A nucleoporin, Nup60p, affects the nuclear and cytoplasmic localization of *ASH1* mRNA in *S. cerevisiae*

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ABSTRACT

The biogenesis of a localization-competent mRNP begins in the nucleus. It is thought that the coordinated action of nuclear and cytoplasmic components of the localization machinery is required for the efficient export and subsequent subcellular localization of these mRNAs in the cytoplasm. Using quantitative poly(A)$^+$ and transcript-specific fluorescent in situ hybridization, we analyzed different nonessential nucleoporins and nuclear pore-associated proteins for their potential role in mRNA export and localization. We found that Nup60p, a nuclear pore protein located on the nucleoplasmic side of the nuclear pore complex, was required for the mRNA localization pathway. In a *Δnup60* background, localized mRNAs were preferentially retained within the nucleus compared to nonlocalized transcripts. However, the export block was only partial and some transcripts could still reach the cytoplasm. Importantly, downstream processes were also affected. Localization of *ASH1* and *IST2* mRNAs to the bud was impaired in the *Δnup60* background, suggesting that the assembly of a localization competent mRNP ("locasome") was inhibited when *NUP60* was deleted. These results demonstrate transcript specificity of a nuclear mRNA retention defect and identify a specific nucleoporin as a functional component of the localization pathway in budding yeast.

Keywords: ASH1; NUP60; RNA localization; in situ hybridization; mRNA export

INTRODUCTION

In eukaryotic cells, subcellular localization and local translation of specific mRNAs is a widespread mechanism for establishment of cellular polarity and determination of cell fate (Du et al. 2007). Generation of a localization-competent mRNP is initiated in the nucleus by the association of localization specific factors with the mRNP that mark mRNAs for their cytoplasmic localization (Farina and Singer 2002; Kruse et al. 2002; Du et al. 2007), Localized mRNAs are subject to many of the same regulatory steps as non localized mRNAs, as they have to be processed, exported to the cytoplasm, translated, and finally degraded. This has led to two classifications of factors that affect mRNA function and fate: general or transcript-specific. While many examples

of transcript-specific translation and degradation factors have been described, little is known concerning factors that control mRNA export (Hieronymus and Silver 2003; Gerber et al. 2004; Keene 2007).

Under normal cellular conditions, mRNAs are efficiently exported from the nucleus. When detected by in situ hybridization using oligo dT probes, minimal poly(A)$^+$ signal is present within the nucleus. In screening for nuclear poly(A)$^+$ RNA retention using temperature-sensitive mutants as well as single gene deletion strains, initial studies identified many genes involved in mRNA export (Gorsch et al. 1995; Heath et al. 1995; Li et al. 1995; Saavedra et al. 1996; Dockendorff et al. 1997). For the essential genes identified by nuclear poly(A)$^+$ retention, the lethal phenotype was thought to be caused by a general defect of mRNA export. For nonessential genes whose deletions show a nuclear accumulation of poly(A)$^+$ RNA, two possible explanations for their partial poly(A)$^+$ retention phenotype can be proposed. They either affect the export of all mRNAs, although to a lesser extent than the essential export factors, or they have a selective effect on a subset of transcripts.

Determining whether these nonessential genes have a general or a transcript-specific effect on mRNA export is critical for understanding the mechanism of nuclear mRNA export. In addition, it has yet to be established how the disruption of such early processes affects the subsequent steps in the mRNA biogenesis pathway (e.g., for localized mRNAs how mRNA localization is affected).

