

Nucleocytoplasmic transport of fluorescent mRNA in living mammalian cells: nuclear mRNA export is coupled to ongoing gene transcription

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In eukaryotic cells, export of mRNA from the nucleus to the cytoplasm is one of the essential steps in gene expression. To examine mechanisms involved in the nucleocytoplasmic transport of mRNA, we microinjected fluorescently labeled *fushi tarazu* (*ftz*) pre-mRNA into the nuclei of HeLa cells. The injected intron-containing *ftz* pre-mRNA was distributed to the SC35 speckles and exported to the cytoplasm after splicing by an energy-requiring active process. In contrast, the injected intron-less *ftz* mRNA was diffusely distributed in the nucleus and then presumably degraded. Interestingly, export of the *ftz* pre-mRNA was inhibited by treatment with transcriptional inhibitors (actinomycin D, α -amanitin or DRB). Cells treated with transcriptional inhibitor showed foci enriched with the injected mRNA, which localize side by side with SC35 speckles. Those nuclear foci, referred to as TIDRs (transcriptional-inactivation dependent RNA domain), do not overlap with paraspeckles. In addition, *in situ* hybridization analysis revealed that the export of endogenous poly(A)⁺ mRNA is also affected by transcriptional inactivation. These results suggest that nuclear mRNA export is coupled to ongoing gene transcription in mammalian cells.

Introduction

In eukaryotic cells, the sites of transcription (the nucleus) and translation (the cytoplasm) are separated by the nuclear membrane. Thus, transport of proteins and RNAs between the nucleus and the cytoplasm via nuclear pores is an essential cellular process. In recent years, through the identification of factors required for nucleocytoplasmic transport, understanding of transport mechanisms for proteins and different classes of RNA, including rRNAs, tRNAs and snRNAs, has increased significantly (for reviews see Cullen 2000, 2003; Lei & Silver 2002).

However, the export mechanism for cellular mRNA was found to be much more complicated than for other types of RNA. Most mRNAs are initially transcribed as precursors (pre-mRNAs) that need to be processed and assembled into ribonucleoprotein particles before export to the cytoplasm. The co- or post-transcriptional maturational processing events of pre-mRNAs include 5'-capping, removal of introns by splicing, and 3' polyadenylation at a defined site within a 3' untranslated region. The complexity of processing and the absence of a reliable *in vitro* mRNA export assay have hampered the biochemical characterization of mRNA export.

Genetic studies in yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been an effective approach for identifying components involved in mRNA export (for a review see Str  ber & Hurt 1999). Temperature-sensitive mutants defective in mRNA export were isolated by screening with fluorescence *in situ* hybridization (FISH) using an oligo dT as a probe. Characterization of those mutants has implicated a variety of proteins in

Communicated by: Yoshikazu Nakamura

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DOI: 10.1111/j.1365-2443.2006.00936.x

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mRNA export. A synthetic lethal screen in yeast also worked effectively to identify factors required for mRNA export. *mex67*, the wild-type gene of which encodes an export receptor, was identified in a synthetic lethal screen with the *nup85Δ* allele (Segref *et al.* 1997). Yra1p, a conserved nuclear RNA binding protein, which acts as a bridge between mRNA and Mex67p, was also identified as an adapter protein for mRNA export in a synthetic lethal screen with a temperature-sensitive allele of *MEX67* (Sträßer & Hurt 2000). The conserved spliceosomal component Sub2p was proposed to recruit Yra1p to the mRNA during pre-mRNA splicing, thereby performing its role in mRNA export in yeast (Luo *et al.* 2001; Sträßer & Hurt 2001). Yra1p and Sub2p are members of the TREX (transcription and mRNA export) complex, which is thought to link transcription and mRNA export in yeast (Sträßer *et al.* 2002).

Fischer *et al.* (2002) reported a genetic interaction between Yra1p and Sac3p. Sac3p was shown to interact with Sub2p *in vivo* and form a stable complex with Thp1p involved in transcriptional elongation (Fischer *et al.* 2002). Sac3p was also shown to associate with Mex67p and nucleoporin Nup1p. Recently, it was found that the Sac3p-Thp1p complex interacts with SAGA, a large intranuclear histone acetylase complex involved in transcription initiation, via a novel protein Sus1p (Rodríguez-Navarro *et al.* 2004). These findings also support a connection between transcription and mRNA export in yeast. In addition to yeast genetics, microinjection of RNAs or proteins into *Xenopus laevis* oocytes has been taken as a complementary approach to analyze the mechanisms of mRNA export. Early studies using this technique showed that the cap structure and polyA tail can facilitate mRNA export from the nucleus. A ³²P-labeled mRNA synthesized with the m⁷G cap structure was exported from the nucleus to the cytoplasm in *X. laevis* oocytes more rapidly than an mRNA synthesized with an adenosine (A) or tri-methylguanosine (m³G) cap (Hamm & Mattaj 1990; Jarmolowski *et al.* 1994). In addition, co-injection of excess m⁷GpppG dinucleotide into the oocyte nucleus effectively inhibited mRNA export (Hamm & Mattaj 1990; Dargemont & Kühn 1992). The export of injected mRNA lacking the polyA tail was also slower than that of mRNA with the polyA tail in *X. laevis* oocytes (Jarmolowski *et al.* 1994).

Using the *X. laevis* oocytes system, Reed and coworkers (Luo & Reed 1999; Zhou *et al.* 2000) showed that mRNAs generated through splicing are exported at high rates, whereas the corresponding mRNAs transcribed from the intron-less cDNAs are poor substrates for export. They also found that Aly, the metazoan homolog of the yeast Yra1p, is recruited to mRNP complexes generated

by splicing and injection of excess recombinant Aly increases both the rate and efficiency of mRNA export *in vivo* (Zhou *et al.* 2000). These results suggest that pre-mRNA splicing is coupled to mRNA export. Drawing upon data from genetic screens in yeast and microinjection studies in *Xenopus* oocytes, it is clear that there exists a complex relationship among the steps in eukaryotic gene expression including transcription, splicing and mRNA export.

In the present study, we analyzed nucleocytoplasmic transport of fluorescently labeled pre-mRNA microinjected into the nuclei of mammalian cells (HeLa cells). The injected pre-mRNAs were exported to the cytoplasm from the nucleus via an energy-dependent process after splicing. Interestingly, we found that inhibition of gene transcription impairs export of the injected pre-mRNA and causes the accumulation of the fluorescent mRNA in nuclear domains named TIDR (transcriptional-inactivation dependent RNA domain), which are localized adjacent to the SC35 speckles. Our results suggest a linkage between nucleocytoplasmic transport of mRNA and ongoing gene transcription in mammalian cells.

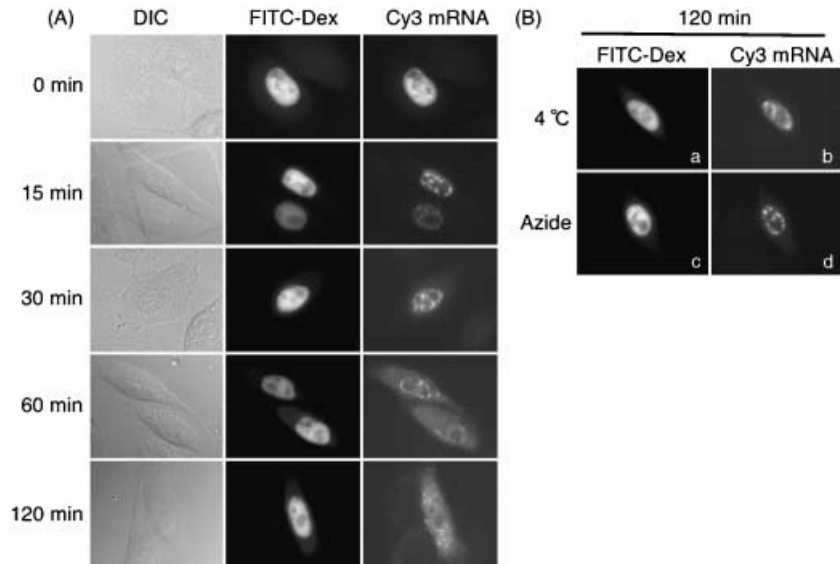
Results

The injected fluorescent *ftz* pre-mRNA is exported to the cytoplasm by an active process

To examine nuclear export of mRNA in mammalian cells, we synthesized a m⁷G capped Cy3-labeled *fushi tarazu* (*ftz*) pre-mRNA containing one intron and a 30 nt polyA tail (497 nt, see Experimental procedures) and microinjected it into the nuclei of HeLa cells. After incubation for 15, 30, 60 or 120 min at 37 °C, the distribution of Cy3-labeled mRNA was examined using fluorescence microscopy. FITC-Dextran (70 kDa), which cannot move to the cytoplasm through nuclear pores by passive diffusion, was co-injected into the nuclei to confirm the nuclear injection of the fluorescent mRNA. Figure 1A shows results of a typical experiment. The injected *ftz* pre-mRNA was initially distributed throughout the nucleus, except for the nucleolus (Fig. 1A, 0 min), and then foci enriched with injected mRNA were observed in the nucleus at 15 min (15 min). The fluorescence in the cytoplasm increased with incubation time, suggesting that the injected mRNA was transported to the cytoplasm (60 min). In some cells, the intensity of the mRNA fluorescence decreased 120 min after injection, suggesting that degradation of the injected mRNA had occurred.

