Nuclear export of single native mRNA molecules observed by light sheet fluorescence microscopy

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Nuclear export of mRNA is a key transport process in eukaryotic cells. To investigate it, we labeled native mRNP particles in living Chironomus tentans salivary gland cells with fluorescent hrp36, the hnRNP A1 homolog, and the nuclear envelope by fluorescent NTF2. Using light sheet microscopy, we traced single native mRNA particles across the nuclear envelope. The particles were observed to often probe nuclear pore complexes (NPC) at their nuclear face, and in only 25% of the cases yielded actual export. The complete export process took between 65 ms up to several seconds. A rate-limiting step was observed, which could be assigned to the nuclear basket of the pore and might correspond to a repositioning and unfolding of mRNPs before the actual translocation. Analysis of single fluorescent Dbp5 molecules, the RNA helicase essential for mRNA export, revealed that Dbp5 most often approached the cytoplasmic face of the NPC, and exhibited a binding duration of approximately 55 ms. Our results have allowed a refinement of the current models for mRNA export.

Dbp5 ATPase cycle | nucleocytoplasmic transport | single molecule microscopy | translocation time

Eukaryotic cells store most of their genetic information on nuclear chromosomes and translate it into proteins in the cytoplasm. Thus, scores of RNA-encoded blueprints must be transported from the nucleus into cytoplasm through the supramolecular nuclear pore complexes (NPCs), which are embedded in the nuclear envelope (NE). Messenger RNA (mRNA) export is considerably more complex than protein transport through NPCs due to its tight connection to nuclear mRNA processing (1, 2). Nascent mRNA is cotranscriptionally loaded with mRNA-binding proteins, which are either transient components of the resulting ribonucleoprotein particle (mRNP) or, like the Chironomus tentans hrp36 (hnRNP A1 in mammals), escort the mRNA to its cytoplasmic destination (3). The key task in mRNA export is the remodeling of the mRNP by the ATP-dependent RNA helicase Dbp5. This process takes place at the cytoplasmic interface of the NPC, and presumably goes along with unloading of transport receptors from the mRNA. After remodeling, the mRNA cannot reenter the NPC and is released into the cytoplasm (2, 4). The most detailed analyses of mRNA export were accomplished by electron microscopy (EM) using C. tentans salivary gland cells (5, 6). By following the large 32–40 kb Balbiani ring 1 and 2 (BR) mRNAs, which encode salivary polypeptides, Daneholt and coworkers visualized native mRNPs during their NPC translocation and obtained a detailed view on this highly ordered process. The pivotal advantage of this system is the direct observation of a completely unmodified endogenous mRNA. However, EM provided only a static view to this highly dynamic cellular process. In vivo analysis of mRNA export requires fluorescence microscopy, which implies the generation of fluorescent mRNPs in situ. In recent studies on mRNA trafficking, this was accomplished by expressing modified mRNAs carrying multiple binding sites for loading with numerous copies of GFP-fused MS2, a bacteriophage coat protein (refs. 7–10) or for molecular beacons (11). To avoid introducing any artificial transcript or modifying an endogenous mRNA, we adopted the C. tentans system for light microscopy. We succeeded in visualizing single native mRNPs labeled by fluorescent hrp36. Light sheet fluorescence microscopy provided the required image contrast for tracing single mRNPs during nuclear export in the large nuclei. In addition, we resolved the reaction kinetics of single Dbp5 molecules at the NPC.

Results

Labeling Native mRNPs In Vivo. During transcription, the growing pre-mRNA associates with heterogeneous nuclear ribonucleoproteins (hnRNPs) to form mRNPs. It is known for the BR mRNPs that some hnRNPs leave the particle during intranuclear trafficking (6), while others escort the mRNA through the NPC. Hrp84 and hrp36 even remain bound to the mRNA when the polyosomes start translation (3, 6). This stable association was also shown for the mammalian hrp36 homolog, hnRNP A1. Therefore, hrp36 constitutes a perfect probe for in vivo labeling of native endogenous mRNAs. C. tentans hrp36 (M, ∼32,000) comprises two RNA recognition motifs (RRM) and a C-terminal glycinerich domain (GRD; Fig. 1A). Previously, we demonstrated that microinjected fluorescently labeled recombinant hrp36 can be used to label and track single mRNPs in living salivary gland cell nuclei (12). After cytoplasmic microinjection the protein was rapidly translocated to the nucleus due to its C-terminal M9 transport signal, and became enriched at BR1 and 2 on chromosome IV (Fig. 1B, filled arrow heads) and other active transcription sites (arrows). To test whether the recombinant protein was stably incorporated in and not only transiently attached to mRNPs, we blocked transcription by either coinjection of actinomycin D or preincubation of the glands in hemolymph containing α-amanitin. Afterwards, the microinjected hrp36 was no longer seen at transcription sites (Fig. 1D and E), and we concluded that the recombinant labeled protein, just like the endogenous hrp36, was cotranscriptionally integrated into mRNPs. This fact was further verified by microinjection of a C-terminal deletion mutant, hrp36ΔGRD, lacking the GRD. The GRD is not directly involved in mRNA binding, but its deletion abrogates the hrp36-mRNA interaction of the Drosophila homolog in vivo (13). As expected, hrp36ΔGRD did not label any transcription sites (SI Appendix, Fig. S1 A–C). Moreover, the transportin-dependent import of the protein was abolished because the M9 signal was also located in the GRD. Vice versa, a GST-GRD fusion protein without RRM domains was transported to the nucleus after cytoplasmic microinjection displaying the characteristic shuttling between nucleus and cytoplasm, but could not bind RNA any more (SI Appendix, Fig. S1 D–F).

Author contributions: J.P.S. and U.K. designed research; J.P.S. and T.K. performed research; J.P.S. and T.K. contributed new reagents/analytic tools; J.P.S. and T.K. analyzed data; and J.P.S. and U.K. wrote the paper.

