Kinetics of CrPV and HCV IRES-mediated eukaryotic translation using single-molecule fluorescence microscopy

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ABSTRACT

Protein synthesis is a complex multistep process involving many factors that need to interact in a coordinated manner to properly translate the messenger RNA. As translating ribosomes cannot be synchronized over many elongation cycles, single-molecule studies have been introduced to bring a deeper understanding of prokaryotic translation dynamics. Extending this approach to eukaryotic translation is very appealing, but initiation and specific labeling of the ribosomes are much more complicated. Here, we use a noncanonical translation initiation based on internal ribosome entry sites (IRES), and we monitor the passage of individual, unmodified mammalian ribosomes at specific fluorescent milestones along mRNA. We explore initiation by two types of IRES, the intergenic IRES of cricket paralysis virus (CrPV) and the hepatitis C (HCV) IRES, and show that they both strongly limit the rate of the first elongation steps compared to the following ones, suggesting that those first elongation cycles do not correspond to a canonical elongation. This new system opens the possibility of studying both IRES-mediated initiation and elongation kinetics of eukaryotic translation and will undoubtedly be a valuable tool to investigate the role of translation machinery modifications in human diseases.

Keywords: eukaryotic translation; IRES; RNA; single molecule

INTRODUCTION

During translation, the ribosome decodes the mRNA sequence in discrete codon steps; tRNAs move from the A- and P-sites to the P- and E-sites, which are universally conserved in all kingdoms of life (Valle et al. 2003). Although the ribosome inner structure is well conserved, eukaryotic translation remains more complicated. The structures of the 80S eukaryotic ribosome, and of its small (40S) and large (60S) subunits, have been elucidated only very recently and show extended RNA segments together with several eukaryote-specific proteins (Ben-Shem et al. 2011). It is thought that these changes compared to prokaryotic ribosomes are for regulatory purposes to interconnect the ribosomes with several biological pathways (Klinge et al. 2012).

The dynamics of translation are difficult to study using traditional biochemical and biophysical tools, because it is a highly stochastic process, involving multiple steps and factors. Single-molecule techniques circumvent the need to synchronize the process and have become more and more popular over the last 10 years to study prokaryotic translation kinetics (Cornish et al. 2008; Marshall et al. 2008; Katranidis et al. 2009; Chen et al. 2013, 2014; Rosenblum et al. 2013). Many of them involve specific labeling of the prokaryotic ribosome, which has been achieved thanks to the knowledge of its atomic structure (Yusupov et al. 2001). Recently, Puglisi’s group reported the labeling of eukaryotic ribosomes, either by the hybridization of labeled oligonucleotides (Petrov and Puglisi 2010; Fuchs et al. 2015) or by the labeling of eS25 protein (Petrov and Puglisi 2010; Fuchs et al. 2015), without affecting ribosome assembly capacity. However, by fluorescently labeling the ribosome, the tRNA substrates or the elongation factors may modify their behavior in an unpredictable way. Furthermore, most single-molecule fluorescence techniques are limited to a very low concentration of labels in a solution (a few 10 nM typ.), which can also dramatically modify the kinetics of the process (Uemura et al. 2010). Although several clever approaches have been developed to study eukaryotic translation by single-molecule FRET (Ferguson et al. 2015), all of them rely on modifying...
either the ribosome itself or translational factors like tRNA to attach a fluorescent dye. An approach with unmodified translational components is of great interest to analyze eukaryotic translation dynamics, as shown by the recent in vivo quantification of translational speed (Morisaki et al. 2016; Murray et al. 2016; Wang et al. 2016; Wu et al. 2016).

Here, we report on eukaryotic translation kinetics starting from an internal ribosome entry site (IRES)-mediated initiation and ongoing over several elongation cycles using a single-molecule total internal reflection fluorescence microscopy (sm-TIRFM) with unmodified mammalian translation machinery, ensuring that labeling will not denature translation. Only the mRNA is labeled through the annealing of short fluorescent RNA probes (see Fig. 1A), which can be detached by the ribosome because of its helicase activity, as shown previously (Takyar et al. 2005).