To exclude the possibility that the injected mRNA simply diffused to the cytoplasm, two criteria were

Figure 1 Nuclear export of Cy3-labeled *ftz* pre-mRNA in HeLa cells. (A) Time course of the export of injected *ftz* pre-mRNA from the nucleus. Cy3-labeled pre-mRNA was co-injected with FITC-Dextran (70 kDa) into the nuclei of HeLa cells. The injected cells were then incubated at 37 °C for the indicated times before fixation. (B) Fluorescent mRNA was exported by an energy-dependent process. (a, b) HeLa cells injected with mRNA were incubated at 4 °C for 2 h before fixation. (c, d) Cells injected with mRNA were also incubated at 37 °C in a medium containing sodium azide (15 mM) and deoxyglucose (9 mM) for 2 h to be metabolically poisoned before fixation.



examined. As shown in Fig. 1B-b, when the cells were incubated at 4 °C for 2 h after injection, migration of the injected *ftz* mRNA to the cytoplasm was completely inhibited. In addition, when the injected cells were incubated at 37 °C for 2 h in a medium containing azide and deoxyglucose which antagonize energy metabolism, the export of mRNA was also severely diminished (Fig. 1B-d). Therefore, we conclude that translocation of the injected Cy3-labeled *ftz* mRNA to the cytoplasm in HeLa cells is done by an energy requiring active process, as is the case with the nuclear export of cellular mRNAs.

The injected *ftz* pre-mRNA is co-localized with the splicing factor SC35 and spliced

It was previously shown that the splicing factor SC35 localizes to nuclear speckles (Fu & Maniatis 1990). Here, we examined whether the nuclear foci, which were detected in the nucleus of the injected cells 15 min after injection, are nuclear SC35 speckles. The injected cells were stained with anti-SC35 antibody as shown in Fig. 2. Most of the *ftz* foci co-localized with the SC35 nuclear speckles, suggesting that the Cy3-labeled pre-mRNA associates with splicing factors.

To test whether the injected fluorescent *ftz* pre-mRNA is indeed spliced, total RNA was isolated from the injected cells either immediately or 120 min after injection and subjected to RT-PCR analysis. An amplified product corresponding to the spliced *ftz* mRNA was detected after 120 min incubation (Fig. 3A). Sequence analysis of the product revealed that it is amplified from a precisely spliced *ftz* mRNA. However, a larger band corresponding to the unspliced pre-mRNA was also observed for 120 min.

The spliced *ftz* mRNA is transported to the cytoplasm

Since the above result indicated that a portion of the injected fluorescently labeled pre-mRNA can be spliced, we next wondered whether the cytoplasmic fluorescence resulted from export of the spliced mRNA as opposed to the unspliced intron-containing pre-mRNA. To this end, unlabeled *ftz* pre-mRNA was injected into the nucleus and *in situ* hybridization was performed using two different oligonucleotide probes. These probes distinguish between unspliced (P-probe) and spliced (M-probe) products (Fig. 3B). RT-PCR analysis revealed that the injected unlabeled *ftz* pre-mRNA was spliced more efficiently than the fluorescently labeled *ftz* pre-mRNA (Fig. 3C). As shown in Figure 3D, P-probe and M-probe gave nucleoplasmic staining with a speckled pattern at 15 min after injection. After 60 min incubation, significant cytoplasmic staining was observed with the M-probe, whereas no hybridization signal was detected in cells with the P-probe. These results suggest that the majority of the injected unlabeled *ftz* pre-mRNA is spliced completely after 60 min incubation and the spliced *ftz* mRNA is transported to the cytoplasm.

The intron-less *ftz* mRNA is diffusely distributed in the nucleus and is unstable

To investigate whether splicing has an effect on the export of the injected mRNA, we injected a Cy3-labeled intron-less *ftz* mRNA with a 30nt polyA tail (346 nt) into the nuclei of the HeLa cells. In contrast with intron containing *ftz* pre-mRNA, the intron-less *ftz*

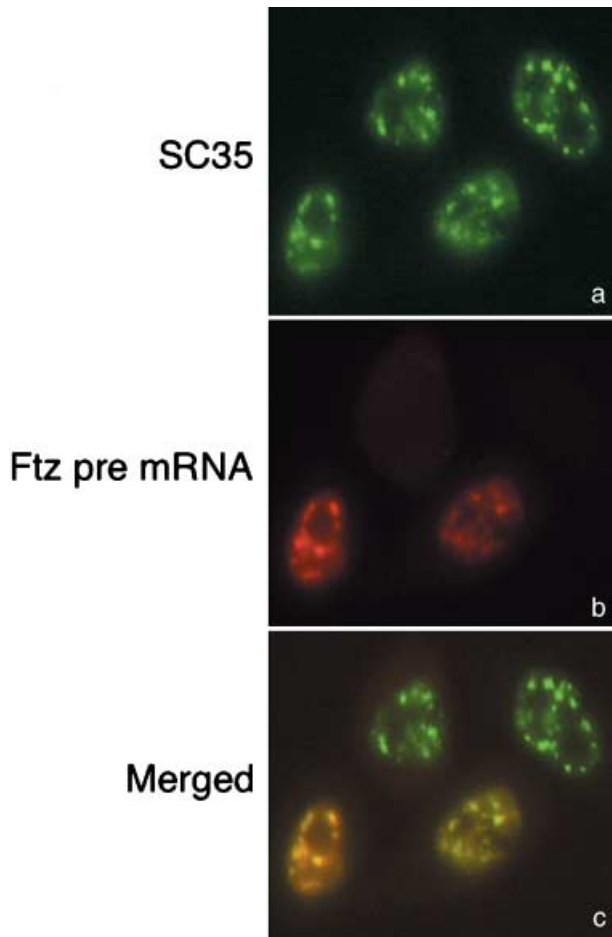


Figure 2 Co-localization of the injected *ftz* pre-mRNA with the SC35 splicing factor. Fifteen minutes after injection of the Cy3-labeled *ftz* pre-mRNA into the nuclei, cells were treated with the anti-SC35 antibody, followed by staining with the anti-mouse IgG antibody conjugated with FITC.

mRNA was diffusely distributed throughout the nucleus with the exception of the nucleolus and no speckle-like distribution was observed (Fig. 4A). The intensity of the Cy3 fluorescence was found to decrease gradually. We could not detect significant quantities of amplified product corresponding to the intron-less *ftz* mRNA by RT-PCR at 2 h after injection (Fig. 4B). These data suggest that injected intron-less *ftz* mRNA is degraded and is less stable than its intron-containing counterpart. Some cells showed a faint cytoplasmic fluorescent signal at 60 min, suggesting that a small amount of intron-less *ftz* mRNA could be exported to the cytoplasm (Fig. 4A). However, we could not exclude the possibility that the increase in cytoplasmic fluorescence in these cells was due to simple diffusion of the degraded mRNA fragments to the cytoplasm.