Once exported to the cytoplasm, a subset of mRNAs in *Saccharomyces cerevisiae* is localized to the bud tip of daughter cells in an active process that is dependent upon the cooperation between *cis*-acting elements within the mRNA and *trans*-acting protein factors. Initially, five *trans*-acting factors were identified genetically. These *SHE* genes are required for the bud-tip localization of the *ASH1* mRNA (Jansen et al. 1996; Beach et al. 1999; Long et al. 2001). Three of these factors have been extensively studied: She2p binds directly to the *cis*-acting elements in *ASH1* mRNA, She1p/Myo4p transports the mRNA on actin cables to the bud tip, and She3p links She2p with She1p/Myo4p. She2p can be found in the nucleus and the cytoplasm but She1p/Myo4p and She3p are only found in the cytoplasm (Kruse et al. 2002; Niessing et al. 2004; Shen et al. 2009, 2010). Subsequently, other proteins have been identified that also affect RNA localization in yeast. One (Puf6p) was found to be a component of the localizing mRNP (locasome) (Gu et al. 2004), while others (Loc1p, Khd1p, Puf5p, Scp160p) (Long et al. 2001; Irie et al. 2002) clearly show a role in localization though they appear not to be a part of the locasome. It is believed that locasome assembly begins in the nucleus sometime between transcription and export. For example, the RNA-binding protein She2p can accumulate in the nucleus when RNA export is inhibited in a *mex67-5* strain or when the N-terminal 70 amino acids of She2p are deleted, suggesting that it binds to the mRNA at a very early step, maybe even cotranscriptionally (Kruse et al. 2002). Further evidence indicates that She2p is associated with RNAP II before ''jumping'' to the *ASH1* nascent chains (Shen et al. 2010). Other proteins required for localization (e.g., Puf6p, a predominantly nuclear protein that serves as a translational repressor), are also believed to bind *ASH1* mRNA while still within the nucleus (Gu et al. 2004). These nuclear events may play a critical role in the identification of a localized transcript. In this study we surmised that identification of a localized mRNA may occur at the nuclear pore, since that is a possible point for the differentiation of transcripts (Bystricky et al. 2009). We therefore chose to examine nonessential nuclear pore and nuclear pore-associated proteins with regard to mRNA export. We show that one of these, Nup60p, specifically affects the fate of localized mRNAs in *S. cerevisiae*.

Nup60p is a nonessential nucleoporin that is exclusively localized to the nucleoplasmic side of the nuclear pore and is part of the nuclear basket structure (Rout et al. 2000). Structurally, it is a FG nucleoporin (Nup), that also contains a putative RNA-binding RRM motif (Devos et al.

2006). Deletion of *NUP60* has been previously reported to retain poly(A)$^+$ RNA and has been proposed to be involved in retention of pre-mRNA in the nucleus (Galy et al. 2004; Palancade et al. 2005). Nup60p also acts as a tether for the localization of proteins to the nuclear periphery. One example of this is the mobile nucleoporin Nup2p, which is localized to the perinuclear region by its association with Nup60p (Dilworth et al. 2005). Additionally, the myosin-like proteins (MLPs) are also tethered to the nuclear periphery through their association with Nup60p (Zhao et al. 2004). A role for Nup2p in the perinuclear localization of actively transcribing *GAL* loci has previously been described (Schmid et al. 2006). The perinuclear localization of the *GAL* transcription sites, as well as those for *KHK1* and *INO1*, has also been correlated with an increase in the transcription of these genes (Brickner and Walter 2004; Schmid et al. 2006; Taddei et al. 2006; Sarma et al. 2007), lending support to the view that the NPC basket affects the process of gene expression.

Here we report a method for the quantification of mRNA export based on fluorescent in situ hybridization (FISH) data. We examine the poly(A)$^+$ retention defects of several nonessential gene deletions and show that retention strength varies for different genes. Subsequently, using a *NUP60* deleted strain as a model, we employ single molecule sensitivity FISH to examine if a mild poly(A)$^+$ retention phenotype is caused by affecting the export of specific mRNAs. Our analysis reveals that She2p-localized mRNAs are inefficiently exported in *Δnup60* cells. Furthermore, those mRNAs fail to properly localize when exported. Our data suggests a functional role for a NPC component in the localization of a subset of transcripts, underlying the importance of nuclear events on the cytoplasmic fate of mRNAs.