Translation initiation usually requires the correct positioning of the aminoacyl-tRNA in the ribosomal P-site. In eukaryotes, this is achieved by a large number of initiation factors, allowing the 40S together with the ternary complex (eIF2-tRNA–GTP) to scan the 5’ part of the mRNA until the initiator tRNA is correctly base-paired with the start codon in the P-site. Viruses have developed numerous translational strategies to express viral proteins or to divert host translation machinery for their own benefit (Firth and Brierley 2012). Among them, internal initiation is commonly used to bypass the requirement of the eIF4 complex (Thompson 2012; Wang et al. 2013). IRES are grouped into four categories that differ with respect to their secondary structure, the translation initiation factors they require, and whether or not they recruit the ribosome upstream of the initiation codon or directly at it.

To initiate translation, we first use the intergenic region of cricket paralysis virus IRES (hereafter simply referred to as CrPV IRES). This is a type IV IRES, enabling the ribosome to translate its mRNA independently of any cellular initiation factors (Wilson et al. 2000). A large number of genetic, biochemical, and structural studies have contributed to characterize this IRES (Wilson et al. 2000; Deniz et al. 2009; Fernandez et al. 2014). Since the first description of the CrPV IRES, it has been thought that it would enable binding of the ribosome directly in an active state by mimicking mRNA to bypass the requirement of the eIF4 complex (Thompson 2012; Wang et al. 2013). IRES are grouped into four categories that differ with respect to their secondary structure, the translation initiation factors they require, and whether or not they recruit the ribosome upstream of the initiation codon or directly at it.

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Although the dynamics of the 40S and 80S subunits’ recruitment to the CrPV-IRES has been recently described (Petrov et al. 2016), how the very first cycles of kinetics compare to the subsequent elongation cycles, especially in a homogeneous system, remains unknown. Using a single-molecule strategy, we were able to quantify the elongation rate of an unmodified single mammalian ribosome and calculate the kinetics of translation during several elongation steps. This
work confirms that the first one, or possibly two, translocation steps are unusual and inefficient in the presence of the CrPV-IRES, with a characteristic time almost 30 times longer than for a classical elongation cycle.

To investigate the applicability of this method to any mRNA with an IRES, we also performed experiments with the hepatitis C virus (HCV) IRES. We show that this IRES also slows down the first cycle. This methodology opens the possibility of studying the behavior of different IRES as well as the kinetics of eukaryotic translation in conditions where changes in translation are suspected to be responsible for genetic diseases or cancers (Stumpf and Ruggero 2011; Reschke et al. 2013).

**RESULTS**

An mRNA was labeled with two small RNA fluorescent probes along its coding sequence and to a biotinylated one at its 5’ end. Depending on the experiments, a single 80S ribosome, purified from RRL, was bound to this mRNA or not. The mRNA was then attached to a coverslip through its 5′ end (Fig. 1A). This coverslip formed the bottom part of a microfluidic chamber. Translation started when total rabbit reticuloctye lysate (RRL), which contains all the factors needed to perform translation, was injected into the chamber. Translation kinetics data were obtained by measuring the departure times out of the evanescent wave for each probe. The ribosomal mRNA entry channel can only admit a single-strand RNA (Yusupova et al. 2001), so the ribosome must unfold mRNA secondary structures (Takyar et al. 2005; Qu et al. 2011) to continue translation until it reaches the stop codon. Small RNA probes have been previously used to map this helicase activity (Takyar et al. 2005). We selected two probes linked to two spectrally distinct fluorophores (ATTO647N for R-primers and ATTO565 for G-primers, see an example in Fig. 1B). Based on the work by Takyar et al. (2005) and the fact that canonical ribosomal footprints from eukaryotes and prokaryotes are both 28 nt in length, we assume that eukaryotic helicase activity is located at the same position as for prokaryotic ribosomes (Martens et al. 2015). We conclude that five elongation cycles are needed to fully detach the UP-(+5)-primer, as shown in Figure 1B. This system allows us to synchronize the departure of the downstream probe (DOWN-primer) using the upstream probe (UP-primer) departure as a reference.

