A) tail. As described for certain class II ARE-containing mRNAs (e.g., GM-CSF, IL-3) [22,23], IFN-β mRNA is deadenylated asynchronously with the formation of poly(A)− intermediates. However, IFN-β mRNA deadenylation is not solely under the control of the ARE. Indeed, poly(A)− shortening is abolished upon deletion of both the ARE and the CRID (Fig. 3). The CRID was identified previously as an instability determinant that, in combination with the 3′UTR, mediates the rapid decay of human IFN-β mRNA in NDV-infected NIH/3T3 cells [13]. Both sequences were shown by UV-crosslinking experiments to recruit a cytosolic 65-kDa protein of unknown identity.
These and our observations suggest that the binding of this 65-kDa protein to one of these elements might be required to induce IFN-β mRNA deadenylation and subsequent degradation of the RNA body.

c-fos, c-myc and plasminogen activator inhibitor (PAI-2) messenger RNAs are other ARE-containing mRNAs bearing instability determinants in their coding region [24–27]. Moreover, in the case of c-fos, it was shown that ARE mediates mRNA deadenylation by a translation-independent mechanism, while the coding region instability determinant facilitates mRNA deadenylation by a mechanism coupled to translation [25,28]. Here, we show that IFN-β mRNA deadenylation occurs independently of the translational status of the mRNA. This observation correlates with the fact that IFN-β mRNA destabilization at later times of infection occurs even when mRNA translation is abrogated by the insertion of a stop codon immediately after the initiation codon [13]. It remains to be established, however, whether any of the two elements taken separately is translation-dependent in promoting mRNA deadenylation.

IFN-β mRNA deadenylation is a constitutive mechanism. Indeed, the IFN-β transcript derived from a constitutively transcribed gene cassette undergoes deadenylation in absence of viral infection. However, poly(A) shortening is detectable only after addition of actinomycin D that blocks the accumulation of newly synthesized polyadenylated IFN-β mRNA (Fig. 5). This observation suggests that the deadenylation machinery is pre-existing in the cells and deadenylates IFN-β mRNA as soon as its synthesis is induced by stimulating agents. The fact that the 65-kDa protein binds ARE and CRID in UV-crosslinking experiments, performed with cytosolic extracts from both non-infected and infected cells [13], further supports the involvement of this protein in the deadenylation process of IFN-β mRNA. Interestingly, IFN-β ARE does not recruit other ARE-binding factors (data not shown), thereby emphasizing the role of this 65-kDa RNA-binding protein [13] whose identity and function remain to be investigated.

IFN-β mRNA deadenylation seems to be conditioned by a nuclear event. Indeed, a synthetic IFN-β mRNA bearing a 100 nucleotide poly(A) tail escapes the deadenylation process when transfected in HeLa cells (Fig. 6). The nondeadenylation of this synthetic transcript does not, however, protect it from rapid decay, thereby suggesting that it becomes a target of an alternative poly(A)-independent degradative pathway. Although the nuclear event conditioning IFN-β mRNA deadenylation remains to be established, we provide the first evidence indicating such requirement for this mRNA degradative process. It seems however, possible that such a nuclear event might also be required for other mRNAs undergoing specific deadenylation. Indeed, most RNA-binding proteins mediating mRNA deadenylation/degradation shuttle between the nucleus and the cytoplasm [29]. The association of specific transcripts with such factors in the nuclear compartment might thus condition their cytoplasmic fate.

Altogether, our results and previous observations [12,13,30] demonstrate clearly that IFN-β mRNA behaves similarly to class II ARE-containing mRNA prototypes (e.g., GM-CSF, IL-3). However, the deadenylation and degradation of IFN-β mRNA is under the control of two independent elements, one of which is located in the mRNA coding region.

The coexistence of two independent but apparently redundant instability determinants might reflect the need for stringent control of IFN-β gene, whose prolonged expression might be detrimental to the organism.

Acknowledgements

This work was funded by the EC contract (QLK3-2000-00721), the Fund for Medical Scientific Research (Belgium, grant 3.4618.01), and the ‘Actions de Recherches Concertées’ (grant 00-05/250).

References


