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Uncapped mRNA introduced into tobacco protoplasts can be imported into the nucleus and is trapped by leptomycin B

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Abstract The mechanism of nuclear export of RNAs in yeast and animal cells is rapidly being uncovered, but RNA export in plants has received little attention. We introduced capped and uncapped fluorescent mRNAs into tobacco (*Nicotiana plumbaginifolia*) protoplasts and studied their cellular localization. Following insertion, capped transcripts were found in the cytoplasm, while uncapped messengers transiently appeared in the nucleus in about one-quarter to one-third of the cells. These mRNAs were trapped by the nuclear export-inhibiting drug leptomycin B, pointing to an export mechanism in plants similar to Rev-NES-mediated RNP export in other organisms.

Keywords mRNA · Nuclear export · Leptomycin B · *Nicotiana* protoplasts

Introduction

Cytoplasmic messenger RNAs reside in mRNA-protein complexes (mRNPs) that contain a number of proteins, including poly(A) binding proteins and a set of proteins in the 50–60 kDa size range coined core-mRNPs, which have been studied extensively in animal cells. These proteins are involved mainly in posttranscriptional regulation and transport of RNA (reviewed by Evdokimova and Ovchinnikov 1999). Intriguingly, there are hardly any data on core-mRNPs and their role in mRNA handling in plants, which is surprising considering the widespread use of wheat germ lysate for in vitro translation. It is still unknown whether nuclear export in plants is mediated by mechanisms similar to those used in other eukaryotic cells. In animal cells and yeast, nuclear export of mRNA is mainly mediated by two different processes (recently reviewed by Cullen 2003). Most mRNA is exported by a cargo system that recruits a Ran GTPase (Clouse et al. 2001; Izaurralde 2002; Perez-Alvarado et al. 2003). These mRNAs are fully processed (Zenkhusen et al. 2002). A second pathway uses exportin 1, also termed Xpo1-export or Crm1-export (Fornerod et al. 1997a,b; Stade et al. 1997; Neville and Rosbash 1999; Brennan et al. 2000; Libri et al. 2002). This process can be blocked by the *Streptomyces* metabolite leptomycin B (LMB; Hamamoto et al. 1983), which binds specifically to exportin 1 (Nishi et al. 1994; Fukuda et al. 1997). By binding to exportin 1, LMB prevents interaction of leucine-rich nuclear export signals (NES) with their export receptor (Fukuda et al. 1997; Kudo et al. 1998), thus retaining proteins and RNPs in the nuclei of yeast and other eukaryotes (Stade et al. 1997; Fornerod et al. 1997a,b; Wolff et al. 1997; Kudo et al. 1998).

It is not clear why multiple mRNA export pathways exist, and how RNAs select their carrier (or vice versa), although certain peptide motifs of RNA binding proteins might be involved in targeting mRNA to either the Crm1- or the Ran-driven pathway (Gallouzi and Steitz 2001). Using cell permeable peptide fragments derived from HuR on the one hand, as well as pp32 and APRIL, which mediate CRM1-mediated import, on the other hand, it could be shown that these particular motifs are necessary and sufficient to access one or the other nuclear export mechanism. There are no reports on the existence of, or requirement for, either of these export pathways for mRNA export in plants. Synthetic mRNAs delivered to plant cells are translated (e.g., Gallie 1991; Gallie et al. 1995; Tanguay and Gallie 1996), indicating that behavior of exogenous mRNA is physiologically relevant. While studying localization of fluorescent mRNAs in plant cells during heat stress (Stuger et al. 1999) we observed tran-

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sient localization of uncapped transcripts in the nucleus. The LMB sensitivity of mRNA export from plant nuclei strongly suggests the presence and necessity of the exportin system in plants. We loaded tobacco protoplasts with different mRNAs and found that introduced messengers could be UV-crosslinked to different proteins. Uncapped, but not capped, transcripts transiently appeared in the nucleus immediately after transformation. LMB trapped the message inside the nucleus; a role for exportin1-like proteins in nuclear mRNA export in plants is therefore likely.