**The mRNA labeling is sequence-specific**

Both primers efficiently bind to the same mRNA, as shown in Figure 1C. We were able to dual label ~80% of the mRNA from pCH2 or pCH4 with R-UP-(+5) and G-DOWN-(+14) in our annealing conditions. To further demonstrate the absence of primers mispairing, we created mRNAs with mutations on either the UP or the DOWN primer binding sites, called MUTUP and MUTDOWN mRNAs hereafter, respectively. The mRNAs should then not be able to attach to either the UP or the DOWN labeled primers. We then performed the hybridization with the UP and DOWN primers, with the rest of the experiment in the same conditions as with the nonmutated mRNAs. In our single-molecule experiment, as expected, we observed 0.1%–1% spots in the color of the primer corresponding to the mutated site compared to the number of spots in the other color, where the mRNA region was not mutated (Fig. 1D). These very few remaining spots correspond most likely to inhomogeneities in the background fluorescence from the coverslip or its surface chemistry used to attach the mRNAs. Furthermore, we observe almost no colocalized spots: 0% green spots when the red primer site is mutated, and 2% when the green primer site is mutated, due to more background noise in the green channel. This demonstrates that primers specifically bind their targets without mispairing.

**The CrPV-IRES supports ribosome binding in the presence of the labeled primers**

One major difficulty to set up such a system was the reconstruction of the translation initiation step in vitro. This step involves more than 40 factors in eukaryotes, several of them being modified post-translationally (Aitken and Lorsch 2012). To circumvent this difficulty, we first used the CrPV IRES intergenic region to initiate translation. This IRES, which enables binding of the 80S ribosome in the absence of initiation factors and initiator tRNA, has been very well characterized, both functionally and structurally (Jan and Sarnow 2002; Pestova et al. 2004; Spahn et al. 2004; Petrov et al. 2016). We first demonstrated that despite the binding of the UP-(+5)-primer close to the ribosome assembly region on the mRNA, CrPV IRES was still able to specifically recruit the 40S subunit (or possibly directly the 80S ribosome) from puriﬁed ribosomes (Fig. 2; Petrov et al. 2016). This demonstrated that CrPV-IRES supports ribosome binding in the presence of primers positioned as in our single-molecule experiments.

**The ribosome efficiently translates mRNAs with CrPV IRES in the microfluidic chamber**

We first used mRNAs with CrPV-IRES, preincubated with 80S ribosomes. We observed the individual initiation complexes using a home-built, objective-based, two-color total-internal reflection fluorescence microscope, allowing localization of dual labeled mRNAs in real time during translation. We first plotted the total number $N$ of remaining R-UP-(+5)-primers versus time, normalized by the initial number of R-UP-(+5)-primers $N_0$ (see Fig. 3A) [same for G-DOWN-(+14)-primer, data not shown]. The total number of analyzed data points is given in Table 1. In order to check that the observed departure phenomenon was not a pure photobleaching effect, well known for organic dyes, we recorded the same movie on a different area of the sample, 30 min after...
During injection and to the salinity difference when injecting RRL, although we reduced it by optimizing the salt concentration of the initial buffer. In any case, the departure rate of the R-UP-(+5)-primer is clearly significantly higher with IRES and no cycloheximide (Fig. 3A, red curve), and we conclude that this increase in the departure rate is due to translating ribosomes.

mRNAs with HCV IRES are also efficiently translated

To further confirm these results, we performed the same experiment using an mRNA carrying the HCV IRES with and without a stop codon before the R-DOWN fluorescent primer (Fig. 3D). The presence of the stop codon prevents the departure of R-DOWN primers, to the same extent as the absence of the IRES. These tests performed with a different mRNA demonstrate that translation is responsible for primer departure.

FIGURE 2. Specific binding of the ribosome to the IRES. For each gel shift assay, concentrations of R-(UP)+5-primers, mRNA, and ribosomal subunits (40S and 60S) are indicated. Mobility shifts were visualized using the primer fluorescence, as indicated in Materials and Methods. (A) Increasing the concentration of ribosomal subunits induces a gel shift only in the presence of mRNA. The 30 nM concentration of ribosomal subunits is selected for further experiments. (B) Increasing the concentration of a nonspecific RNA competitor (tRNA) does not change the gel shift. (C) Increasing the concentration of a specific but nonfluorescent RNA competitor (CrPV IRES) reduces the binding of the ribosomal subunits to the mRNA. Images have been obtained by fluorescence scanning with a 633 nm laser and a 670BP30 filter (Typhoon, GE Healthcare). Contrast and brightness have been modified if necessary using Photoshop CS6. These changes have been applied equally across the entire images.

RRL injection, i.e., long after translation was over. The photobleaching curve \(N^{\text{obl}}(t)\) (Fig. 3A, gray curve) decreases to only 79 ± 6% after 50 sec, which is much higher than the 49 ± 7% observed in the translation test, \(N^{\text{exp}}(t)\) (Fig. 3A, red curve). This indicates that most departures in the first 50 sec after the RRL injection cannot be attributed to photobleaching.