The authors declare no conflict of interest.

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to be performed in PBS. The FISH experiments revealed that transcription and export of BR1, BR2 or total poly(A) mRNA were only maintained if the glands were incubated and microscopically examined in hemolymph after dissection (SI Appendix, Fig. S4). In hemolymph transcription and export were functional for at least 60 min, but not in PBS. Thus, salivary glands were dissected and microinjected in PBS, but for microscopic transport measurements the sample chamber was filled with hemolymph to ensure that data were collected under physiological conditions.

**Single Molecule Analysis of mRNP Export.** Fluorescence imaging of single molecules with high speed and the precise determination of single molecule positions is critically dependent on high signal-to-noise ratios. To achieve optimal signal-to-noise ratios, we used light-sheet fluorescence microscopy (17, 18). A thin light sheet was generated by a cylinder-lens optics, which illuminated the sample from the side perpendicular to the detection pathway of an inverted microscope (SI Appendix, Fig. S5). Thus, fluorophores near the focal region of the observation objective were selectively excited, which minimized fluorescence background, photobleaching, and phototoxicity (19). Very low amounts of AlexaFluor647-labeled hrp36 molecules were injected into the cytoplasm of salivary gland cells imaged after 15 min incubation in hemolymph with α-amanitin to selectively block RNA polymerase II.

**Characterization of Wild-Type and Mutant Dbp5 In Vivo.** To resolve the binding kinetics of Dbp5 in comparison to mRNP export, we prepared fluorescence-labeled recombinant Dbp5. Microinjected Dbp5 was evenly distributed between cytoplasm and nucleus after microinjection, and strongly accumulated at the NE (Fig. 2 A and B). This characteristic localization was seen for endogenous Dbp5 in *C. tentans* (14), human (15), and yeast cells (16). Blocking transcription by actinomycin D in salivary gland cells abolished this localization, as shown previously for endogenous Dbp5 (14) (Fig. 2 C and D). Finally, we analyzed the subcellular distribution of Dbp5 point mutants defective in NUP214 binding (R252A, Fig. 2E) or both NUP214 and mRNA interaction (D216R; SI Appendix, Figs. S2 and S3). We found that the interaction of wild-type *C. tentans* Dbp5 with NUP214 was sufficient to achieve the characteristic NPC localization. We concluded that the recombinant Dbp5 bound specifically and could functionally replace endogenous Dbp5 at the NPC.

**FISH Analysis of Transcription and mRNP Export.** *C. tentans* salivary gland cells represent an elegant but intricate biological system. In FISH experiments using various hybridization probes, we identified the conditions for successful mRNP export experiments before we began with the actual light microscopic analysis of mRNP export (see SI Appendix). Salivary gland dissection had been performed according to ref. 20). In order to visualize the NE as the locus of mRNP export, we coinjected Alexa546-labeled NTF2 molecules. NTF2 is the transport receptor for RanGDP import and interacts with FG repeats of nucleoporins with high selectivity (21). NTF2- and hrp36-Alexa647 signals were sequentially acquired using exactly the same imaging path to minimize alignment problems in the dual color measurements. In movies that were acquired with an image integration time of 20 ms at...
50 Hz frame rate, single hrp36-labeled mRNPs could well be discerned in the salivary gland cell nucleoplasm, cytoplasm, and near or at the NE. Unbound hrp36 molecules could not be resolved with this time resolution because they diffused too fast. Numerous complete mRNP export events were observed in which hrp36-labeled mRNPs approached the NE from the nucleoplasm, bound to it, and left it on the cytoplasmic face (e.g., Fig. 3 A and Movie S1). In experiments aimed at the analysis of the interaction of single Dbp5 molecules with the NE, we proceeded in a similar manner using Alexa647-labeled Dbp5 molecules instead of hrp36. It was immediately obvious that Dbp5 showed exclusively short interactions with the NE (Fig. 3 B and Movie S2).

**Kymograph-Based Dwell Time Analysis.** To efficiently screen the data for export events and to quantify the duration of mRNA export and Dbp5-NPC interactions, we evaluated the movies by kymographs which displayed the Alexa647-fluorescence signal of the linearized curve along the NE as a function of time in x-direction. The fluorescence along the NE was plotted in y-direction (21). Example kymographs are shown for hrp36-labeled mRNPs (Fig. 3 C) and for wild-type Dbp5 (Fig. 3 D). The kymographs revealed the recording of an interaction with the NE in a whole movie at a single glance. Also, the duration of such interactions could directly be determined in intensity plots of respective image lines (Fig. 3 E and F). We recorded several thousand movies, and their evaluation in terms of kymographs revealed numerous observations of hrp36 and Dbp5 interactions with the NE.

**Analysis of hrp36 and Dbp5 Interaction with the NE.** For all events detected in the kymographs, we carefully inspected the corresponding raw images. In these we determined the respective time-resolved single molecule movement in relation to the NE position that showed the approach, binding, and release of hrp36-labeled mRNPs and Dbp5 molecules to and from the NE. All observations were sorted into categories by discriminating among all possible interaction types for particles moving near the NE (Fig. 4 A). Exemplary trajectories for all these types from the mRNP dataset are shown in SI Appendix, Fig. S6. We restricted the further analysis to the categories that comprised explicit NE approach, binding, and release. Trajectories of molecules approaching from nucleoplasm and hitting the NE but returning to the nucleus were designated as nuclear probing (np), and trajectories beginning and ending in the cytoplasm correspondingly as cytoplasmic probing (cp). The duration of the immediate membrane interaction was determined for all events in a category and plotted in histogram form. This procedure revealed that for Dbp5 cytoplasmic probing was prevailing (Fig. 4 B and C), whereas for hrp36-labeled mRNPs nuclear probing was observed most frequently ($N_{np} = 313$ and $N_{cp} = 184$) (Fig. 4 D and E).