Fluorescent β -globin mRNA is also exported to the cytoplasm

To determine whether the nuclear export of fluorescently labeled mRNA is general or unique to the *ftz* mRNA derived transcript, we carried out similar experiments using another substrate derived from the human β -globin sequence. When a m⁷G capped Cy3-labeled β -globin pre-mRNA containing one intron and a 30nt polyA tail (543 nt, see Experimental procedures) was injected into the nuclei of HeLa cells, the cells showed a non-nucleolar nucleoplasmic distribution at 15 min (Fig. 5A). Unexpectedly, the nuclear speckled pattern of the fluorescent mRNA was not detected at that time. However, a weak speckled distribution began to appear and an increase in cytoplasmic signal was observed after 60 min (Fig. 5A). These observations suggest that the kinetics of the splicing reaction of the β -globin pre-mRNA is slower than that of the *ftz* pre-mRNA. RT-PCR analysis of the β -globin pre-mRNA showed that a certain amount of pre-mRNA remains even after 120 min incubation, suggesting that splicing of the injected β -globin pre-mRNA is inefficient (data not shown).

On the other hand, when the intron-less β -globin mRNA (413 nt) was injected, it was distributed diffusely in the nucleus except for the nucleolus, much like the intron-less *ftz* mRNA (Fig. 5B). Also, the fluorescent signal became weaker in cells as the incubation time increased and no significant cytoplasmic signal was detected within 2 h after injection.

Taken together with the results of the *ftz* mRNA microinjection, the rate and the efficiency of mRNA export seem to be dependent on the sequence. Splicing may also affect the mRNA export and stability of RNA transcripts. These observations are consistent with the data obtained from previous *Xenopus* oocyte microinjection assays.

Treatment with transcriptional inhibitors affects export of the injected mRNA

Next, we investigated effects of several drugs on the export of the fluorescently labeled mRNA in injected HeLa cells (Fig. 6A). HeLa cells injected with Cy3-labeled *ftz* pre-mRNA were incubated in medium containing either leptomycin B (LMB; an inhibitor for nuclear export of proteins containing a leucine rich nuclear export signal (NES)) or cycloheximide (a translational inhibitor). As shown in Figure 6A–b,d, neither LMB nor cycloheximide significantly inhibited mRNA export. In contrast, when the injected cells were treated with transcriptional inhibitors, such as actinomycin D, α -amanitin,

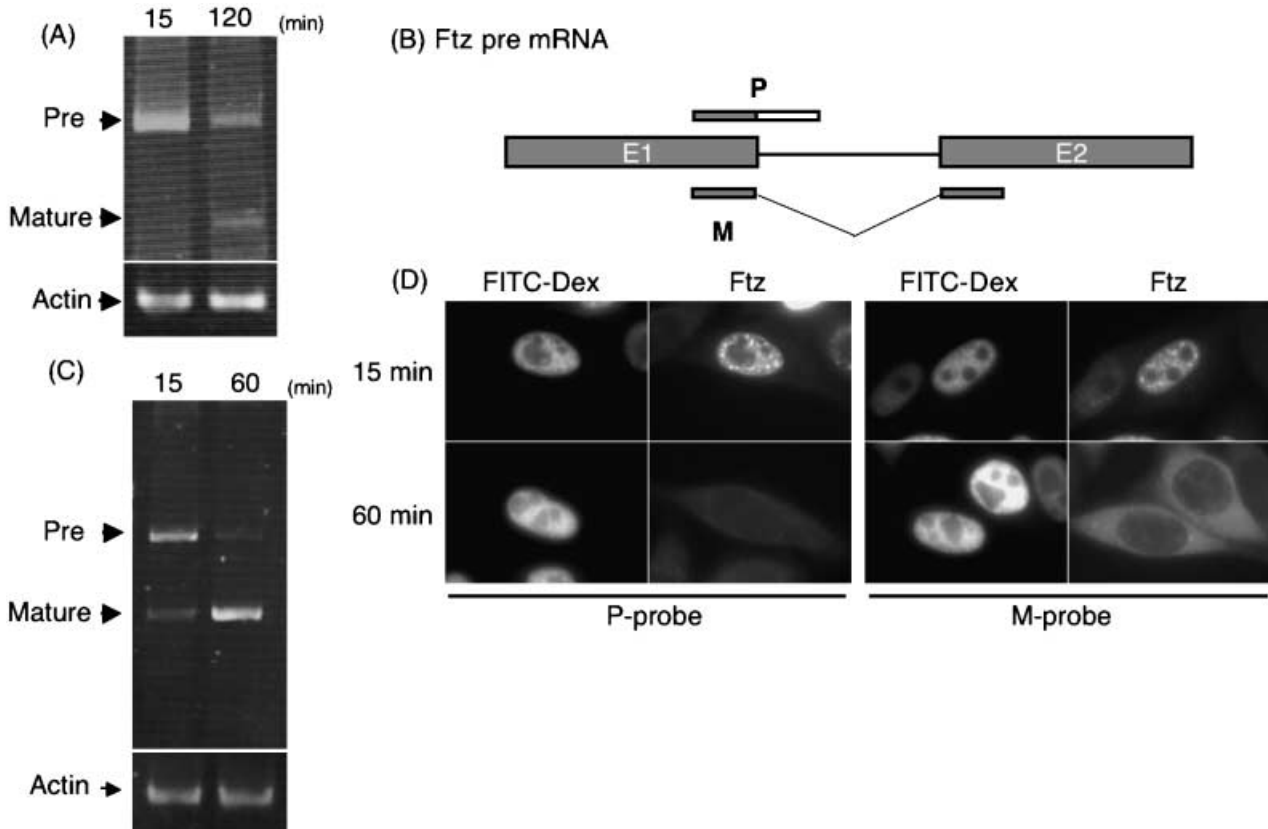


Figure 3 The injected pre-mRNA is spliced and exported to the cytoplasm. (A) Splicing of the injected fluorescent pre-mRNA. Total RNA from the cells injected with the fluorescent *ftz* pre-mRNA was subjected to RT-PCR analysis to identify the spliced *ftz* mRNA. As a control of RT-PCR, endogenous actin mRNA was amplified from the isolated total RNA. (B) Schematic representation of the probe structures. P and M denote the probes hybridizing to *ftz* pre- and mature mRNAs, respectively. (C) Splicing of the non-labeled *ftz* pre-mRNA. Total RNA from the cells 15 min (15) or 60 min (60) after the injection of the non-labeled *ftz* pre-mRNA was subjected to the RT-PCR analysis to detect the spliced *ftz* mRNA. (D) *In situ* hybridization using the probes for pre- and mature *ftz* mRNAs. Cells injected with the non-labeled *ftz* pre-mRNA were incubated for 15 or 60 min and subjected to *in situ* hybridization with the digoxigenin-labeled probes shown in (B). The spliced *ftz* mRNA was predominantly detected in the cytoplasm in cells incubated for 60 min.

or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), respectively, no increase in cytoplasmic fluorescence was observed (Fig. 6A-f,h,j). The number of the nuclear *ftz* speckles decreased and the size of each speckle increased relative to those in untreated cells. These observations suggest that transcriptional inactivation causes inhibition of fluorescently labeled mRNA export and alters the subnuclear structure.

Figure 6B shows a result of RT-PCR analysis to monitor the splicing reaction of the injected *ftz* pre-mRNA in the presence of actinomycin D. We detected efficient splicing of *ftz* pre-mRNA in cells treated with actinomycin D for 60 min (Fig. 6B, lane 3), suggesting that transcriptional inactivation does not affect splicing, at least for injected pre-mRNAs.

To examine if the transcriptional inhibition also causes defects in protein import and export, we transfected HeLa cells with pcDNA-R1GLFPA6 that expresses a GST-NES-NLS-GFP fusion protein, and then intracellular distribution of the expressed fusion protein was observed. As shown in Fig. 6C, the fusion protein distributed predominantly in the cytoplasm in the absence of LMB, whereas addition of LMB led to the accumulation of the fusion protein in the nucleus. These observations suggest that the expressed fusion protein is shuttling between the nucleus and the cytoplasm in HeLa cells. The same phenomenon was observed in the presence of actinomycin D (Fig. 6C-e-g), suggesting that protein import and export are not affected by transcriptional inactivation, in contrast to the export of the injected mRNA.