Other biological activities, such as RNase, could also explain fluorophore departures. However, the fact that there is no mRNA degradation is demonstrated by the observation of a nonilluminated area after RRL injection in the presence of an mRNA with no IRES. As there was no laser excitation, photobleaching cannot account for fluorophore departure in this area and the absence of IRES prevents initiation from occurring. We observed many spots in the nonilluminated area, the same as before the injection of the RRL. This indicates that there was no mRNA degradation during the experiment.

To identify the departures due to translation, we performed two additional control experiments. First, we preincubated RRL with cycloheximide. This antibiotic binds to the ribosomal E-site and blocks translation after two cycles (Pestova and Hellen 2003). Adding cycloheximide clearly slowed down fluorophore departures but did not prevent them completely (Fig. 3A, blue curve). In a second control experiment, we used an mRNA without the IRES sequence (\(\Delta\)IRES mRNA) to prevent translation initiation. The corresponding curve \(N^{\Delta\text{IRES}}(t)\) (Fig. 3A, purple curve) is very close to the cycloheximide control, indicating that indeed in both situations translation is inhibited. We believe that the few remaining departures in both controls were due to the flow during injection and to the salinity difference when injecting the RRL, although we reduced it by optimizing the salt concentration of the initial buffer. In any case, the departure rate of the R-UP-(+5)-primer is clearly significantly higher with IRES and no cycloheximide (Fig. 3A, red curve), and we conclude that this increase in the departure rate is due to translating ribosomes.

**Determination of the ribosome speed during elongation**

We then focused on the R-UP-(+5) and G-DOWN-(+14)-primers, hybridized on a CrPV-IRES mRNA, that were colocalized on the first red and green images. We calculated the histograms \(H^{\text{exp}}\) and \(H^{\Delta\text{IRES}}\) of the departures of both primers as a function of the difference \(t_{\text{DOWN}} - t_{\text{UP}}\), with and without the IRES sequence. The times \(t_{\text{UP}}\) and \(t_{\text{DOWN}}\) correspond to the departures of R-UP-(+5) and G-DOWN-(+14) from a given mRNA strand. The histograms \(H^{\text{exp}}\) and \(H^{\Delta\text{IRES}}\) were normalized by the initial number of colocalized spots. The difference \(H^{\Delta\text{trans}} = H^{\text{exp}} - H^{\Delta\text{IRES}}\) is plotted in Figure 3B, and a Gaussian fit shows a maximum at 12.5 ± 2.1 sec. Thus, on average, G-DOWN-(+14) departs from a given mRNA strand 12.5 sec after R-UP-(+5). Because the lifetime before photobleaching of the red fluorophore is three times larger than that of the green one, departures due to photobleaching would, on average, occur in the inverse order. Thus, the fact that this histogram is centered at \(t_{\text{DOWN}} > t_{\text{UP}}\) is yet another confirmation that we observed translation. As nine elongation cycles are needed between the two fluorophore departures, we conclude that one elongation cycle takes an average of 1.4 ± 0.2 sec.

**CrPV IRES-bound ribosomes are responsible for the translation events observed in single-molecule experiments**

Because the cell extract used for the single-molecule experiments contains ribosomes, we performed control

Eukaryotic translation at the single-molecule level
experiments without preincubating purified 80S ribosomes on CrPV IRES mRNAs to quantify the contribution of free RRL ribosomes to the signal observed in the experiments. In both cases, the same mRNA was used. We analyzed the departure times for each primer. Figure 3C shows the derivatives $D^{\text{trans}}(t) = d[N^{	ext{UP}}(t)/N^{	ext{IRES}}(t)]$, which represent the probability for the UP-(+5) probe to disappear at time $t$ in two cases: with preincubated purified ribosomes (red curve) or without (black curve). The area under each curve in Figure 3C corresponds to the fraction of the probe carrying mRNAs that were translated. This translation efficiency is much larger (50%) in the experiment with preincubation of the 80S than without (10%). This indicates that hardly 10% of the mRNAs were translated without preincubation of the ribosomes. So free ribosomes are not efficient to initiate translation at the CrPV IRES.