RESULTS

Differential poly(A)$^+$ RNA retention in deletion mutants of nuclear periphery-associated proteins

In order to identify nonessential genes with partial mRNA export defects we performed FISH using an oligo dT probe on eight deletion strains of nonessential nucleoporins and nuclear pore-associated proteins and quantified their nuclear poly(A)$^+$ RNA retention phenotype (Fig. 1). None of the strains displayed significant growth defects compared to wild-type cells. Wild-type (K699) and *mex67-5* strains were used as negative and positive controls, respectively (Fig. 1A). To quantify the mRNA export defect, we measured the total fluorescence intensities (TFI) of the poly(A)$^+$ signal for the entire cell and of the nucleus (as determined by DAPI staining, see Materials and Methods). Using these values, we determined the nuclear content of poly(A)$^+$ RNA for individual cells of a particular strain. The wild-type strain showed that ~8% of the total cellular poly(A)$^+$ RNA signal the nucleus. Levels of mRNA retention were
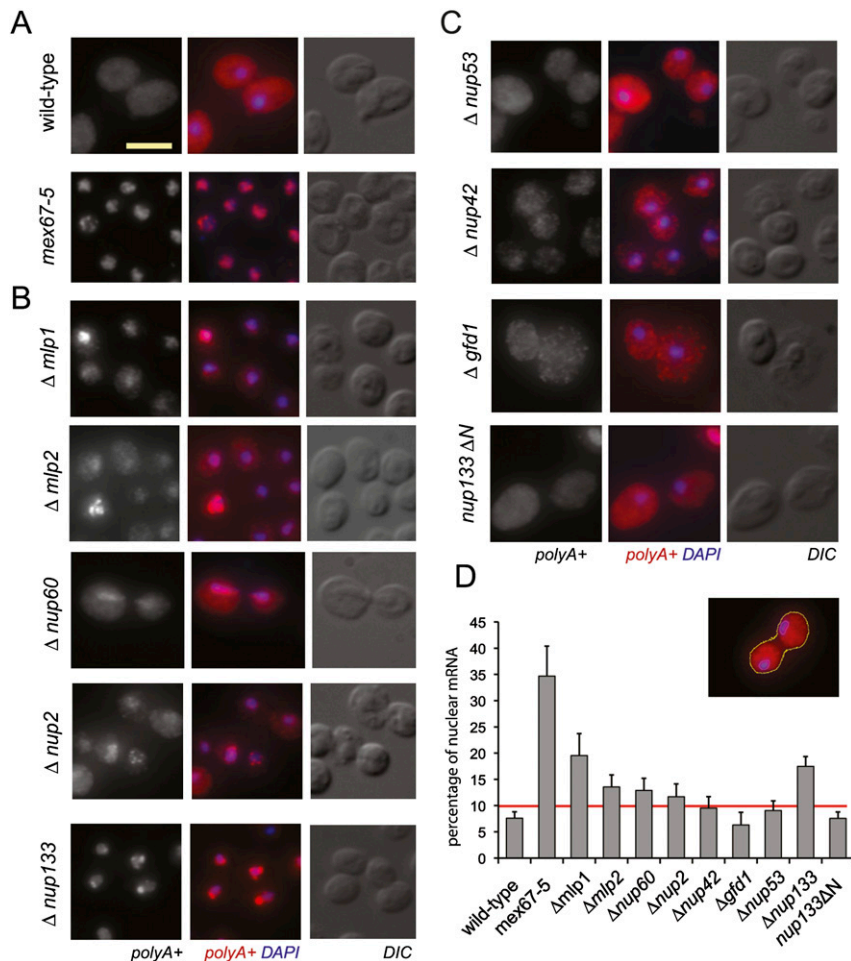
FIGURE 1. Differential poly(A)$^+$ RNA retention in nonessential nucleoporin deletion strains. Fluorescent in situ hybridization using a Cy3 labeled oligo-dT probe of strains deleted for specific nuclear pore and associated proteins to determine poly(A)$^+$ RNA distribution. Cells were grown at 30°C. (A) Wild type and mex67-5 (37°C). (B) Strains showing poly(A)$^+$ retention phenotype. (C) Strains without significant nuclear retention of poly(A)$^+$ RNA. (D) Histogram showing the relative amount of nuclear poly(A)$^+$ RNA. The nuclear poly(A)$^+$ signal was determined by segmenting nuclear and cytoplasmic boundaries and quantifying the ratio of nuclear-to-cytoplasmic signal. (Inset) Borders are drawn segmenting out the DAPI (magenta border) from the whole cell (yellow border). The line drawn at 10% retention indicates detectable enrichment of poly(A)$^+$ signal in the nucleus. Scale bar is 5 µm.