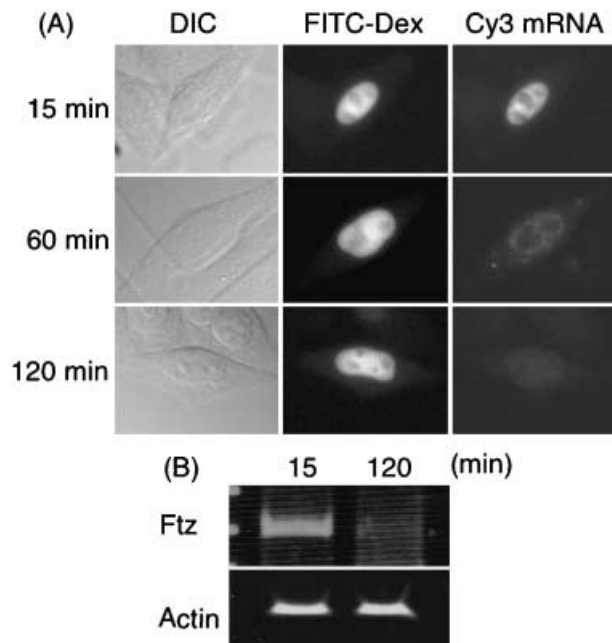


Figure 4 (A) Nuclear export of the intron-less *ftz* mRNA. The intron-less *ftz* mRNA transcribed from the cDNA was injected into the nuclei of HeLa cells and then intracellular distribution of the injected mRNA was observed after incubation at 37 °C for indicated times. (B) The intron-less *ftz* mRNA was degraded after injection. RT-PCR analysis was done using total RNA isolated from cells injected with intron-less *ftz* mRNA, as described in Experimental procedures.

Injected *ftz* mRNA is enriched in novel nuclear domains, TIDRs, in the transcriptionally inactivated cells

To identify the precise localization of the reorganized nuclear *ftz* speckles, actinomycin D treated cells were stained with several antibodies against components of known nuclear bodies, including splicing SC35 speckles, Cajal bodies and PML (promyelocytic leukemia protein) bodies. Immunostaining with antibodies against p80 coilin (a marker for Cajal bodies, Andrade *et al.* 1991) and PML (Santa Cruz, PG-M3) showed no significant stained signals in actinomycin D treated HeLa cells, although those nuclear bodies were clearly stained by the antibodies in non-treated cells (data not shown). These results suggest that Cajal and PML bodies disassemble in transcriptionally inactivated HeLa cells. On the other hand, reorganized *ftz* speckles mostly co-localize with the SC35 speckles, but are also found to be more intense in areas adjacent to the SC35 speckles after actinomycin D treatment (Fig. 7A, upper panels). The same phenomenon was observed in actinomycin D treated

HeLa cells injected with β -globin pre-mRNA (Fig. 7A, lower panels).

Recently, novel nuclear compartments, paraspeckles, were shown to localize adjacent to SC35 speckles and alter their localization in a transcription-dependent fashion (Fox *et al.* 2002). To test the possibility that the injected *ftz* pre-mRNA was accumulating in the paraspeckles after transcriptional inactivation, HeLa cells were stained with an antibody against PSP1, a component of the paraspeckle (Fox *et al.* 2002). The injected cells did not show co-localization of reorganized paraspeckles and *ftz* speckles, including the foci enriched with *ftz* mRNA, after actinomycin D treatment (Fig. 7B, merged). Although at present we cannot determine exactly where and what these reorganized *ftz* foci are, we designated these unknown transcription-dependent nuclear domains as transcriptional inactivation dependent RNA domains, TIDRs.

Endogenous poly(A)⁺ mRNA is also retained in the nucleus after treatment with a transcriptional inhibitor

We wondered whether transcriptional inactivation inhibits the nuclear export of endogenous poly(A)⁺ mRNAs leading to their accumulation in TIDRs, as was observed for injected Cy3 labeled *ftz* pre-mRNA. To this end, Cy3-labeled *ftz* pre-mRNA without a polyA tail [poly(A)⁻ *ftz* pre-mRNA] was injected into the nuclei of the HeLa cells and *in situ* hybridization was performed with an oligo dT probe to detect endogenous poly(A)⁺ mRNA, after actinomycin D treatment (Fig. 8A). This assay allowed us to label specifically both the endogenous poly(A)⁺ mRNA and injected *ftz* pre-mRNA. Similar to the poly(A)⁺ transcript, poly(A)⁻ *ftz* pre-mRNA was found to accumulate in TIDRs, adjacent to SC35 speckles, after 2 h treatment with actinomycin D (Fig. 8A). However, we detected no drastic enrichment of endogenous poly(A)⁺ mRNA in TIDRs. Due to the sensitivity of *in situ* hybridization, we cannot exclude the possibility that a small amount of poly(A)⁺ mRNA accumulated in TIDRs after the transcriptional inactivation.

We treated the injected cells with actinomycin D for 8 h and performed *in situ* hybridization with the oligo dT probe to observe the distribution of endogenous poly(A)⁺ mRNA. If mRNA export is not affected by treatment with transcriptional inhibitors, then poly(A)⁺ mRNAs transcribed prior to the treatment should be exported to the cytoplasm after the inhibition of transcription. As shown in Fig. 8B,C, a certain amount of poly(A)⁺ mRNA remained in the nucleus 8 h after treatment with the transcriptional inhibitor actinomycin D.

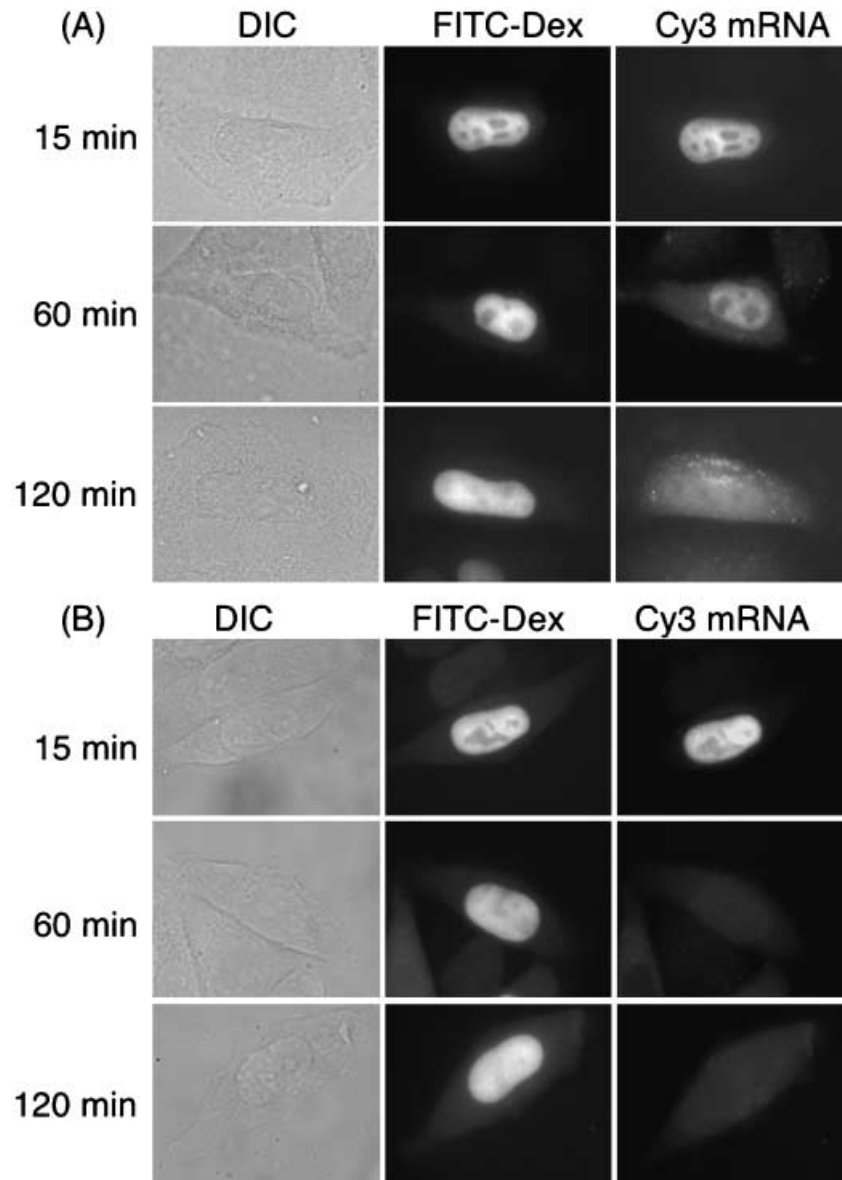


Figure 5 Nuclear export of (A) the β -globin pre-mRNA and (B) the intron-less mRNA. The Cy3-labeled mRNAs were injected into the nuclei of HeLa cells and the intracellular distribution was observed after incubation at 37 °C for the indicated time. Fluorescent mRNA was co-injected into the nuclei with FITC conjugated dextran (FITC-Dex).