![Fig. 3. Binding time analysis of mRNPs and Dbp5 at the NE.](image)

(A) Time series showing a single hrp36-labeled mRNP during its export from nucleus (n) to cytoplasm (c). The 1st and 3rd rows show raw images of hrp36 fluorescence; the 2nd and 4th rows show filtered data (red) and the NTF2-stained NPCs (green). Because the mRNP was observed at the NE for 230 frames ($4.6\ \text{s}$), only the first and last 10 frames were shown. The nuclear approach to and cytoplasmic departure from the NE were marked. Single image size, 4.5 $\mu\text{m}^2$. (B) A single Dbp5 molecule binds to the NE. The upper row shows raw data, and the lower row the corresponding filtered frames (red) and the NTF2-stained NPCs (green). The Dbp5 molecule hit the NE at two different spots dwelling for 1 and 3 frames, respectively. Image size 5.25 $\mu\text{m}^2$. (C) Kymograph of the mRNP export shown in (A). Hrp36-fluorescence along the NE was plotted vs. time ($19\ \text{s}$). When the mRNP left the NPC at the cytoplasmic face the signal showed a characteristic wobbling (see Movie S1). Only the membrane-bound phase (arrows) was considered for dwell time determination. (D) Kymograph of the membrane binding of Dbp5 from (B) (arrow). Fluorescence intensity along one row of the respective kymographs. (E) mRNP export shown in (A), (C), and (F). Dbp5 binding shown in (B) and (D). Fluorescence intensity was plotted in red, and the threshold in gray. The dwell time was determined as the time until the signal intensity fell below the threshold for three subsequent frames.

![Fig. 4. mRNP and Dbp5 binding time distributions.](image)

(A) Classification of NE binding events (np: nuclear probing; cp: cytoplasmic probing; exp: export; ne: nucleus to envelope; ec: envelope to cytoplasm; env: only envelope). Frequency histograms of (B) Dbp5 nuclear probing, (C) Dbp5 cytoplasmic probing, (D) nuclear probing of hrp36-labeled mRNPs, and (E) cytoplasmic probing of hrp36-labeled mRNPs.
Obviously, only mRNP trajectories starting in nucleoplasm, hitting the NE, and ending in cytoplasm were unambiguous nuclear export processes (exp). The interaction times were compiled in a histogram (Fig. 5A). In comparison to nuclear probing, the capture of complete export events succeeded less often. Nevertheless, the kymograph analysis made it possible to extract quite a number of export events ($N_{exp} = 121$). In order to determine the mean duration of mRNP translocation across the NPC, and also the mean interaction times of cytoplasmic and nuclear probing by mRNPs and Dbp5, respectively, we translated the above histograms into normed cumulative probability distributions. These distributions allowed us to directly quantify the interaction time constants of the examined molecules with the NE (for a similar analysis, see ref. 9) by fitting exponential decay functions to the data. The resulting cumulative probability distribution for mRNP export events required a double-exponential decay function to yield a satisfactory description of the data (Fig. 5B). The fit yielded two time constants, $\tau_1 = 65 \pm 5$ ms (87%) and $\tau_2 = 350 \pm 25$ ms (13%). The occurrence of more than a single time constant was expected, because we observed export of the complete mRNA pool comprising genes of differing size. Time constants $\tau_1$ and $\tau_2$ presumably corresponded to the export of mRNA with a size of up to a few kilobases. However, some export events lasted conspicuously long. We observed eight bona fide export events with durations longer than 0.5 s, up to 6 s. We assume that these rare long events referred to the export of extremely large mRNA like the BR transcripts (5, 22). To support this assumption, we estimated the expected export duration for BR mRNA, and our chances to observe such an export (see SI Appendix). We found that we could expect to observe the export of a fluorescently labeled BR mRNA only rarely. Considering the transcription rate of BR mRNPs and the number of simultaneous export processes that can be deduced from electron microscopic data, we estimated the mean export event duration of BR mRNPs to be about 20 s. Hence, the long observed export events could well correspond to the observation of BR mRNPs.

Like the complete export, the duration of nuclear probing of mRNPs required a biexponential decay fit, and yielded NE interaction times of $\tau_1 = 50 \pm 6$ ms (94%) and $\tau_2 = 275 \pm 15$ ms (6%) (Fig. 5C). We suspect that the short time corresponded to mRNP collisions with the NE, whereas the long one corresponded to interrupted or unsuccessful exports, when the particles entered the nuclear basket but did not proceed to the cytoplasm. In contrast, hrp36-labeled particles that approached the NE from the cytoplasm resided at the NE with a single binding time of $\tau = 95 \pm 6$ ms. This longer duration compared to the short nuclear probing time probably just reflected the lower mobility of mRNPs in cytoplasm compared to nucleoplasm. Cytoplasmic NE probing of Dbp5 had a time constant of $\tau = 54 \pm 5$ ms (Fig. 5C). Due to the very low number of events, we did not further analyze nuclear probing of Dbp5. All time constants are summarized in Table 1.

Trajectories of Single mRNPs within the NPC. Finally, we focused on the actual transit of single mRNA molecules through the NPC. For this purpose, we traced single mRNPs labeled by hrp36 with nanometer precision during their pore transit (see Materials and Methods). Remarkably, such trajectories did not show reproducible signs of a progressive movement through the NPC (Fig. 6A). However, in long trajectories a pause in the movement was seen. Obviously the respective particles halted for a certain time period. In the shown example, the stop lasted for 4 s. We used a trajectory analysis introduced by Lowe et al. (23), who examined the nuclear import of quantum dots. Seventeen trajectories with binding durations between 0.3 s and 5.6 s were rotated onto a common axis corresponding to the export direction to account for the various positions of the events on the curved NE. Then we searched for the best overlap of these trajectories with a virtual NPC scheme that was designed according to the NPC geometry of salivary gland cell nuclei of C. tentans (24) (Fig. 6B). The excellent correspondence of the coordinate distribution and the NPC dimensions suggested that the mRNA lingered predominantly in the nuclear basket and the nuclear entrance of the NPC, probably before the actual translocation took place.