$\Delta nup133$ strain showed accumulation of poly(A)$^+$ RNA at one or two foci within the nucleus. A NUP133 deletion strain displays clustering of nuclear pores at one to two foci, suggesting that transcripts might be localized specifically to the clustered nuclear pores (Heath et al. 1995; Li et al. 1995; Pemberton et al. 1995). However, pore clustering was not sufficient for RNA retention, as an N-terminal truncation of NUP133, which retained the pore clustering phenotype, did not retain the poly(A)$^+$ mRNA. This was reflected in our results as the nuclear poly(A)$^+$ levels were wild type (7.5% ± 1.9%) in the NUP133ΔN strain (Fischer et al. 2002; Libri et al. 2002; Dieppois and Stutz 2010). While many of the retention defects were quantitatively similar, we observed variation within single mutant populations. Specifically, $\Delta mlp1$, $\Delta mlp2$, $\Delta nup60$, and $\Delta nup2$ strains showed a mild export defect in most cells but a more severe defect in a subset of cells. Such sporadic retention of poly(A)$^+$ RNA was never seen in wild-type cells. This might suggest partial penetrance of the deletion phenotype in some cells. Alternatively, it could signify that a particular class of mRNAs expressed in these cells is dependent on a specific nucleoporin for export. To determine if specific classes of mRNAs are affected by the deletion of nonessential nucleoporins (Heath et al. 1995; Pemberton et al. 1995), we employed a FISH-based approach that allowed for the detection of single mRNA molecules in the context of a NUP60 deletion strain.

quantified for each deletion strain (Fig. 1D). Strains with an obvious retention of poly(A)$^+$ RNA had nuclear signal that was >10% of the total cellular fluorescence (Fig. 1B). Strains in which the nuclear content of poly(A)$^+$ RNA was <10% of the total poly(A)$^+$ were categorized as nonretaining (Fig. 1C). As described previously, the nuclear poly(A)$^+$ signal in many strains affecting mRNA export was not uniformly distributed in the nucleus, but showed a granular staining. This granular poly(A)$^+$ staining, commonly seen in the mex67-5, $\Delta mlp1$, $\Delta mlp2$, $\Delta nup60$, and $\Delta nup2$ strains, might likely be the result of mRNAs retained at the site of transcription, a phenotype that has been described for a subset of mRNA export and processing mutants (Fischer et al. 2002; Libri et al. 2002; Dieppois and Stutz 2010). The

Localized mRNAs are specifically retained in a NUP60 deletion

One difficulty in determining the specificity of mRNA export mutants in the past has been the inability to quantify, precisely and accurately, the distribution of specific mRNAs. This has especially been a problem in yeast, where most mRNAs are present in very low copy numbers (Holstege et al. 1998; Zenklusen et al. 2008; Larson et al. 2009). Therefore, we employed a single molecule counting approach using oligonucleotide probes labeled with multiple fluorescent dyes against a single gene (Zenklusen et al. 2008; Zenklusen and Singer 2010). FISH was performed on wild-type and $\Delta nup60$ strains using gene-specific probe sets.

To determine the extent of nuclear retention, the number of nuclear and cytoplasmic transcripts within each individual cell was quantified and expressed as the percent of total cellular transcripts contained within the DAPI signal (Fig. 2; see Materials and Methods).