Huang *et al.* (1994) reported previously similar results using α -amanitin that inhibits transcription by RNA polymerase II. These results suggest that export of endogenous mRNAs is also impaired by transcriptional inactivation.

Discussion

In this study, we showed that fluorescent pre-mRNAs injected into the nuclei of HeLa cells are distributed to the SC35 speckle domains, spliced to form mature mRNAs and then exported to the cytoplasm from the nucleus via an energy-dependent active process. Using

fluorescent pre-mRNAs as injection substrates, we can analyze a process of nuclear mRNA export in living mammalian cells. Development of such an experimental system is of great importance since the knowledge regarding RNA export mechanisms came so far from *Xenopus* oocyte system or yeast genetics. We obtained evidence that inactivation of gene transcription affects the nuclear export of mRNAs in mammalian cells with this assay system.

We found that the intron-less *ftz* and β -globin mRNAs transcribed from the cDNAs are not distributed to the SC35 speckle domains in contrast to the cases of pre-mRNAs (Figs 4 and 5B). They degraded more rapidly

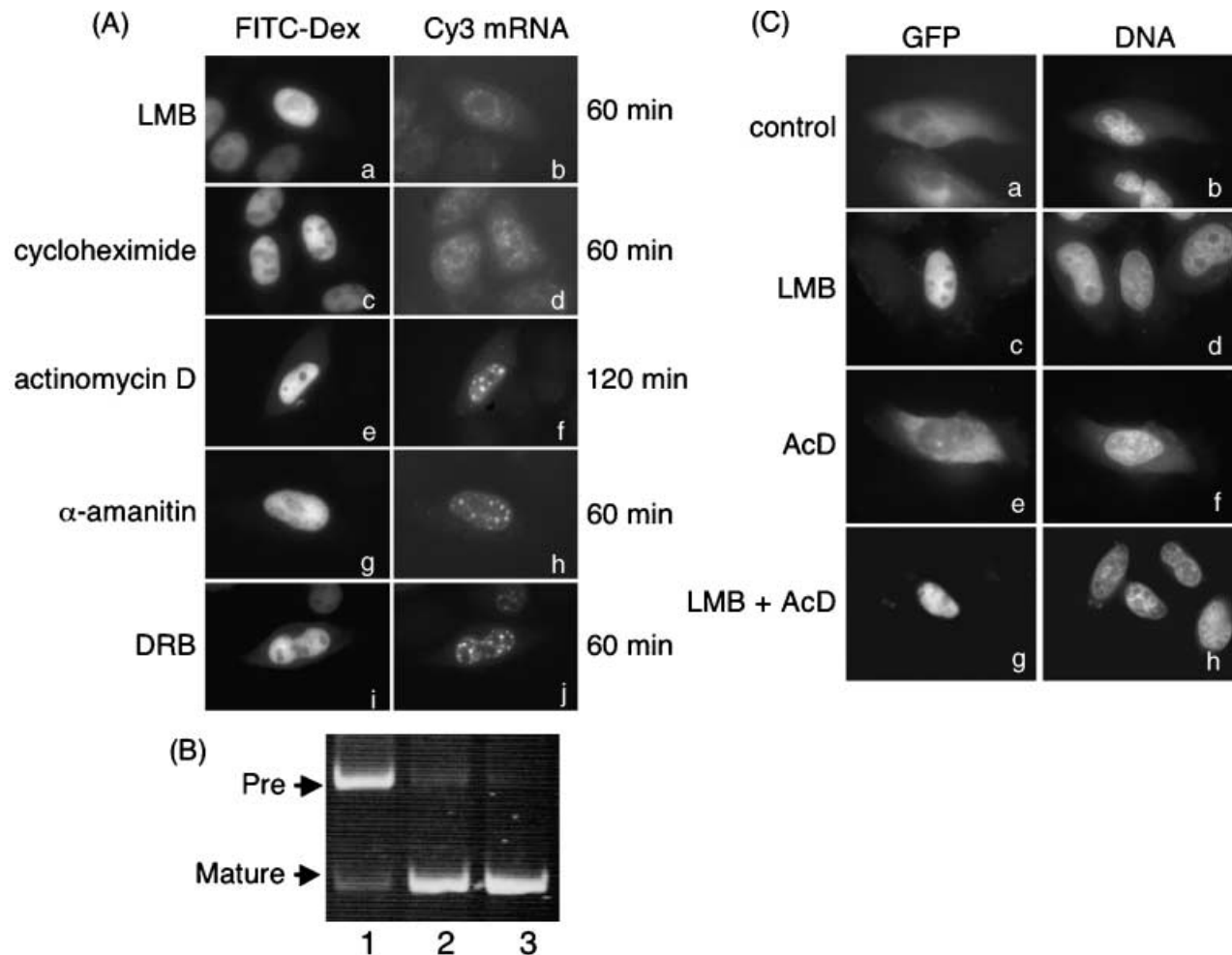


Figure 6 (A) Effects of drug treatments on the export of mRNA. HeLa cells injected with the fluorescent *fitz* pre-mRNA were incubated in medium containing LMB (50 ng/mL), cycloheximide (100 μ g/mL), actinomycin D (5 μ g/mL), α -amanitin (50 μ g/mL) or DRB (25 μ g/mL) at 37 °C for the indicated time before fixation. (B) Splicing of the *fitz* pre-mRNA in the presence of actinomycin D. The non-labeled *fitz* pre-mRNA was injected to the nuclei of HeLa cells, and incubated for 15 min (lane 1) or 60 min (lanes 2 and 3) in the absence (lanes 1 and 2) or presence of 5 μ g/mL of actinomycin D (lane 3). (C) Transcriptional inactivation had no effects on transport of a protein with NES and NLS. The plasmid expressing the GST-NES-NLS-GFP was introduced into cells and then localization of the expressed fusion protein was examined in the absence (control) or presence of LMB (50 ng/mL), actinomycin D (AcD; 5 μ g/mL) or both at 37 °C.

than pre-mRNA after injection into the nucleus. Formation of the exon junction complex (EJC) on the spliced mRNAs during pre-mRNA splicing (Le Hir *et al.* 2001) might enhance the stability of the injected pre-mRNAs in cells. It was not apparent that degradation of the injected intron-less mRNA occurred in the nucleus or in the cytoplasm. Thus, we could not determine if splicing reaction promotes the export of the injected mRNA in mammalian cells, as reported in the *Xenopus* oocyte system by Reed and coworkers (Luo & Reed 1999; Luo *et al.* 2001).

It was previously reported that fluorescently labeled β -globin or adenovirus pre-mRNA microinjected into the nuclei of cultured mammalian cells localizes in 30–60 discrete nuclear sites (Wang *et al.* 1991; Melcák *et al.* 2001). In those experiments, however, neither splicing of the injected pre-mRNA nor its export from the nucleus was analyzed.

Watanabe *et al.* (1999) also examined nuclear export of digoxigenin-labeled, capped mature TAK1 mRNA (1.7 kb) injected into the nuclei of rat fibroblastic 3Y1 cells. They found that it takes 5 h until the majority of the