Materials and methods

RNA synthesis

Messengers were transcribed with the mCap mRNA capping kit (Stratagene, Amsterdam, The Netherlands) in the presence of recombinant RNase inhibitor (Promega, Mannheim, Germany) according to standard protocols using either α-(^{32}P)-UTP (NEN, Boston, Mass.) or fluorescein-12-UTP (Roche Biochemicals, Mannheim, Germany), respectively. To synthesize uncapped RNA, the capping reagent was omitted from the reaction and the amount of GTP was increased to match the concentration of the other nucleotides.

In situ localization of RNA

Fluorescein-labeled RNA (approximately 40 pg/cell) was inserted into 15,000 tobacco (*Nicotiana plumbaginifolia*) leaf protoplasts using polyethylene glycol, and cells were fixed and collected by sedimentation as described previously (Stuger et al. 1999). The experiment was performed with three different mRNAs and was repeated twice. After attachment to polylysine-coated coverslips, cells were washed twice with 50 mM Tris/HCl, pH 7.8; 150 mM NaCl (PBS), stained with DAPI (4′,6-diamino-2-phenylindole) for 10 min, then washed with PBS again. Cells were mounted in PBS containing 75% glycerol and 0.1% phenylenediamine. DAPI- and fluorescein-labeled mRNA was photographed under a Zeiss Axiophot fluorescence microscope (Zeiss, Jena, Germany).

UV crosslinking

Radiolabeled mRNA (luc-A_{50}) was inserted into 50,000 tobacco protoplasts according to Stuger et al. (1999). After a 2 h incubation, cells were collected by 5 min centrifugation at 500 *g*, transferred to lids clipped from microcentrifuge tubes floating on ice water, irradiated with 2 J/cm^2 of 254 nm light using a UV-crosslinker (Stratagene), and lysed with 10 mM Triton X-100. The lysate was incubated with RNase A (40 μg/ml, 30 min at 37°C), proteins were precipitated with acetone and separated on a 12% SDS-PAGE gel (Laemmli 1970), which was vacuum-dried and autoradiographed. Similarly, 20 μl of a cleared tomato (*Lycopersicon peruvianum*) cell lysate (Stuger et al. 1999) was used for crosslinking with and without 100 ng yeast tRNA.

Results and discussion

Introduction of mRNA into tobacco protoplasts and association with cytoplasmic proteins

We introduced radiolabeled mRNAs into protoplasted tobacco mesophyll cells and protoplasts from tomato and