Transcripts of seven different genes were assayed, chosen based on diversity of function and regulation. *ASH1* and
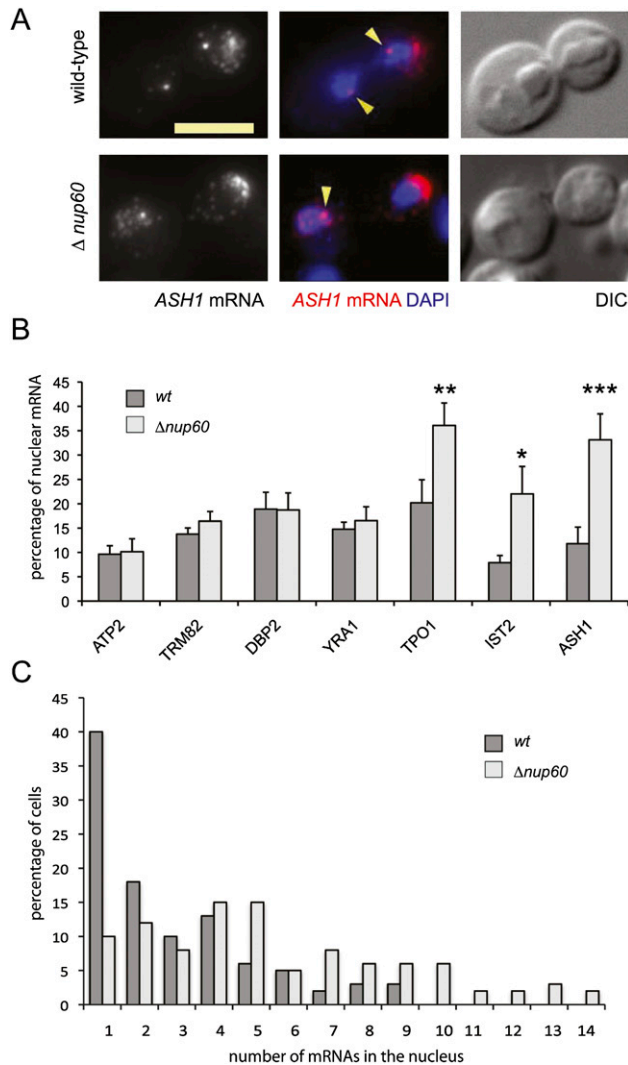
FIGURE 2. Deletion of *NUP60* leads to nuclear retention of localized transcripts. (*A*) In situ hybridization of *ASH1* mRNA in wild-type and *Δnup60* strains. Retention of transcripts in the nucleus is observed in the *Δnup60* mother cell. Arrowheads indicate transcription sites. Retention is not seen in the *Δnup60* daughter cell, where the *ASH1* gene is not being transcribed. (*B*) Quantification of transcript-specific in situ hybridizations for seven genes with different expression profiles and cellular localization in wild-type and *Δnup60* strains. Only the localized *IST2*, *TPO1*, and *ASH1* mRNAs show enhanced accumulation of nuclear transcripts in *Δnup60* over wild-type strains (*P = 0.011, **P = 0.0007, ***P = 0.0001). (*C*) Wild-type cells clear transcripts from the nucleus faster than *Δnup60* cells. Number of total nuclear *ASH1* mRNAs were counted in wild-type or *Δnup60* cells (n = 61 cells for each strain). The percent of total cells containing one to 14 mRNAs per nucleus is shown.