Ribosomes present in RRL efficiently translate HCV-IRES mRNAs

To confirm that the type of CrPV-IRES prevents efficient initiation of the ribosome in RRL, we used HCV-IRES. This IRES is a type III IRES that only requires eIF3 and eIF2 as initiation factors and has been shown to be very efficient in RRL (Borman et al. 1995). Using this IRES, we show that translation efficiency is 30% without ribosome preincubation (Fig. 3E).

Both HCV and CrPV IRES poise the first translational steps

The primer departure due to translation occurs when the ribosome has performed $n$ elongation cycles [$n = 5$ for R-UP-(+5)]. If all the elongation cycles were identical, the fitting function for $D^{\text{trans}}(t)$ would be a $\gamma$ law $p(t) = (t^{-\alpha} \exp(-t/t_{\alpha}))/((t_{\alpha}(n - 1)!)$ with $t_{\alpha}$ the characteristic time for one elongation cycle. However, this function,
for \( n = 5 \), does not fit our experimental data, indicating that one time parameter is not sufficient to describe the dynamics observed. We considered that the ribosome translation is well described by a rapid phase corresponding to the first part of the \( D_{\text{trans}}(t) \) curve, and a slow one, corresponding to the tail of \( D_{\text{trans}}(t) \). The simplest fitting function with two time parameters that could describe these data is the convolution of one slow event happening with an exponential probability of characteristic duration \( t_0 \), and a rapid phase described by an exponential probability of characteristic duration \( t_1 \):

\[
D_{\text{fit}}(t) = \frac{A}{t_0 - t_1} (e^{-t/t_0} - e^{-t/t_1}),
\]

where \( A \) corresponds to the fraction of translated mRNAs.

For CrPV IRES, we performed the same fitting procedure to the \( D_{\text{trans}}(t) \) curves for different primers, hybridized at different locations on the mRNA, and included a bootstrapping process to evaluate the mean value and the error bars on the fitting parameters \( A, t_0 \) and \( t_1 \). In addition to the R-UP-(+5) and G-DOWN-(+14) primers, we tested an mRNA from pCH5 with nine additional nucleotides upstream of the R-UP-primer hybridization site. In this situation, the ribosome must translate eight codons (instead of five previously) before the R-UP-(+8)-primer departure (same R-UP-primer). The results of all the fits are summarized in Table 2. \( t_0 \) describes a slow phase lasting typically 40 sec, independently of the primer. \( t_1 \) corresponds to the fast phase. Within the error bars, its value is proportional to \( n - 1 \) or \( n - 2 \). Furthermore, the elongation time \( t_{\text{el}} \) obtained by dividing \( t_1 \) by \( n - 1 \) or \( n - 2 \) is consistent with the elongation time of 1.4 ± 0.2 sec previously measured between the R-UP-(+5) and G-DOWN-(+14)-primer departures. To test whether melting the double strand formed by the primer and the mRNA slowed down the ribosome, we added a third fitting time parameter and found it to be consistent with zero within our error bars. We conclude that unzipping the primer has a negligible effect on our measured elongation time. The fact that the time \( t_0 \) for the slow phase is independent of the primer location, and that it is longer than the time interval between the two primer departures, proves that it is not due to the unzipping of the RNA probes but corresponds to an event that occurs only between the translation starting

**TABLE 1.** Values of the initial number \( N_0 \) of primers for each experiment

| Experiment | CrPV IRES |
|------------|-----------|
| R-UP-(+5)  | 7112/3627 |
| G-DOWN-(+14)| 1754/930 |
| Colocalized primers | 1494/651* |
| R-UP-(+8)  | 5756/3511 |
| ΔIRES mRNA control | 6991/1888 |
| Cycloheximide control | 2924/877 |
| Photobleaching | 1486/312 |

**HCV IRES**

| Experiment | CrPV IRES |
|------------|-----------|
| R-DOWN-(+24) | 4111/1398 |
| ΔIRES mRNA control | 4303/473 |
| mRNA with STOP control | 8182/1309 |

The value indicated in italics is the total number of departures after 50 sec, except for the colocalized primers (*) where it is given at the end of the experiment (after 250 sec).