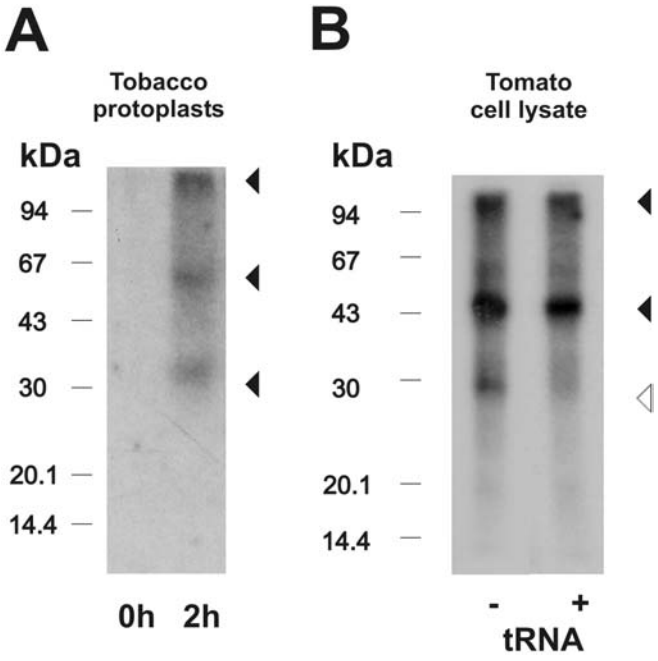

Fig. 1a, b In vivo and in vitro crosslinking. **a** After transformation with radioactive labeled luciferase (luc) mRNA (luc-A_{50}), tobacco protoplasts were harvested immediately (*left lane*) or incubated for 2 h (*right lane*). Cells were collected and the mRNA covalently attached to proteins by UV-crosslinking. **b** A nucleus-free tomato cell lysate was incubated with ^{32}P-labeled mRNA (luc-A_{30}) in the presence (*right lane*) or absence (*left lane*) of 100 ng yeast tRNA. After crosslinking, proteins were separated by SDS-PAGE. The position and size (kDa) of marker proteins is indicated on the left of each panel. *Arrowheads* Cross-linked proteins (note that tRNA competes with a protein at 30 kDa (*open arrowhead* in **b**), while the other prominent protein at 50 kDa was not affected)

Arabidopsis cell cultures. For polyethylene glycol-mediated transformation, we used about 40 pg radiolabeled capped firefly luciferase (luc) mRNA per cell. While no mRNA could be re-isolated from tomato or Arabidopsis cells, half of the RNA could be recovered from the tobacco protoplasts after transformation. Approximately 15–20% of the added RNA (i.e., 30–40% of the RNA that entered the cells) was still intact after 2 h, as judged from gel electrophoresis and autoradiography (not shown). This is in agreement with a half-life of 100 min for luc mRNA as reported by Gallie (1991). To check the quality of the re-isolated mRNA, we analyzed its ability to bind RNP proteins. To detect binding of cellular proteins to the introduced messengers, cells loaded with radioactive mRNA were irradiated with UV light, lysed, and treated with RNase A. Three labeled bands were visible on an SDS-PAGE gel (Fig. 1a), at about 30, 50, and over 90 kDa. When labeled mRNA was crosslinked to proteins in a tomato cell lysate, the 30 kDa band disappeared upon addition of excess tRNA (Fig. 1b). SDS-PAGE gels of purified mRNP particles from *Xenopus* oocytes contained protein bands of similar size. A protein of about 110 kDa was identified as nucleolin, the 50 kDa band contained mRNP 3+4, a known translational regulator (Yurkova and Murray 1997). The 30 kDa protein bound any nucleotide