Discussion

The first observation of a single mRNA traversing the nuclear pore succeeded 20 y ago by electron microscopy of BR mRNPs

![Fig. 5.](image-url) mRNP and Dbp5 kinetics binding to the NE. (A) Analysis of mRNP export kinetics. Membrane events that represented export events were collected and their duration plotted as histogram. (B) The normed cumulative counts of the histogram in (A) were determined and fitted by a mono-(dashed gray curve) and biexponential (solid black curve) function. The residual plot (insert) demonstrated that the export data were better described by a biexponential (black columns) than a monoexponential function (gray columns). (C) Comparison of the kinetics of cytoplasmic probing of Dbp5 (triangles), nuclear probing (squares) and export (circles) of hrp36-labeled mRNPs. Solid curves indicate the mono- (nuclear/cytoplasmic probing) and biexponential (for export only) fits.

<table>
<thead>
<tr>
<th>Probe</th>
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<th>$\tau_2$ (ms)</th>
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<td>54 ± 5</td>
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<td>Nuclear probing (np)</td>
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<td>50 ± 6</td>
<td>275 ± 15</td>
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<tr>
<td>hrp36</td>
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<td>65 ± 5</td>
<td>350 ± 25</td>
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<tr>
<td>hrp36</td>
<td>Cytoplasmic probing (cp)</td>
<td>184</td>
<td>95 ± 6</td>
<td>-</td>
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</table>

Table 1. Summary of mRNP and Dbp5 Kinetics
in salivary glands of *C. tentans* (25). The BR transcripts are embedded in doughnut-shaped granules of 50 nm diameter, which dock at the nuclear basket, rearrange therein, and enable the mRNA to enter the pore with the 5′ end first. Recently several researchers used the MS2 system to produce GFP-labeled mRNPs to study mRNA export. In order to create particles sufficiently bright for single molecule detection, multiple stem loops were introduced (7–9), which bound up to 48 MS2 coat proteins (MCPs) fused to GFP. The total molecular mass of the MCP/RNA tag was roughly 3,000,000 (9). Considering this molecular mass as a compact protein the MCP load alone corresponded to a particle of 16 nm diameter. Native mRNPs on the average have elongated shapes of 5–10 nm thickness and 15–30 nm length (26). Thus, the MS2-derived mRNA tags were as large as entire untagged mRNPs (11). Mor et al. (8) introduced different gene constructs based on human dystrophin cDNA, followed the MCP-GFP tagged mRNAs, and estimated the maximum export duration for a 10 kb mRNA construct with 0.5 s, but could not resolve the actual translocation process with their frame rate of 1 Hz. Grünwald and Singer (9) employed a MCP-tagged β-actin mRNA and sampled its interaction with the NE at 20 ms temporal resolution yielding a total translocation time of their mRNA construct of 180 ms. However, these authors excluded peculiarly slow events from the analysis, and their mRNA probe was preferentially seen at both the nuclear and cytoplasmic faces of the NPC.

To avoid any significant mRNP modification we established a labeling approach based on the endogenous mRNA binding protein hrp36, which is an integral part of *C. tentans* mRNPs. After microinjection, the fluorescence labeled protein was stably incorporated into mRNPs during transcription and allowed visualization of single native mRNPs in the salivary gland cell nuclei during nuclear export. The mRNPs were often perceived interacting with nuclear interface of the NE, but then returning to the nucleoplasm. We observed transient and short-lived (τg ~ 50 ms) and long (τg ~ 275 ms) interactions. Probably the shorter ones can be related to collisions with the NE without an entry into the nuclear basket. The longer ones could reflect unsuccessful docking, where mRNPs dissociate from the NPC before a suitable final position or conformation for translocation could be achieved.

About 25% of the encounters with the NE resulted in a successful mRNP export. These actual export events showed widely differing durations spanning two orders of magnitude in time. We derived two time constants (65 ms and 350 ms) describing the dissociation kinetics, but detected also events lasting up to almost 6 s. This time was not limited by bleaching of the fluorescent probe, because the actual approach to the NPC, binding, and dissociation into the cytoplasm were observed. Considering the fact that we labeled the complete mRNP pool comprising small and large particles, we suspect that a continuous distribution of binding times ranging from about 50 ms up to several seconds would appropriately describe the data, but it is not possible to prove the existence of such a distribution from real data.

Several times we observed mRNPs, possibly large BR transcripts, that arrested for several seconds at the NPC. The spatial region of this arrest was very well defined, and corresponded to a region with an extension of ±25 nm only. While the colocalization precision was not good enough to directly relate this region to a specific NPC domain, we speculate that it might correspond to the nuclear basket, and that the pause might reflect the rearrangement and unpacking of the RNP fibril that had previously been made out by electron microscopy (22, 25). A best-fitting overlay of especially long export trajectories and the NPC structure of *C. tentans* supported this notion (Fig. 6 B). Interestingly, the overlay indicated an accumulation of positions in the basket region and adjacent entry of the central framework of the NPC. Thus, the rate-limiting step of the translocation process is probably located in that domain and not at the cytoplasmic NPC face, where the mRNA is remodeled by the RNA helicase Dbp5 (4, 27, 28). An important cofactor of Dbp5 is Gle1, which is also tethered to the NPC via a specific NUP interaction. Gle1 binds Dbp5 together with soluble Inositol-6-phosphate, IP6, which might trigger ATP hydrolysis (4, 29). It was postulated that this reaction is the rate-limiting step in the Dbp5 ATPase cycle (29). Recently Hodge et al. (16) analyzed the interaction of Dbp5-GFP with the NE by photoobleaching and determined a recovery time of <1 s, suggesting a rapid exchange kinetics of the molecule at the NPC. These authors proposed a model wherein single Dbp5 go through multiple ATP-dependent remodeling cycles before being replaced. We found that single Dbp5 approached the NPC predominantly from the cytoplasm, and dissociated into this compartment after a transient binding with a mean duration of 55 ms. This duration was significantly longer than the binding times of protein transport receptors (10), and it is tempting to speculate that the 55 ms correspond to a full meachanochemical cycle of Dbp5 that was characterized by Montpetit, et al. (29). Future experiments using respective Dbp5 mutants are required to clarify this issue.