**TABLE 2.** Summary of the results obtained from the fits

| Experiment | CrPV IRES |
|------------|-----------|
| A (%)      | 51 ± 1    |
| \( t_0 \) (sec) | 38 ± 3 |
| \( t_1 \) (sec) | 4.2 ± 0.8 |
| Hyp. 1 : \( t_{\text{el}} = t_1/(n - 1) \) (sec) | 1.1 ± 0.2 |
| Hyp. 2 : \( t_{\text{el}} = t_1/(n - 2) \) (sec) | 1.4 ± 0.3 |
| \( t_{\text{el}} \) (sec) from histogram | 1.4 ± 0.2 |

| HCV IRES |
| R-DOWN-(+24) |
| A (%)      | 30 ± 1    |
| \( t_0 \) (sec) | 29 ± 4 |
| \( t_1 \) (sec) | 11.6 ± 4.6 |
| \( t_{\text{el}} = t_1/(n - 1) \) (sec) | 0.5 ± 0.2 |
point and the UP-primer departure. Using the same fitting procedure for HCV IRES, we obtained $t_0 = 29 \pm 4$ sec, indicating that both IRES-mediated translations display the same kinetics scheme (compare Fig. 3C,E).

**DISCUSSION**

Our approach allows quantification of elongation speed in two ways. Purified and preincubated ribosomes translate at a rate of $1.4 \pm 0.2$ sec per codon. This value is slightly slower than the one obtained by the ribosome profiling approach (0.2 sec per codon) (Ingolia et al. 2012). However, with non-purified ribosomes, the translation rate increases threefold ($0.5 \pm 0.2$ sec per codon), which is in good agreement with the kinetics already obtained in vivo (Morisaki et al. 2016; Wang et al. 2016; Wu et al. 2016; Yan et al. 2016). Although the kinetics of initiation has recently been addressed in a heterogeneous cell-free system (Zhang et al. 2016), the consequences of the IRES-dependent initiation for the elongation cycles have never been evaluated in an unmodified translation system. Interestingly, comparison between both systems gives different results. Zhang and coworkers observed four slow translocation cycles, whereas our data fit with only two slow translocations that could correspond to the slowest cycles described by Zhang and coworkers. (see Table 2). One may suspect that heterogeneity in the translation system leads to kinetics issues. Indeed, they describe four very slow steps (between 80 and 200 sec for one translocation) and even the faster cycles are at least 30-fold slower than the translocation speed obtained in vivo ($0.1$ translocation/sec versus three translocations/sec, respectively). Such values cannot represent physiological speed of translation and reflect problems due to the heterogeneity of their system (shrimp ribosomes with yeast elongation factors in the presence of E. coli tRNA). Actually, it is true that CrPV IRES is well known to support translation initiation using various ribosomes (yeast, human, shrimp) (Thompson et al. 2001; Spahn et al. 2004); however, this does not imply that initiation kinetics is conserved in all these artificial systems. Using the CrPV IRES, we measured the speed of eukaryotic ribosomes and were able to identify two characteristic times: a very long time (40 sec) and a short time (1.4 sec) for all remaining translocations. This translocation time is in good agreement with recent in vivo experiments that indicated a ribosome translocation rate around $3 \pm 1$ amino acids per second (Morisaki et al. 2016; Wang et al. 2016; Wu et al. 2016; Yan et al. 2016).

Our data support the model proposed by Ramakrishnan’s team (Fernandez et al. 2014; Murray et al. 2016). They observed, by high-resolution cryoEM, the exact position of the intergenic CrPV IRES in interaction with the ribosome and showed that this IRES enables the ribosome to assemble one codon upstream than what was previously anticipated. Moreover, they showed that eEF2 is able to translocate the PKI part of the IRES (mimicking the tRNA in the A-site) from the A- to the P-site, allowing the A-site to accept the next cognate tRNA. Our results indicate that the translocation of PKI from the A- to the P-site is a rate-limiting event (almost 30 times longer than a canonical elongation cycle). One may anticipate that this translocation is very unusual, because the ribosome has to deal with a very large secondary structure. Once this first step has occurred, the second elongation cycle takes place with a tRNA in the A-site and the IRES bound between P- and E-sites. This second elongation cycle is probably also kinetically slow (see Table 2); however, our data indicate that once these translocations are over, all further translocations occur at the same speed. This suggests that during the next translocation step, IRES is no longer a limiting factor. Owing to time resolution limits, we cannot exclude that with PKI in the P-site and a cognate tRNA in the A-site, the translocation occurs at a normal speed due to a quick release of the PKI from the ribosome (Fig. 4).