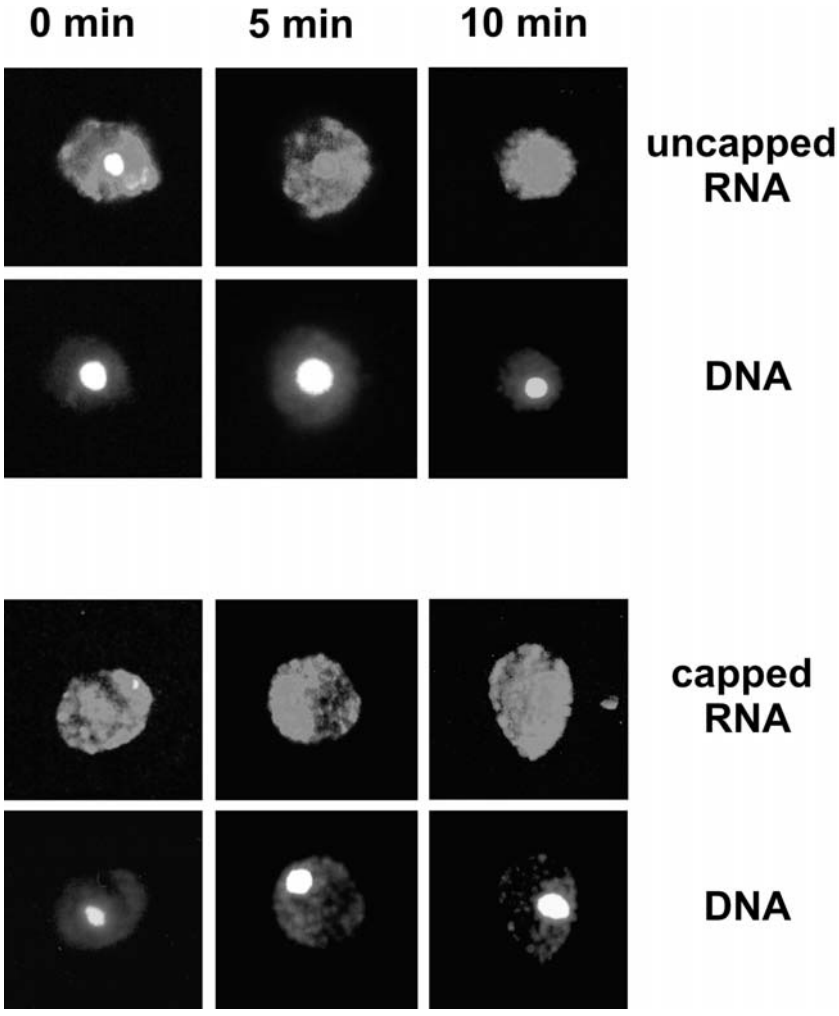

Fig. 2 Cap-dependent nuclear import of mRNA. Fluorescein-labeled luciferase mRNA without (*upper panels*) and with (*lower panels*) a 5′ rr^{5}GpppG cap was introduced into tobacco protoplasts. Cells were fixed and analyzed after transformation at the times indicated. *Top rows*: RNA fluorescence. *Bottom rows*: DNA (DAPI) fluorescence. Background in DAPI images is due to staining of chloroplast and mitochondrial DNA. DAPI images were slightly overexposed to visualize chloroplast DNA in addition to nuclei

triphosphate in *Xenopus* oocytes (Meric et al. 1997), which may explain why unlabeled tRNA competed with mRNA for binding to the 30 kDa protein in the tomato cell extract. The UV crosslinking patterns, together with translation of luc mRNA introduced in tobacco protoplasts (Gallie 1991), indicate that synthetic mRNAs introduced into tobacco protoplasts bind cytoplasmic mRNA-binding proteins in a physiologically relevant way and therefore might behave like endogenous messengers.

Uncapped but not capped mRNA is rapidly imported into the nucleus of tobacco protoplasts

The cellular localization of inserted messengers was resolved within the first 10 min after introduction using fluorescein-labeled transcripts. Introduction of fluorescent RNA into cells has been successfully employed to study nuclear import of RNase MRP RNA (Jacobson et al. 1995) and sn(o)RNAs (Jacobson and Pederson 1998a; Lange et al. 1998a,b), nuclear localization of srpRNA (Jacobson and Pederson 1998b), and cytoplasmic distribution of mRNAs during heat stress (Stuger et al. 1999). Figure 2 shows the location of luciferase RNA (fluorescein) and DNA (DAPI) inside tobacco protoplasts. Virtually all cells showed sufficient fluorescence to assess subcellular localization. Uncapped mRNA entered the nucleus in around one-quarter to one-third of transformed cells immediately following introduction (Fig. 2, top row), while transcripts with a 5′m7-GpppG cap remained in the cytoplasm (Fig. 2, third row). We observed the same for tomato histone H4 and HSP17 mRNAs (not shown). We do not know the mechanism by which uncapped mRNAs enter the nucleus. However, transfer of RNAs from cytoplasm to nucleus is not uncommon, and has been extensively studied for 5S rRNA and snRNAs (DeRobertis et al. 1982; Fischer et al. 1991; Michaud and Goldfarb 1992). Since the presence or absence of the cap was the only initial difference between the RNAs, interaction with cap-binding proteins may have prevented nuclear import of the capped messenger. Within 5 min most of the RNA was found in the cytoplasm, after 10 min hardly any nuclear localization was visible anymore, both for capped and uncapped transcripts (Fig. 2). Similar results were obtained with messengers coding for tomato histone H4 protein and HSP17.6 (not shown). It is possible that the uncapped RNA acquired a cap inside the nucleus. However, experiments using tobacco and other