TPO1 are cell-cycle regulated genes, peaking in mitosis (Cho et al. 1998; Spellman et al. 1998). *DBP2* and *YRA1* encode genes with introns (Barta and Iggo 1995; Strasser and Hurt 2000; Dong et al. 2007). *ASH1*, *IST2*, and *TPO1* mRNAs localize to the bud tip of daughter cells using the She2p-dependent localization pathway (Shepard et al. 2003). *ATP2* is a constitutively expressed nuclear-encoded mitochondrial protein, and *TRM82* is a constitutively expressed housekeeping gene. Messenger RNAs that showed a significant nuclear retention defect were all transcripts that are localized to the bud tip of dividing yeast cells, whereas the other genes showed no effects. *ASH1*, *IST2*, and *TPO1* mRNAs showed significant increases in the percentage of total transcripts retained in the *Δnup60* nucleus (Fig. 2) (*ASH1* threefold [12% ± 3 in wild type, 37% ± 6* in *Δnup60*], *IST2* threefold [6% ± 1.3 in wild type, 19% ± 7.8 in *Δnup60*], and *TPO1* 1.5-fold [25% ± 3.8 in wild type, 39% ± 5.9 in *Δnup60*]). This indicated that Nup60p is required for efficient export of this specific class of mRNAs.

To test if this retention phenotype was specific for *Δnup60*, or if deletion of other nuclear proteins required for RNA localization show a similar phenotype, we analyzed the mRNA distribution of *ASH1*, *IST2*, and *TPO1* in a *puf6* deletion background (Gu et al. 2004). Puf6p is a predominantly nuclear protein required for efficient *ASH1* mRNA localization and translation, however, it is not known at what step of locasome assembly Puf6p associates with *ASH1* mRNAs (Gu et al. 2002; Thomsen et al. 2003; Gu et al. 2004; Deng et al. 2008). Nuclear retention of *ASH1*, *IST2*, and *TPO1* mRNAs was not affected in a *Δpuf6* background. Similarly, retention of the nonlocalized *ATP2* and *YRA1* mRNAs was not affected in wild type, *Δnup60*, and *Δpuf6* strains. For example, *ATP2*'s mRNA retention value is 9.6 ± 1.7, 10.1 ± 2.7, and 9.0 ± 1.8 for wild type, *Δnup60*, and *Δpuf6*, respectively. This suggests that the phenotype is specific for *Δnup60* in combination with localized mRNAs. Since *ASH1* is the best-studied example of a localizing transcript in yeast, we focused our analysis on characterizing the effect of a *Δnup60* deletion on *ASH1* mRNA biogenesis and localization.

mRNA retention does not occur at the site of transcription

Mutations in different mRNA processing and export mutants have been shown to cause accumulation of nascent mRNAs at the site of transcription. This phenotype has been extensively studied using galactose-induced (GAL) and heat-shock genes, for which an intense nuclear mRNA dot colocalizing with the GAL or heat-shock gene loci had been observed in different mutant strains. The mechanism by which this retention occurs is not yet fully understood (Dieppois and Stutz 2010). To characterize whether the mRNA export phenotype of a *Δnup60* deletion strain was

similar, we compared the *ASH1* mRNA nuclear accumulation phenotype of a wild-type and a *Δnup60* strain to a *mex67-5* strain, which has been shown to block mRNA export and lead to the accumulation of mRNAs at their site of transcription (Segref et al. 1997; Jensen et al. 2001). We therefore counted the number of mRNAs in the nuclei of wild type and *Δnup60* (Fig. 2C). In most wild-type cells, a single, bright nuclear *ASH1* mRNA signal was present, representing the site of transcription where multiple nascent mRNAs are associated with the *ASH1* gene. We rarely detected mRNAs in the nucleoplasm, indicating that export of *ASH1* mRNA was rapid in wild-type cells (Fig. 2C). However, in *Δnup60*, a distribution of nuclear mRNAs ranging from one to 14 transcripts was seen. Approximately 40% ± 7.3% of the total cellular mRNAs was contained within the nucleus. In the *mex67-5*, this increases to 68% ± 2.6% and can be detected throughout the nucleus indicating that transcripts were released from the site of transcription into the nucleoplasm, but not exported to the cytoplasm and not retained at the site of transcription (Supplemental Fig. 1). By contrast, *SSA4* mRNAs are retained at the site of transcription in a *mex67-5* strain (Thomsen et al. 2003). One possible explanation for this phenotype could be that heat-shock mRNAs, such as *SSA4*, utilize a different export mechanism (Saavedra et al. 1997). However, it is also possible that the inability to identify nucleoplasmic *SSA4* mRNAs in the earlier studies was due to the lack of sensitivity of the FISH protocol used, which only allowed detection of multiple mRNAS at the site of transcription, but not the detection of single mRNAs within the nucleoplasm. Furthermore, *ASH1* mRNAs are detected in the cytoplasm of a *Δnup60* deletion stain, suggesting that export kinetics were not blocked, but slower in the *Δnup60* strain, selective for localized mRNAs.