It has been shown that HCV IRES assembles an 80S ribosome that is poised at the initiator codon (Filbin et al. 2013). This takes place through the interaction of part of the IRES with the β-hairpin of the ribosomal protein uS7 and with the N-terminal helix-turn-helix motif of eS25 (Quade et al. 2015). Both IRES share common features; in particular, they both interact with uS7 as described by cryo-EM studies (Spahn et al. 2004; Boehringer et al. 2005; Filbin et al. 2013). However, unlike CrPV, the HCV IRES requires several initiation factors (Lukvavsky 2009). A recent cryoEM study revealed that the swine fever virus IRES (similar to HCV IRES) displaces the core of eIF3 from its position on the 40S subunit (Hashem et al. 2013). This binding would reduce eIF3 availability to form a canonical 43S preinitiation complex. From these data, it seems clear that both IRESs induce similar conformational changes in the ribosome despite utilizing distinct mechanisms for translation initiation. In both cases, IRES represent a hindrance to ribosome movement and explain our observation that both CrPV and HCV IRES strongly slow down ribosome translocation during the first elongation cycles. One may postulate this is a general feature of IRES initiation.

More generally, our results demonstrate that the system we have developed is well suited to study translation kinetics at the single-molecule level during multiple elongation cycles. It can be applied to numerous translational questions. For example, it is now well established that the translation machinery is a target for deregulation in diseases such as cancer (Stumpf and Ruggiero 2011; Marcel et al. 2013). The ability to quantify translation kinetics in such environments will provide great improvements in the understanding of how the translational machinery is involved in these pathologies.

**MATERIALS AND METHODS**

**Plasmids and oligonucleotides**

pCH1 contains the CrPV IGR IRES (coordinates 6028–6219 from the viral mRNA) downstream from the SP6 promoter as well as a
region where the chimeric biotinylated DNA/2′ O-allyl-modified RNA oligonucleotide (Namy et al. 2006) binds upstream of an HpaI site. The plasmid pCH2 is identical to pCH1 except that in pCH2, the coding sequence of the PB1 gene (from pPS0 plasmid) (Somogyi et al. 1993) without stop codon was inserted into the HpaI site. This allowed us to extend the initial coding sequence to make the polypeptide visible in gel during in vitro translation assay. The pCH3 plasmid also is derived from pCH1 with the insertion of the sequence TCTACTGCTGAACTCGCT at the HpaI site to insert the UP-primer binding site. The DOWN-primer site has been chosen to have a melting temperature compatible with the UP-primer. The plasmid pCH4 is derived from pCH3 with a deletion of the IRES named ΔIRES in the manuscript. The plasmid pCH5 is derived from pCH3 with three additional codons inserted upstream of the UP-primer to increase the distance between the IRES and the UP-primer. pHCV is a pUC19 derivative plasmid containing the HCV-type 1b IRES followed by the first six codons of the core protein coding sequence (NCBI accession number AJ238799 from nucleotide 1 to 360). All other features are identical to pCH1.

RNA oligonucleotides coupled to an ATTO fluorophore were synthesized by Eurogentec. The UP-primer (5′-AAAGAGUUCA GCAGU-3′) is identical to the oligonucleotide H12 from Takyar et al. 2005, except for the 3 A at the 5′ end used to space the fluorescent label from the hybridized region. The label is either ATTO647N or ATTO565, excited in the red and in the green part of the spectrum, respectively. The associated nomenclature is thus R-UP-primer and G-UP-primer, respectively.

Purification of ribosomal subunits

Ribosomal subunits were prepared from nuclease-treated rabbit reticulocyte lysate (RRL; Promega). One milliliter of RRL was mixed with 3 mL buffer A [20 mM Hepes, pH 7.8, 150 mM K(OAc), 6 mM Mg(OAc)2, and 2 mM dithiothreitol (DTT)] and incubated with 1 mM puromycin (Sigma-Aldrich) for 10 min at 4°C and then for 10 min at 37°C. Ribosomal subunits were isolated by the centrifugation of this suspension through a 3 mL 20% sucrose cushion in buffer A for 4 h at 4°C and 44,000 rpm, using a Beckman TLA110 rotor. Pellets were resuspended in buffer A to a concentration of 12 A260 U/mL.