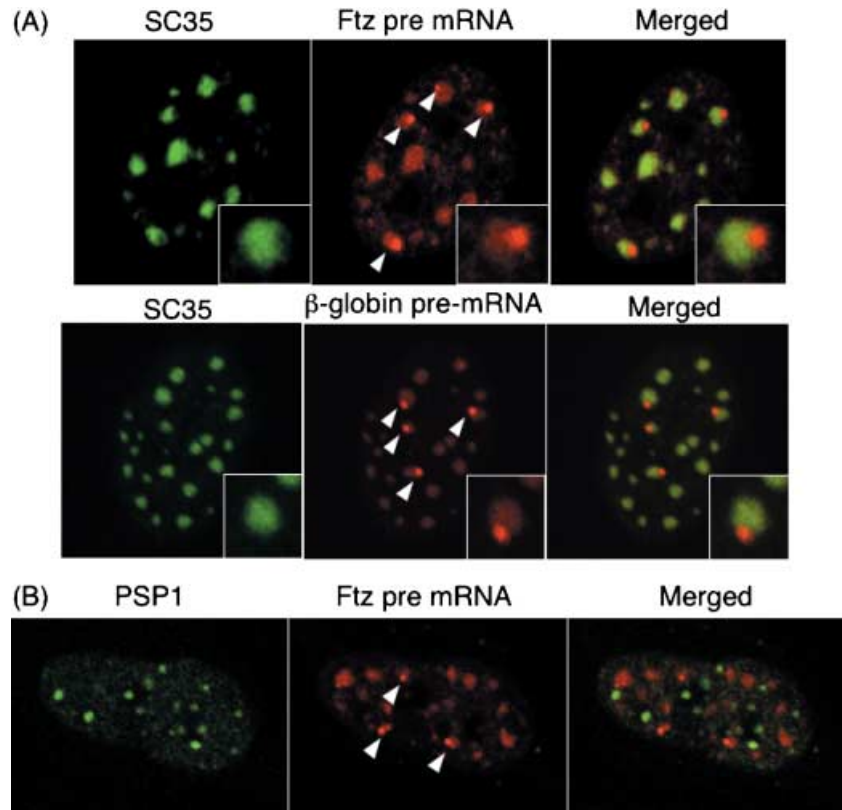


Figure 7 (A) Injected *ftz* or β -globin pre-mRNAs accumulated side by side with a few SC35 speckles in cells treated with actinomycin D for 2 h. In the merged image, red denotes the injected mRNA and green SC35, respectively. Arrowheads indicate the foci enriched with Cy3-mRNA (TIDRs). Insets show the enlarged photographs of SC35 speckles and TIDRs. Confocal images were taken by a Nikon Digital Eclipse confocal microscopy system. (B) Paraspeckle protein PSP1 does not localize in TIDRs. After treatment with actinomycin D (5 μ g/mL), HeLa cells were injected with Cy3 labeled *ftz* pre-mRNA and incubated further for 2 h. The cells were then fixed and subjected to immunostaining with anti-PSP1 antibody (Fox *et al.* 2002). Green denotes PSP1 and red the injected *ftz* pre-mRNA, respectively. Arrowheads indicate TIDRs.

injected mRNA becomes cytoplasmic. The slow export of the injected mRNAs in their system is probably due to their use of the intron-less mRNA as a substrate. In their assay system, export of the injected mRNA was inhibited by treatment with leptomycin B. In contrast to their results, leptomycin B had no significant effect on the export of injected fluorescent *ftz* pre-mRNA, while at the same concentration NES-mediated protein export was completely inhibited (Fig. 6C). Previous experiments in *S. cerevisiae*, *X. laevis* oocytes and HeLa cells suggested that CRM1-mediated export pathway is not a major mRNA export route (Fornerod *et al.* 1997; Neville & Rosbash 1999; Ossareh-Nazari *et al.* 2000). Our results also support the notion that CRM1 is not involved in the major mRNA export pathway in mammalian cells.

Unexpectedly, we found that treatment of cells with a transcriptional inhibitor, such as actinomycin D, DRB or α -amanitin, leads to a drastic inhibition of nucleocytoplasmic transport of the injected fluorescent mRNA (Fig. 6A). Those transcriptional inhibitors block the transcription by totally different mechanisms. Actinomycin D exerts its effects by binding to DNA templates, thereby interfering with the elongation of growing RNA chains, and inhibits transcription by the RNA

polymerases I to III (Kersten *et al.* 1960). DRB inhibits RNA polymerase II transcription specifically via inhibition of RNA polymerase II phosphorylation (Sehgal *et al.* 1976). α -amanitin blocks transcription by binding stoichiometrically to RNA polymerases forming a tight complex (Lindell *et al.* 1970), and inhibited the export of the injected mRNA at the concentration required for specific inhibition of the RNA polymerase II transcription. Thus, inhibition of mRNA export by the transcriptional inhibitors seems to be a result of RNA polymerase II inactivation.

In situ hybridization of HeLa cells with the oligo dT probe showed that the amount of poly(A)⁺ mRNAs localized in speckled regions does not drastically decrease after treatment with actinomycin D (Fig. 8B,C). If there are no defects in mRNA export in cells treated with the transcriptional inhibitors, then poly(A)⁺ mRNAs present in the speckled regions would decrease rapidly by virtue of their transport to the cytoplasm. It is likely that the nuclear export of endogenous poly(A)⁺ mRNAs is also blocked by inactivation of gene transcription. Shoplund *et al.* (2002) showed that, by using *in situ* hybridization, mRNAs do not disperse from SC35 domains in the nucleus when transcription is arrested. Thus, inhibition of mRNA export by transcriptional inactivation

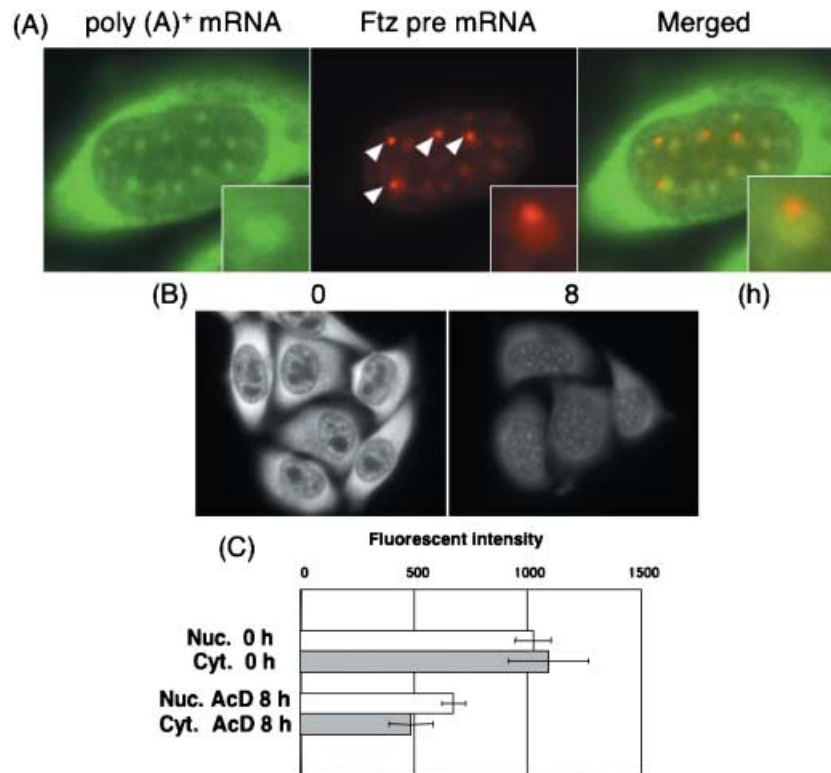


Figure 8 (A) Localization of the injected mRNA and endogenous poly(A)⁺ mRNA in cells treated with actinomycin D. The cells injected with Cy3-labeled, poly(A)⁻ *ftz* pre-mRNA were incubated at 37 °C with actinomycin D for 2 h and then subjected to *in situ* hybridization with the oligo dT probe. The poly(A)⁻ *ftz* pre-mRNA was prepared by transcribing the template DNA digested with *SacI*. (B) Nuclear export of endogenous poly(A)⁺ mRNAs was also prevented by transcriptional inactivation. HeLa cells incubated (a) without or (b) with actinomycin D for 8 h were subjected to *in situ* hybridization with the oligo dT probe. Nuclear signals in a speckled pattern remained significantly 8 h after transcriptional inactivation, whereas the cytoplasmic signals decreased drastically. (C) Quantitation of the intensities of the hybridized signals for poly(A)⁺ RNA in the nuclear speckles (Nuc.) or in the cytoplasm (Cyt.) in the absence (0 h) or presence of actinomycin D for 8 h (AcD 8 h). Fluorescent intensities were measured by IPLab Imaging Software (Scanalytics, Inc.). For quantitation of the nuclear and cytoplasmic fluorescent signals, 60 nuclear speckles selected at random and 60 spots, of which an area of a measuring circle is the same with that for a nuclear speckle, in the cytoplasm were measured using *in situ* hybridization images shown in (B). Error bars indicate standard deviation of the mean.

seems to be a general phenomenon that is not specific for injected mRNA.

As a high concentration of the translational inhibitor, cycloheximide, had no effects on mRNA export, blockage of mRNA export by the inhibition of transcription is not a secondary effect of decreased translation of factors required for mRNA export. Inactivation of gene transcription itself seems to affect the mRNA export process, raising the possibility of a functional cross-talk between gene transcription and mRNA export in mammalian cells.