Among the genes sampled, *ASH1* is the highest in its expression. Possibly, the *Δnup60* created a rate-limiting step that affected highly expressed genes most obviously. To test whether other highly expressed genes show a partial export defect in a *Δnup60* background due to a decrease in export rates, we determined mRNA export efficiency of four highly transcribed mRNAs in wild-type and *Δnup60* strains. *ENO1* and *CCW12*, encoding a glycolytic enzyme and a cell wall component, respectively, are constitutively expressed at very high levels (61 and 81 mRNAs per cell); and *GAL1* and *GAL10* mRNAs are strongly induced by galactose. Due to the very high expression of these mRNAs, single mRNA counting was difficult, so we determined if nuclear mRNAs were detected in wild-type or a *Δnup60* background (Supplemental Fig. 2). Despite the high expression, an increased nuclear mRNA signal was not observed, confirming that mRNA export is an efficient process in yeast and that nuclear accumulation of mRNAs in *Δnup60* is specific for localized mRNAs.

Deletion of *NUP60* results in decreased expression of *ASH1* and movement of its transcription site away from the nuclear periphery

The retention of *ASH1* mRNA in the nucleus may be related to a perturbation of the site of transcription. Nup60p is responsible for the peripheral localization of Nup2p, as well as Mlp1p and Mlp2p (Hood et al. 2000; Dilworth et al. 2001, 2005; Zhao et al. 2004). Nup2p is also responsible for perinuclear tethering and transcriptional activity of the *GAL* genes (Zhao et al. 2004; Schmid et al. 2006; Taddei et al. 2006). *ASH1*, like the *GAL* genes, contains a SAGA promoter (Huisinga and Pugh 2004), suggesting that the active *ASH1* locus might anchor to the nuclear periphery by the same mechanism as the *GAL* genes. We therefore investigated whether the intranuclear position of the *ASH1* transcription site was altered in a *Δnup60* strain. The position of transcription sites was scored as peripheral, subperipheral, or internal (Fig. 3; see Materials and Methods) according to the three-zone analysis of gene loci localization that has been used previously to determine the position of genes relative to the nuclear periphery (Dilworth et al. 2001; Hediger et al. 2002; Casolari et al. 2004; Abruzzi et al. 2006; Cabal et al. 2006; Taddei et al. 2006). We observed that in wild-type cells, *ASH1* transcription sites were predominantly peripheral (Fig. 3). However, upon deletion of *NUP60*, *ASH1* transcription sites were approximately distributed evenly between the three zones.