Nondenaturing gel mobility shifts

Conditions for native gel shift assays are as follows: pCH2-derivated mRNA (500 nM) was incubated with 100 nM R-UP-primer in a 10 µL reaction in buffer B (20 mM Tris pH 7.8, 5 mM MgCl2, 50 mM DTT, and 0.25 mM ATP) for 5 min at 68°C, 5 min at 37°C, and 5 min in ice. mRNA/oligonucleotide complexes were diluted 20 times in buffer C [20 mM Hepes, pH 7.8, 100 mM K(OAc), 2.5 mM Mg(NO3)2, 2 mM DTT, and 0.25 mM spermidine]. This complex (5 nM) is incubated in buffer C with an increasing concentration of ribosomal subunits, from 0 to 40 nM, at 37°C for 10 min. For controls, the mRNA/oligonucleotide complex (5 nM) was incubated with 30 nM ribosomal subunits and with an increasing concentration of either competitor or noncompetitor RNA from 0 to 100 nM. Competitor RNA only contains the CrPV IRES sequence. Yeast tRNA (Sigma-Aldrich) is used as a noncompetitor RNA. Gel shifts were performed in 0.8% (w/v) agarose gels in THM buffer (66 mM Heps, 34 mM Tris, 2.5 mM MgCl2, final pH 7.5) at room temperature. Gel-mobility shifts were analyzed by fluorescence scanning with a 633 nm laser and a 670BP30 filter (Typhoon, GE Healthcare).

In vitro transcription

Plasmids were linearized with the appropriate restriction enzyme (KpnI or XhoI) and transcribed in vitro with SP6 RNA polymerase (Promega). RNA is purified using RNA-Spin columns (Roche).

Single-molecule experiments

A home-built objective-based TIRF microscope was used. ATTO-647N- and ATTO-565-labeled molecules were excited using a
640-nm laser diode or a 532-nm diode-pumped solid-state laser (both Oxxius). Fluorescence emission was collected by a 1.45 NA oil-immersion objective (PlanApo, Olympus) and imaged onto an electron-multiplying charged coupled device (EMCCD) camera (iXon+897 Andor) at 100- or 200-msec exposure. The excitation and detection of ATTO-647N and ATTO-565 fluorescence were alternated using home-built electronic. The two lasers were reflected on the same dichroic mirror (Chroma z532/633rpc) and a motorized filter wheel was used to choose the appropriate emission filter (Chroma HQ665lp and Semrock FF01-580/60, respectively). A laboratory-built microscope-based focus drift correction system was used to keep the focus during the whole experiment. Cellular extract delivery was achieved using a modified syringe pump (Harvard Apparatus) (dead time 2–3 sec). In RRL, for an exposure time of 200 msec every 5 sec, the lifetime before photobleaching of ATTO647N (resp. ATTO565) was $\tau_R = 85 \pm 16$ sec ($\tau_C = 38 \pm 6$ sec).

**In vitro translation on a surface**

The mRNA from pCH3, pCH4, pCH5, and pHCV plasmids (0.5 $\mu$M) were annealed to the chimeric biotinylated DNA2/ O-allyl-modified RNA oligonucleotide (0.5 $\mu$M) and 2 $\mu$M fluorescent-labeling oligonucleotides in a 1 $\mu$L reaction in 0.5 $\mu$L ligase buffer (NEB) for 5 min at 68°C, 5 min at 37°C, and 5 min at 4°C. The mRNA/oligonucleotide complexes were diluted 100 times in buffer E ($[20$ mM Hepes, pH 7.8, $100$ mM Na(OAc), $100$ mM K(OAc), $2.5$ mM Mg(OAc)${}_2$, $2$ mM DTT, and 0.25 mM spermidine]. The mRNA/primer complexes were further diluted in buffer E to 0.8 $\mu$L of amino acid–Cys mix provided) previously incubated at 30°C for 10 min. Control experiments were carried out in the presence of 355 $\mu$L cytochrome oxidase (Euromex) in each solution. When the RRL reached the excitation area, data acquisition was launched. Every 5 sec (unless otherwise stated), two images were acquired, first in the red channel and 500 msec later in the green one.

**Data analysis**

For each color, analysis was performed on individual spots, provided that they were diffraction limited and that they were present in the first two images and disappeared abruptly (after one frame) during the movie. Error bars on the primer departure probability curves $D(t)$ and on the fitting parameters were obtained by a bootstrapping procedure (Press et al. 1995). For each set of $N_o$ experimental data points, 1000 samples of $N_o$ bootstrapped points were computed. The same analysis procedure was performed on each bootstrapped sample: Each one was fitted, leading to the average fitting parameters and their standard deviations given in the text.

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