Our results suggest different scenarios for mRNA export. The mRNP trajectory data suggest that there is a rate-limiting step at the nuclear basket, which could refer to finding a suitable mRNP configuration for entering the pore interior. The duration of this step could vary largely for mRNPs of different sizes. The actual translocation could be achieved by the action of Dbp5. An alternative hypothesis is suggested by comparison of the Dbp5 binding time (55 ms) with the export duration of the larger mRNPs (350 ms up to several seconds). Several Dbp5 molecules might act in sequence on a single mRNP to achieve the required export factor release and mRNP translocation. In this case the observed translocation pause is just valid for the specific incorporation site of the labeling hrp36 that remains in the basket until it is finally

![Fig. 6. Binding site distribution at the NPC of mRNP...](image-url)
moving through the pore. Furthermore, several Dpb5 molecules could act at different locations on a single mRNP simultaneously. Thus, we speculate that the action of several dozen of Dpb5 molecules might be required to achieve translocation. This hypothesis is compatible with the characteristic and strong labeling of the NE by fluorescent Dpb5, indicating the presence of a large number of Dpb5 molecules at the NE.

In summary, we made the well known C. tentans system usable for light microscopic study of mRNA export at the single molecule level. Notably, this system allows the analysis of nuclear export of fully native mRNA. Additional single molecule experiments using Förster resonance energy transfer between the translocating mRNA and Dpb5 will probably allow further insight into the mRNA translocation process.

**Materials and Methods**

**Preparation and Microinjection of C. tentans Salivary Glands.** C. tentans midges were cultivated as described before (12). Salivary glands were transferred to PBS, which was replaced either to cover slides or custom made cuvettes designed for light sheet microscopy. For injection, the glands were kept in PBS, which was replaced afterwards with 20–40 μl hemolymph. Hemolymph was prepared freshly or used as premade stock flash frozen in liquid nitrogen and stored at −80 °C.

**Preparation of Fluorescent Proteins.** Dpb5 cDNA was amplified from an epithelial C. tentans cell line. All proteins were expressed as GST fusion proteins in E.coli BL21(DE3),pLysS), and purified using Glutathione agarose (Sigma). The N-terminal deletion mutant hrp36ΔRRM (aa 201–297) was used as GST fusion protein. From all other proteins the GST moiety was removed by thrombin digestion. For labeling of hrp36, an N-terminal tetra-cystein tag [Cys-Cys-Pro-Gly-Cys-Cys, tc (30)] was introduced. Th-crp36 proteins were labeled with AlexaFluor467 maleimide, and Dpb5 and NUP214 were labeled with AlexaFluor488 and AlexaFluor546 NHS-ester, respectively.

**Light Microscopy.** Confocal images were made with a Zeiss LSM-510-META using a 63x (LCI Plan Neofluor, NA 1.2, Carl Zeiss Microlmaging) water immersion objective. For light sheet fluorescence microscopy of single fluorescent mRNPs, a modified Axiosvert 200 (Carl Zeiss Microlmaging) was employed. For details of the instrument, see ref. 18 and SI Appendix, Fig. S5.

**Data Evaluation and Analysis.** For single molecule imaging, movies were collected (integration time 20 ms per frame) and split into substacks of 1,000 frames for evaluation. The first fifty frames showed the NTF2-stained NPCs. Movies S1 and S2 were contrast-enhanced and background-subtracted before evaluation. For mRNP export, we examined 140 nuclei from 70 salivary glands. We evaluated 7,180 movies of 20 s duration. For Dpb5, we analyzed 1,300 movies of 20 s duration from 24 nuclei (12 salivary glands). For dwell time analysis of single mRNPs or Dpb5 molecules kymographs were created, which allowed direct read-out of the respective times (21). Kymographs were created automatically using ImageJ (31) and custom-made plugins as described in the SI Appendix. Routines for manual tracking of single molecule signals with subpixel precision were written in Matlab (MathWorks). Further data analysis and handling was done with Origin 8.0 (OriginLab).

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Supplemental Information

Nuclear export of single native mRNA molecules observed by light sheet fluorescence microscopy

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

a) Fluorescence in situ hybridizations

Whole mount fluorescence *in situ* hybridization of *C. tentans* salivary glands was done according to Le Mee et al. (2008), with minor modifications. The gland were dissected in PBS without Tween, fixed for 20 min in PBS with 4% paraformaldehyde (PFA, Sigma) and washed five times with PBS. After treatment with proteinase K the glands were fixed again for 20 min in PBS/PFA, washed in PBS and finally hybridized overnight. For hybridization either BR2.1 specific (ACU UGG CUU GCU GUG UUU GCU UGG UUU GCU), BR1 specific (CUU UCU AGC GCA UUC UUC UGC UUU AGU UC), poly-dU (dU$_{20}$) or non-binding control (AGC AAA CCA AGC AAA CAC AGC AAG CCA AGU) 2’-O-Methyl-RNA oligonucleotides 5’ labeled with ATTO647N (IBATagnology) were used (Siebrasse et al., 2008; Veith et al., 2010).