Recently, functional cross-talk between transcription and mRNA export, as well as cross-talk among splicing, poly(A)⁺ addition and mRNA export, have been reported (reviewed in Reed 2003). Sub2p and Yra1p,

factors involved in nuclear export of mRNA, were shown to associate with multisubunit THO complex required for transcription elongation (Jimeno *et al.* 2002). Yeast null mutants of THO components accumulate poly(A)⁺ RNA and heat shock *SSA1* mRNA in the nuclei at the non-permissive temperature (Sträßer *et al.* 2002). The THO complex with mRNA export factors was designated TREX, which is thought to link between transcription and nuclear mRNA export. Sus1p that functions in mRNA export was shown to interact with a SAGA histone acetylase complex involved in transcription initiation (Rodríguez-Navarro *et al.* 2004). In addition, *ptr6*, one of mRNA transport mutants in *S. pombe*, was found to have a mutation in the gene encoding a transcriptional coactivator TAFII 55 (Shibuya *et al.*

1999). These findings suggest that there is a mechanism for co-transcriptional loading of export factors on to mRNAs. Such a co-transcriptional loading system, however, is not necessarily a prerequisite for formation of export competent complexes, as biologically active transport factors can be loaded on to mRNA post-transcriptionally in the *Xenopus* oocyte system and in our export assay system. Our experiments also suggested that transcription and mRNA export is linked even when mRNAs have been exogenously synthesized and microinjected.

Nup98p, a GLFG (glycine-leucine-phenylalanine-glycine) repeat containing nucleoporin, is thought to aid in directing RNAs to the nuclear pore (Griffis *et al.* 2002). Photobleaching of green fluorescent protein-Nup98p in living cells revealed that Nup98p is mobile within the nucleoplasm and dynamically associates with both the nuclear bodies (Nup98 bodies) and nuclear pore complexes (Griffis *et al.* 2002). The mobility of Nup98p within the nucleus and at the pores was highly dependent on ongoing transcription by RNA polymerases I and II. Similarly, the activity of gene transcription might also affect dynamics of mRNA export factors in the nucleus.

Interestingly, some populations of the injected fluorescent mRNAs accumulated to form foci, which we designated TIDR, in the nucleus upon arrest of gene transcription (Fig. 7A). Those TIDRs localize side by side with several SC35 speckles. Such nuclear localization of TIDRs resembles that of paraspeckles (Fox *et al.* 2002). Paraspeckles contain at least three proteins, namely, PSP1, PSP2 and p54/nrb, which re-localize to the nucleolar periphery when transcription is inhibited. Immunostaining analysis revealed that PSP1 does not reside in TIDRs (Fig. 7B). Thus, it is likely that paraspeckles and TIDRs are distinct nuclear structures. Paraspeckles were suggested to function in RNA metabolism based on the fact that the paraspeckle proteins are RNA binding proteins (Fox *et al.* 2002). Since nuclear localization patterns of TIDRs and paraspeckles are quite similar, those domains might have a functional relationship. Further experiments are underway to clarify the function of TIDRs and its relationships to paraspeckles and SC35 speckles.

Experimental procedures

Cell cultures

HeLa cells were plated on to CELLocate coverslips (Eppendorf Inc.) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in 5% CO₂.

Preparation of fluorescently labeled RNAs

For preparation of *ftz* pre- and intron-less mRNAs, pGEM-pre-*ftz* and pGEM-mature *ftz* (Rio 1988) were digested with *Hind*III and *Sac*I, and fragments containing a part of the *Drosophila fushi tarazu (ftz)* genomic gene or cDNA were inserted between *Hind*III and *Sac*I sites of pSP64-polyA (Promega). *ftz* mRNAs (497 nt) with a polyA sequence (30 nt) was prepared from plasmids digested with *Eco*RI. The linearized plasmids were transcribed in a 50 µL solution containing 40 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 10 mM DTT, 1 mM of ATP, CTP and UTP, 0.2 mM GTP, 0.8 mM m⁷GpppG (Promega), 40 U SP6 RNA polymerase and 20 U RNase inhibitor for 4 h at 40 °C. After the reaction, the template DNA was digested with DNase I.

For preparation of β-globin mRNAs, pSP64-HβΔ6 that contains a part of the human β-globin gene or pSP64-HβΔ6-IVS1,2 that contains a part of the β-globin cDNA (Kraimer *et al.* 1984) was digested with *Hind*III and *Bam*HI, and subcloned between *Hind*III and *Bam*HI sites of pSP64-PolyA.

Labeling of the transcribed mRNAs with a fluorescent dye (Cy3) was carried out using a Label IT Cy3 nucleic acid labeling kit (Pan Vera Corp) according to a manufacturer's instruction. After the reaction, the labeled RNAs were purified using spin columns (Sephadex G-50) and ethanol-precipitated. The concentration of each RNA was measured using a spectrophotometer with absorbance at 260 nm. The amount of Cy3 coupled with mRNA was measured spectrofluorometrically at an excitation wavelength of 550 nm. On the average, each RNA was labeled with ~18 molecules of Cy3 per molecule.

Nuclear microinjection of RNA

All RNA samples were dissolved in microinjection buffer (10 mM NaH₂PO₄ (pH 7.2) and 70 mM KCl) and injected at a concentration of 1.5 µM with 1.5 mg/mL of lysine fixable FITC-conjugated 70 kDa dextrans (Molecular Probes Inc.). For microinjection, we used cells cultured for 2 days after plating, which were ~80% confluent. Microinjection was done using an Eppendorf 5170 microinjector (injection pressure 50 hPa, compensation pressure 45 hPa and injection duration 0.5 s).

Detection of fluorescent signals

After injecting fluorescent mRNA into the nuclei, cells were incubated at 37 °C in 5% CO₂. To detect the fluorescent signal, cells were washed with PBS and fixed for 15 min at room temperature in 3% formaldehyde in PBS. After washing, fluorescence images of the cells were obtained using an Olympus AX70 fluorescence microscope equipped with a Photometrics Quantix cooled CCD camera.

Analysis by RT-PCR

Approximately 50 cells in a culture dish were injected with the fluorescent mRNAs and incubated at 37 °C for the indicated time.

TRIZOL reagent (Invitrogen) was added to the culture dish to lyse the cells and to isolate total RNA. Reverse transcription of the injected mRNA was carried out using Ready-to-Go You-Prime First-Strand Beads (Amersham Biosciences) and primers corresponding to 3' end of the injected *ftz* or β -globin mRNA (for the *ftz* mRNA, *ftz4*: 5'-TGGTAGCGGGTGTACGTCTG-3', and for the β -globin mRNA, 5'-GGGGATCCACGTGCAGCTTG-3'). The PCR reaction was done using the resultant cDNA sample and appropriate primers (for the *ftz* mRNA, *ftz4* and 5'-GCAGAAGCTGAAGAATGGCG-3', and for the β -globin mRNA, 5'-ACACAAGTGTGTCTACTAGC-3' and 5'-CACAGTGCAGCTCACTCAGT-3'). The amplified products were electrophoresed on an 8% polyacrylamide gel.

In situ hybridization

Cells were prepared for fluorescence *in situ* hybridization by fixing with 3% formaldehyde for 15 min at room temperature. After washing with PBS, cells were permeabilized with 0.5% triton X-100 for 5 min on ice, rinsed with PBS and 2 \times SSC. Hybridization was done at 42 °C in a solution containing 2 \times SSC, 20% formamide, 1 mg/mL of tRNA, 10% dextran sulfate and an oligo dT probe labeled with digoxigenin at the 3' end. After hybridization, cells were washed 3 \times 10 min each, in 2 \times SSC, and incubated with anti-digoxigenin antibody followed by treatment with anti-mouse IgG antibody conjugated with FITC.

Analysis of protein import and export

To examine the defects in protein import and export in HeLa cells, the *NdeI/SmaI* fragment of pR1GLFPA6 encoding a GST-NES (Pap1)-NLS (SV40)-GFP (Kudo *et al.* 1999) was subcloned into pcDNA3.1 (Invitrogen). After transfection with the resultant plasmid, HeLa cells were cultured for 20 h and then treated with leptomycin B (LMB; 50 ng/mL), actinomycin D (5 μ g/mL) or both. When cells were treated with both LMB and actinomycin D, actinomycin D was added to the culture 60 min prior to LMB addition. After incubation for 60 min, localization of the GFP fusion protein was examined using an OLYMPUS AX70 fluorescence microscope equipped with a Photometrics Quantix cooled CCD camera.