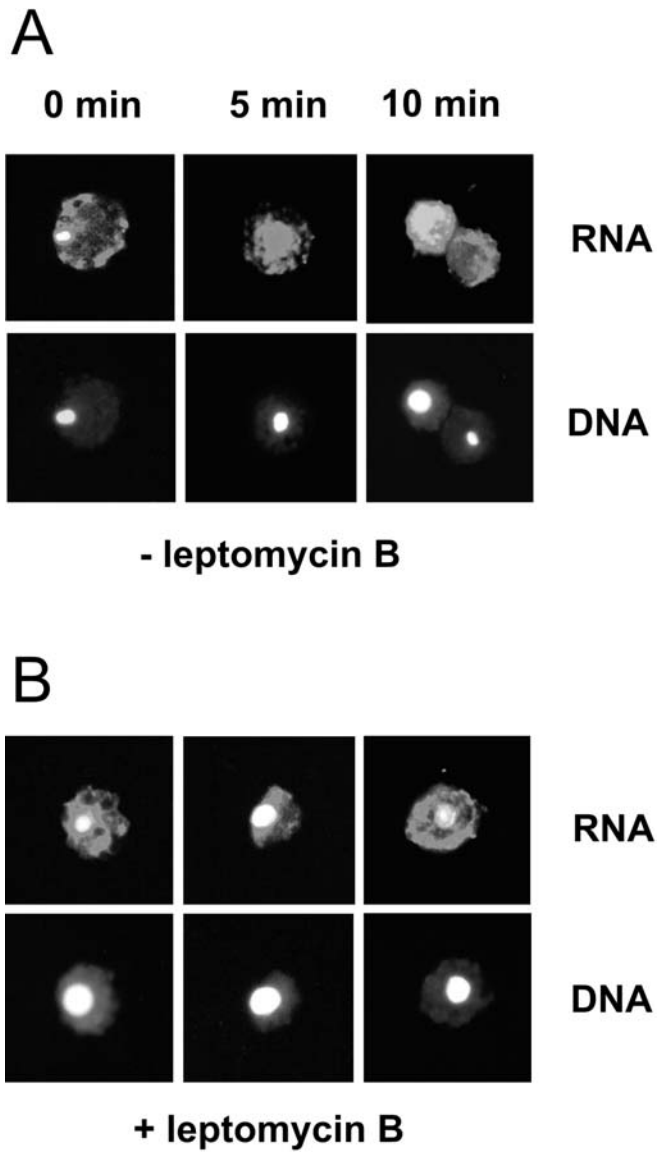

Fig. 3a, b Nuclear retention of uncapped mRNA by leptomycin B (LMB). Fluorescein-labeled luciferase mRNA was introduced into tobacco protoplasts incubated **a** without and **b** with LMB. Cells were fixed and analyzed after transformation at the times indicated

plant protoplasts indicate that translation of uncapped messengers is far less efficient than that of capped transcripts (e.g., Gallie 1991), which argues against such a scenario. Either way, several studies indicate that a cap is not required for nuclear export (reviewed by Izaurralde and Adam 1998).

Nuclear export of mRNA in tobacco protoplasts is blocked by LMB

When tobacco protoplasts were treated with 100 nM LMB, 10 min prior to transformation, we found that nuclear mRNA was trapped (Fig. 3, third row), while untreated cells export labeled transcripts (Fig. 3, top row). Capped transcripts still resided in the cytoplasm (not shown), indicating that these messengers indeed did not enter the nucleus, as opposed to an import/export event too fast to be detected in this experimental system, Since LMB blocks interaction of NESs with their receptors, our results suggest that export of mRNA in tobacco is mediated by NES-carrying proteins and possibly involves a plant counterpart of exportin1/Crm1p. This is in line with results from Stade et al. (1997), who showed that mRNA export in *Saccharomyces cerevisiae* utilizes such a pathway and Brennan et al. (2000), who could demonstrate that human genes containing AU-rich elements were retained in the nucleus after application of LMB. The LMB-sensitivity of mRNA export in our experiments with tobacco protoplasts, the LMB-insensitivity of most mRNA export in mammalian cells and *Xenopus* oocytes, and the conflicting data for yeast suggest that pathways for mRNA export can vary considerably among and within species. The question of which pathways and receptors are used in different systems will probably continue to present a challenge for some time to come.

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