b) In vitro binding analysis

To prove the interaction of recombinant wild type and mutant *C.tentans* Dbp5 with the β-propeller domain of the nucleoporin NUP214 an *in vitro* binding assay was employed. GST-Dbp5 proteins were immobilized on Glutathion beads, excess GST protein was removed by washing with PBS and the Dbp5 loaded beads were then incubated in the presence of fluorescence labeled NUP214 βΔC (final concentration 0,4 mg/ml) in PBS at room temperature for 15 minutes. Finally 20 µl of the suspension was placed on a cover slide in a wet chamber and imaged directly on a confocal microscope using the 63x objective lens (LCI Plan Neofluar, NA 1.2) without any further washing steps. To allow direct comparison of the binding efficiency all samples were imaged with identical microscope settings (laser output, pinhole diameter and photomultiplier voltage) and focusing on the equator of similar sized beads (70 -80 µm diameter) corresponding to ~35 µm above cover slide surface.

c) Estimate of mRNP labeling ratio

Larger mRNPs contain higher numbers of hrp36. E.g., BR mRNPs contain up to 150 per mRNP (Wurtz et al., 1996). Estimating the BR2 mRNA length as 37 kB, we expect on the average 4 hrp36 per 1 kB transcript. Extrapolating this to the average mRNA length of 1 – 2 kB in *C. tentans* cells (Büchner-Vollmers and Hollenberg 1981), we expect 4 to 8 hrp36 molecules only in an average transcript. Is it then possible to discriminate in our experiments the differently sized mRNPs from each other by their fluorescence intensity? The answer is NO: The amount of injected hrp36 in the single particle tracking experiments was so low that each mRNP contained maximally a single hrp36. This can be deduced from the number of observed mRNPs. There are about 3 large BR mRNPs per µm$^3$ as can be estimated from
EM images (e.g., Singh et al, 1999). In our experiments we usually observed only 3 - 10 labeled particles in a complete field of view, which corresponded to a volume of about 20x20x0.5 \textmu{}m$^3$= 200 \textmu{}m$^3$. This corresponds to a density of maximally 10/200=0.05 labeled particles per \textmu{}m$^3$. Thus, alone the probability to label a BR mRNP was 0.05/3=0.017. Since only 1.7\% of all BR mRNPs were labeled, the probability of having more than one labeled hrp36 per BR mRNP was almost zero. If we perform the same estimate using the total number of mRNPs, we yield an even lower overall labeling probability.

d) Theoretical estimate of BR2 mRNP export frequency and duration

A salivary gland cell nucleus contains ~ 8600 BR2.1 transcription units each containing about 120 RNA polymerases on the average (Lamb and Daneholt, 1979). This yields a total number of 1032000 active polymerases. The elongation rate is 31 nucleotides per s at 18°C, and the synthesis takes 1194 s or about 20 min for the 37000 bases of a BR2.1 gene. In summary, all RNA pol II produce 860 BR2.1 mRNAs per second. There are three very similar large genes: BR1, BR2.1 and BR2.2. All three are active in the salivary glands. Consequently, the number of produced large BR mRNPs must be multiplied by 3 assuming the expression level of the three genes is similar. Assuming a constant number of BR particles in the nucleus, 2580 mRNPs must be exported per second on the average.

The NE surface of a salivary gland cell nucleus with a radius of 30 \textmu{}m has an area of ~11.300 \textmu{}m$^2$, what means that the approximate BR2 export rate is ~0.23 transcripts/(\textmu{}m$^2$ s). With our camera and the used 40X objective lens we typically imaged 20 \textmu{}m long sections of the NE with a depth of field of 0.5 \textmu{}m. Therefore we theoretically could observe 2.3 BR mRNP export events per second. Considering that only a small fraction of BR mRNPs was fluorescence labelled – according to our estimation about 1\% – suggested that the overall probability to detect BR mRNP export events was very low. A number of 2.3 x 0.001 events/s might be observable corresponding to a single event in about 7.5 min observation time corresponding to 1 event in 24 movies. The frequency of observing complete BR mRNP export events including approach, binding and release from the NE is obviously significantly lower because it meant that the mRNP had to move exclusively within the focal plane upon approach binding and release. Kylberg et al. (2010) reported an average distance of 3.33 \textmu{}m between two NPCs containing translocating BR mRNPs in thin sections (70 nm) of the NE. This corresponds to ~57 BR mRNPs per equatorial section (see also Zao et al., 2004), and it results in a total number of 51430 simultaneously occurring export processes per nucleus. Since 2580 BR mRNPs must be exported per second, the export duration of a single BR mRNP is about 20 s. Obviously, the chance to observe complete export events of BR mRNPs including approach to and release from the pore using movies with durations of 19 s was quite low.
e) Trajectory overlay to NPC structure

All export trajectories with kymograph-determined binding durations of > 300 ms were selected. These trajectories contained ≥ 17 single positions, because at least one nucleoplasmic and one cytoplasmic position were observed, respectively. These trajectories were rotated according to the curvature of the NE at the respective position, where the trajectories were recorded. Thus, the trajectories were aligned to a common transport direction along the main symmetry axis of the NPC. Then, the single trajectories were moved with 1 nm stepsize iteratively in x- and y-direction over an NPC-shaped mask. The mask was formed according to the *C. tentans* NPC structure determined by Kiseleva et al. (1998), which was included in Fig. 6c. The trajectory position with the highest number of positions inside the NPC was taken as its best fitting position. Finally, all thus repositioned trajectory coordinates were plotted into a common coordinate system. The result was shown in Fig. 6c.

f) Kymograph construction and analysis

For dwell time analysis of single mRNPs or Dbp5 molecules kymographs were created, which allowed direct read-out of the respective times. Based on the NTF2 signal a new stack was created from the raw movies containing the straightened membrane and adjoining cytoplasmic and nuclear regions (15 pixels wide in both directions). Using the new stack a three by three pixel array is moved pixel-wise along the centre of the straightened membrane and the averaged fluorescence intensity plotted in a new frame. For every frame in the original movie a new column is added to the kymograph. Hence, the y-direction of the kymograph corresponds to the line of the NE and its x-width corresponds to the number of frames in the original movie. Due to diffraction limitation single molecules appeared as blurred spot with a full width at half maximum of 2 to 3 pixels under the chosen conditions. Thus, also particles only wandering alongside the membrane might contribute fluorescence to the central membrane kymograph. To exclude these background signals we introduced an additional filtering step by subtracting the kymographs of the adjacent nuclear and cytoplasmic region from the membrane kymograph. These background-corrected membrane kymographs were multiplied and the resulting membrane kymographs allowed simple identification of NE interactions and recovery of their respective dwell time.