Immunofluorescence analysis

For indirect immunofluorescence analysis, cells injected with the Cy3 labeled mRNAs were fixed with 2% formaldehyde for 15 min (5 min for detection of PSP1), treated with a solution containing 0.5% BSA and 0.2% Triton X-100 for 5 min on ice, and incubated with the mouse anti-SC35 antibody (PharMingen) or the rabbit anti-PSP1 antibody (kindly provided by Dr Fox) for 1 h. Subsequently, cells were incubated with FITC-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Cappel) for 2 h and analyzed using a Nikon Digital Eclipse confocal microscopy system. For inhibition of transcription by actinomycin D, a concentration of 5 μ g/mL was used.

Acknowledgements

We thank Drs T. Andoh (Kumamoto University) and Y. Kurihara (Yokohama National University) for discussion, and M. Ohara (Fukuoka, Japan) and Kristine F. O'Brien (Brandeis University) for language assistance. We also thank Drs E.K.L. Chan (The Scripps Research Institute) and A. Fox for providing the antibodies against p80 coilin and PSP1, respectively. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a grant from the Japan Science and Technology Corporation to T.T.

References

- Andrade, L.E.C., Chan, E.K.L., Raska, I., Peebles, C.L., Roos, G. & Tan, E.M. (1991) Human autoantibody to a novel protein of the nuclear coiled body, immunological characterization and cDNA cloning of p80-coilin. *J. Exp. Med.* **173**, 1407–1419.
- Cullen, B.R. (2000) Nuclear RNA export pathways. *Mol. Cell Biol.* **20**, 4181–4187.
- Cullen, B.R. (2003) Nuclear RNA export. *J. Cell Sci.* **116**, 587–597.
- Dargemont, C. & Kühn, L.C. (1992) Export of mRNA from microinjected nuclei of *Xenopus laevis* oocytes. *J. Cell Biol.* **118**, 1–9.
- Fischer, T., Sträßer, K., Racz, A., *et al.* (2002) The mRNA export machinery requires the novel Sac3p-Thp1p complex to dock at the nucleoplasmic entrance of the nuclear pores. *EMBO J.* **21**, 5843–5852.
- Fornerod, M., Ohno, M., Yoshida, M. & Mattaj, I.W. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051–1060.
- Fox, A.H., Lam, Y.W., Leung, A.K., *et al.* (2002) Paraspeckles: a novel nuclear domain. *Curr. Biol.* **12**, 13–25.
- Fu, X.-D. & Maniatis, T. (1990) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature* **343**, 437–441.
- Griffis, E.R., Altan, N., Lippincott-Schwartz, J. & Powers, M.A. (2002) Nup98 is a mobile nucleoporin with transcription-dependent dynamics. *Mol. Biol. Cell* **13**, 1282–1297.
- Hamm, J. & Mattaj, I.W. (1990) Monomethylated cap structures facilitate RNA export from the nucleus. *Cell* **63**, 109–118.
- Huang, S., Deerinck, T.J., Ellisman, M.H. & Spector, D.L. (1994) In vivo analysis of the stability and transport of nuclear poly(A)⁺ RNA. *J. Cell Biol.* **126**, 877–899.
- Jarmolowski, A., Boelens, W.C., Izaurralde, E. & Mattaj, I.W. (1994) Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.* **124**, 627–635.
- Jimeno, S., Rondon, A.G., Luna, R. & Aguilera, A. (2002) The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability. *EMBO J.* **21**, 3526–3535.
- Kersten, W., Kersten, H. & Rauen, H.M. (1960) Action of nucleic acids on the inhibition of growth by actinomycin of *Neurospora crassa*. *Nature* **187**, 60–61.

- Krainer, A.R., Maniatis, T., Ruskin, B. & Green, M.R. (1984) Normal and mutant human β -globin pre-mRNAs are faithfully and efficiently spliced *in vitro*. *Cell* **36**, 993–1005.
- Kudo, N., Taoka, H., Toda, H., Yoshida, M. & Horinouchi, S. (1999) A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor Pap1. *J. Biol. Chem.* **274**, 15151–15158.
- Le Hir, H., Gatfield, D., Izaurralde, E. & Moore, M.J. (2001) The exon–exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. *EMBO J.* **20**, 4987–4997.
- Lei, E.P. & Silver, P.A. (2002) Protein and mRNA export from the nucleus. *Dev. Cell* **2**, 261–272.
- Lindell, T.J., Weinberg, F., Morries, P.W., Roeder, R.G. & Rutter, W.J. (1970) Specific inhibition of nuclear RNA polymerase II by α -amanitin. *Science* **170**, 447–449.
- Luo, M.J. & Reed, R. (1999) Splicing is required for rapid and efficient mRNA export in metazoans. *Proc. Natl. Acad. Sci. USA* **96**, 14937–14942.
- Luo, M.L., Zhou, Z., Magni, K., *et al.* (2001) Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. *Nature* **413**, 644–647.
- Melcák, I., Melcáková, S., Kopsky, V., Vecerová, J. & Raska, I. (2001) Prespliceosomal assembly on microinjected precursor mRNA takes place in nuclear speckles. *Mol. Biol. Cell* **12**, 393–406.
- Neville, M. & Rosbash, M. (1999) The NES-Crm1p export pathway is not a major mRNA export route in *Saccharomyces cerevisiae*. *EMBO J.* **18**, 3746–3756.
- Ossareh-Nazari, B., Maison, C., Black, B.E., Levesque, L., Paschal, B.M. & Dargemont, C. (2000) RanGTP-binding protein NXT1 facilitates nuclear export of different classes of RNA *in vitro*. *Mol. Cell. Biol.* **20**, 4562–4571.
- Reed, R. (2003) Coupling transcription, splicing and mRNA export. *Curr. Opin. Cell Biol.* **15**, 326–331.
- Rio, D.C. (1988) Accurate and efficient pre-mRNA splicing in *Drosophila* cell-free extracts. *Proc. Natl. Acad. Sci. USA* **85**, 2904–2908.
- Rodríguez-Navarro, S., Fischer, T., Luo, M.J., *et al.* (2004) Sus1, a functional component of the SAGA histone acetylase complex and the nuclear pore-associated mRNA export machinery. *Cell* **116**, 75–86.
- Segref, A., Sharma, K., Doye, V., *et al.* (1997) Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)⁺ RNA and nuclear pores. *EMBO J.* **16**, 3256–3271.
- Sehgal, P.B., Darnell, J.E. & Tamm, I. (1976) The inhibition by DRB (5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole) of hnRNA and mRNA production in HeLa cells. *Cell* **9**, 473–480.
- Shibuya, T., Tsuneyoshi, S., Azad, A.K., Urushiyama, S., Ohshima, Y. & Tani, T. (1999) Characterization of the ptr6+ gene in fission yeast: a possible involvement of a transcriptional coactivator TAF in nucleocytoplasmic transport of mRNA. *Genetics* **152**, 869–880.
- Shopland, L.S., Johnson, C.V. & Lawrence, J.B. (2002) Evidence that all SC-35 domains contain mRNAs and that transcripts can be structurally constrained within these domains. *J. Struct. Biol.* **140**, 131–139.
- Sträßer, K. & Hurt, E. (1999) Nuclear RNA export in yeast. *FEBS Lett.* **452**, 77–81.
- Sträßer, K. & Hurt, E. (2000) Yra1p, a conserved nuclear RNA-binding protein, interacts directly with Mex67p and is required for mRNA export. *EMBO J.* **19**, 410–420.
- Sträßer, K. & Hurt, E. (2001) Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. *Nature* **413**, 648–652.
- Sträßer, K., Masuda, S., Mason, P., *et al.* (2002) TREX is a conserved complex coupling transcription with messenger RNA export. *Nature* **417**, 304–308.
- Wang, J., Cao, L.-G., Wang, Y.-L. & Pederson, T. (1991) Localization of pre-messenger RNA at discrete nuclear sites. *Proc. Natl. Acad. Sci. USA* **88**, 7391–7395.
- Watanabe, M., Fukuda, M., Yoshida, M., Yanagida, M. & Nishida, E. (1999) Involvement of CRM1, a nuclear export receptor, in mRNA export in mammalian cells and fission yeast. *Genes Cells* **4**, 291–297.
- Zhou, Z., Luo, M.J., Sträßer, K., Katahira, J., Hurt, E. & Reed, R. (2000) The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. *Nature* **407**, 401–405.

Received: 8 September 2005

Accepted: 24 November 2005