For alignment of trajectories the NE position was determined with sub pixel accuracy. To this end the NTF2 frames were summed up and a line selection was created using the brightest membrane pixel. For every membrane pixel three lines were plotted (one perpendicular and two lines +/- 30°) and the fluorescence intensity along the lines was fitted with a Gaussian. The averaged centre of the Gaussians was then taken as the central membrane position. Membrane registration and creation of kymographs were done automatically using ImageJ
(Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011) and custom-made plugins. For further single particle tracking a Matlab (MathWorks, Natick, USA) based routine was written, which allowed manual tracking of the mRNP at the NE and 2D Gaussian fitting for sub-pixel localization.
Figure S1: Microinjection of hrp36 deletion mutants

(a) Cytosolic microinjection of the C-terminal deletion mutant, hrp36ΔGRD-AF647, which is lacking the glycine rich domain (GRD). This domain not directly involved in mRNA binding, but it is crucial for the steric regulation of RNA recognition motifs. After microinjection hrp36ΔGRD did not label any transcription sites. Since the M9 signal is also deleted by the truncation, the protein can only slowly enter the nucleus by diffusion. Confocal scans were taken after 20 minutes incubation in hemolymph. (b) BSA labeled with AF546 was co-injected to mark the site of injection and as control for the integrity of the NE. (c) Corresponding bright-field image. (d) Cytosolic microinjection of the GST-GRD fusion protein. Like wild type hrp36 the GRD fusion protein is translocated to the nucleus because the M9 domain within the GRD mediates transportin dependent shuttling between nucleus and cytosol. However, no accumulation at the transcription sites is observed without the RRM. (e) Subcellular distribution of the co-injected BSA-AF546. (f) Corresponding bright-field image. All size bars, 25 µm.
Alignment of the C. tentans Dbp5 sequence with that of Dbp5 proteins from Drosophila, human and yeast. Highly conserved residues are labeled in yellow. Point mutants used in this study are indicated with a red box. The Dbp5(R252A) point mutant did not show any binding to the NE (Fig. 2e). The mutated arginine is a highly conserved residue and found at the respective position of Dbp5 from higher eukaryotes to fungi (Napetschnig et al., 2009). It corresponds to R259 in DDX19, the human Dbp5 ortholog, and crystallographic studies of DDX19 revealed that this arginine forms a critical salt bridge in the interface to the NUP214β propeller domain (Napetschnig et al., 2009). When human DDX19(R259A) was transiently expressed in HeLa cells it displayed no detectable NE staining, which was confirmed by our findings with the respective C. tentans mutant Dbp5(R252A). Furthermore, we employed a double negative mutant, Dbp5(D216R) corresponding to the DDX19(D223R) point mutant recently described by von Moeller et al. (2009). D223 contributes another important salt bridge to NUP214 and is directly engaged in mRNA binding. Therefore DDX19(D223R) can neither bind RNA nor interact with NUP214. As expected the corresponding C. tentans Dbp5(D216R) was completely absent from the NPC in vivo (data not shown), and thus displayed the same subcellular distribution like the R252A mutant.
**Figure S3: In vitro binding study of Dbp5 with NUP214**

To demonstrate the interaction of recombinant wild type and mutant *C.tentans* Dbp5 with the β-propeller domain of the nucleoporin NUP214 an *in vitro* binding assay was employed. As shown above immobilized wild type GST-Dbp5 bound isolated NUP214βΔC fragments, i.e. amino acids 1 to 405 of the human NUP214, while neither GST-Dbp5(R252A) nor GST-Dbp5(D216R) did so. GST-Dbp5 proteins were immobilized on Glutathion beads and incubated in the presence of fluorescence labeled NUP214 βΔC. The beads (diameter 70 -80 µm) were visualized by confocal microscopy.
Figure S4: FISH analysis of mRNA export in fixed salivary glands

(a) BR2.1 mRNA FISH of a late fourth instar larva (size bar, 25 µm). (b) BR2.1 mRNA FISH of an early fourth instar larva (bar, 10 µm). (c) BR2.1 mRNA FISH, the gland was fixed directly after dissection without any incubation; sister gland from (d) (bar, 10 µm). (d) BR2.1 mRNA FISH, but gland was fixed after 60 minutes incubation in PBS (bar, 10 µm). (e) BR2.1 mRNA FISH, the gland was directly fixed after dissection without any incubation; sister gland from (f) (bar, 25 µm). (f) BR2.1 mRNA FISH of a gland fixed after incubation for 45 minutes in PBS and afterwards for 15 minutes in hemolymph (bar, 25 µm). (g) BR2.1 mRNA FISH, the gland was directly fixed after dissection without any incubation; sister gland from (h) (bar 10 µm). (h) BR2.1 mRNA FISH, but gland was fixed after 60 minutes incubation in hemolymph (bar, 10 µm). (i) polyA mRNA FISH of a gland directly fixed after dissection without any incubation (bar, 25 µm). (j) BR1 mRNA FISH of a gland directly fixed after dissection without any incubation (bar, 25 µm).
Comments to Fig. S4:

The FISH experiments were performed to control the biological function of nuclear export, and to find conditions how it could be maintained. The overall mRNA amount and the mRNA concentration of specific transcripts may vary between individual larvae during development (e.g. early and late fourth instar; compare Fig. S4a and b). Therefore we always employed the two sister glands of one animal for comparison of different incubation conditions. After an incubation of 60 minutes in phosphate buffered saline (PBS) dissected glands showed a dramatic nuclear BR2 mRNA enrichment (Fig. S4d) compared to the directly fixed sister gland (Fig. S4c). This demonstrated clearly that export did not occur under this condition. Moreover, occasionally the cells displayed formation of cytoplasmic blebs indicating cell damage (see arrow in Fig. S4d). Dissection and microinjection of the glands required to work in PBS. This procedure took 10 to 15 minutes until the PBS could be replaced by hemolymph. However, even when glands were kept for 45 minutes in PBS, but were then transferred to hemolymph, an incubation time of 15 minutes was sufficient to restore the normal state as observed directly after dissection. This can be seen in Fig. S4f compared to the directly fixed sister gland (Fig. S4e). Transcription and export endured for at least 60 min, when the glands were maintained in hemolymph as shown by Fig. S4h in comparison to the directly prepared gland (Fig. S4g). Despite on-going transcription no nuclear mRNA accumulation is observed during hemolymph incubation demonstrating that mRNA export endures. When a polyU-probe was applied a distinct nuclear rim stain could be noticed (Fig. S4i). However, besides their overall abundance neither the BR1 (Fig. S4j) nor BR2 transcripts were visibly enriched at the nuclear envelope (Fig. S4a, b, c, e, f, g and h). Note that the BR1 and BR2 transcription sites can easily be identified due to their characteristic ring-like appearance (Fig.S4 a, b, c, e, f, g h and j). Finally it should be noted that generally the levels of intranuclear fluorescence and the intensity ratio nucleoplasm versus cytoplasm were quite variable even under identical conditions, as could be seen by comparing the three reference nuclei (Figs. S4 c, e and g).
Figure S5:Selective plane illumination microscopy of salivary glands

(a) An elliptical laser beam was focused from the side into a glass sample chamber, which was placed on the stage of a commercial inverse microscope (Axiovert 200, Carl Zeiss MicroImaging). The lateral position of the specimen chamber could be adjusted by micrometer screws, and the axial position by a stepping motor in 100 nm steps. For fluorescence excitation we used a 532 nm DPSS laser (LasNova50 Green, Lasos) and a 640 nm laser diode module (Cube 640-40C, Coherent). The combined laser lines were guided through an acousto-optical tunable filter and via a mono-mode fiber (kineFlex, Point Source) to the microscope. The elliptical Gaussian illumination beam was formed by a cylindrical Galilean beam expander in front of the illumination objective (plan apochromat 106, NA 0.28; Mitutoyo). Fluorescence was collected with the 40x, NA 1.2 water immersion objective lens (C-Apochromat) and imaged with an EMCCD camera with 128x128 pixels (iXon BI DV-860, pixel size 24 µm, Andor Technologies). An additional 4-fold magnifier resulted in an object field pixel size of 150 nm. The sheet illumination created an optical sectioning effect. This strongly reduced fluorescence background and created a superb image contrast. (b) Sketch of the light sheet illumination of a salivary gland cell nucleus. The salivary gland cells exhibit dimensions much larger than that of typical mammalian cells as shown in the lower left side. Single molecule imaging was performed typically in a distance of 125 µm from the glass chamber bottom with the light sheet focus at the nuclear envelope.
Figure S6: Examples of hrp36-labelled mRNPs at the NE

All membrane incidents were classified according to the categories described in the main text. Every time series shows a typical incident of its category as indicated by the sketches on the left hand side.

(a) Nuclear probing, np; (b) only envelope, env; (c) cytosolic probing, cp; (d) nucleus to envelope, ne; (e) envelope to cytosol, ec; (f) envelope to nucleosol, en; (g) cytosol to envelope, ce

Upper panels show raw data, lower panels show the overlays of the mRNPs in the red channel and the NTF2-labelled NE in the green channel (image area 4.65x4.65)
µm²). The trajectories and NE position were plotted on the right hand side. The green dots indicate the start, and the red dots the end of each trajectory.

**Legends to Supplemental Movies**

Supplemental Movie S1:

Export event of hrp36-labeled mRNP. Single frame acquisition time 20 ms, data acquisition rate 50 Hz, display at 10 Hz, field of view 19.2x19.2 µm². The mRNP position is indicated by an arrow. Left, contrast enhanced hrp36 raw data; center, raw data with the mRNP marked in blue; right, rolling ball background-subtracted and filtered data (red) with the NTF2 signal shown in green.

Supplemental Movie S2:

Cytosolic probing of Dbp5. Single frame acquisition time 20 ms, data acquisition rate 50 Hz, display at 5 Hz, field of view 19.2x19.2 µm². The Dbp5 position is indicated by an arrow. Left, contrast enhanced Dbp5 raw data; center, raw data with the Dbp5 marked in blue; right, rolling ball background-subtracted and filtered data (red) with the NTF2 signal shown in green.

**SUPPLEMENTAL REFERENCES**


**Supporting Information**

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**Movie S1**  Export event of hrp36-labeled mRNP. Single frame acquisition time 20 ms, data acquisition rate 50 Hz, display at 10 Hz, field of view 19.2 x 19.2 μm². The mRNP position is indicated by an arrow. Left, contrast enhanced hrp36 raw data; center, raw data with the mRNP marked in blue; right, rolling ball background-subtracted and filtered data (red) with the NTF2 signal shown in green.

*Movie S1 (AVI)*

**Movie S2**  Cytosolic probing of Dbp5. Single frame acquisition time 20 ms, data acquisition rate 50 Hz, display at 5 Hz, field of view 19.2 x 19.2 μm². The Dbp5 position is indicated by an arrow. Left, contrast enhanced Dbp5 raw data; center, raw data with the Dbp5 marked in blue; right, rolling ball background-subtracted and filtered data (red) with the NTF2 signal shown in green.

*Movie S2 (AVI)*