A previous study (Taddei et al. 2006) showed that peripherally localized genes were more highly transcribed when they are properly (peripherally) localized than when the same transcription sites are internalized. In order to determine whether *Δnup60* had an effect on the overall levels of mRNA, we measured the mRNA abundance for the initial
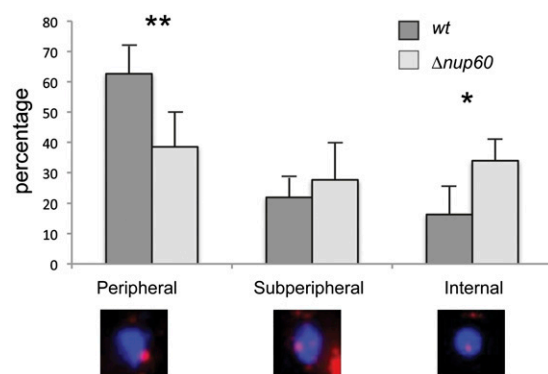
FIGURE 3. Distribution of *ASH1* transcription sites upon deletion of *NUP60*. FISH using *ASH1* mRNA-specific probes were performed in wild-type and *Δnup60* cells. 3D data sets (30 z-planes in 200 nm intervals) were acquired for *ASH1* mRNA and the DAPI signal, merged, and the position of the transcription site relative to the border of the nucleus was determined in 3D. Representative images (z-projections) of the scoring phenotypes are shown on the *bottom* of the graph. One hundred and two wild-type and 90 *Δnup60* transcription sites were scored (*P = 0.024, **P = 0.016).

set of seven genes screened in wild-type and *Δnup60* cells using single molecule sensitivity FISH (Zenklusen et al. 2008). The overall cellular levels of the three localized genes were decreased in the absence of *NUP60* (Fig. 4). *ASH1* mRNA levels were reduced by 49% in *Δnup60* cells (40 ± 11 molecules/cell vs. 20 ± 4 molecules per cell, P = 0.0007). *IST2* and *TPO1* levels were also reduced by 38% and 57%, respectively. The control mRNAs *ATP2* and *TRM82* did not significantly change their transcripts levels per cell. In contrast, the spliced *YRA1* and *DBP2* mRNAs both showed increased numbers of transcripts when *NUP60* was deleted. The average number of *YRA1* mRNAs per cell increased 48% from 14 ± 1 to 20 ± 2 and *DBP2* mRNAs increased 128% from 4 ± 1 to 9 ± 3. Nup60p has been previously implicated in the retention of improperly spliced transcripts within the nucleus (Galy et al. 2004) and as part of a perinuclear mRNA surveillance complex (Skružný et al. 2009). *YRA1* mRNA levels are also regulated by a feedback mechanism involving the export of *YRA1* pre-mRNA mRNAs to the cytoplasm (Dong et al. 2007). It is therefore possible that the deletion of *NUP60* led to an increase in leakage of unspliced or improperly spliced transcripts to the cytoplasm, resulting in the increased mRNA levels observed.

Reduced transcript levels can be caused either by a decrease in the rate of synthesis or an increase in the rate of degradation. In order to distinguish between these possibilities, we calculated the number of *ASH1* transcripts produced in both wild-type and *Δnup60* strains by determining the average polymerase loading on the *ASH1* gene. Transcription sites were defined as nuclear spots containing more than one transcript in order to distinguish the site from single nuclear mRNAs. Both wild-type and *Δnup60* strains showed similar transcript loads at the transcription site (3.14 ± 1.14 for wild type and 2.86 ± 0.96 for *nup60*), indicating a similar transcription frequency in the two strain
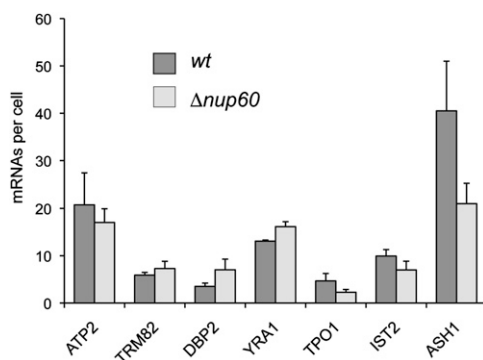
FIGURE 4. mRNA abundance is affected upon deletion of NUP60. The total number of transcripts for genes indicated was measured on an individual cell level from FISH results in wild type (K699) and *Δnup60* and plotted as a histogram. The localized mRNAs show significant decreases in transcripts comparing wild type with the *Δnup60* strains; spliced mRNAs show increases. *P*-values are listed in order of decreasing significance: *YRA1*: P = 0.0001, *ASH1*: P = 0.0007, *IST2*: P = 0.0018, *DBP2*: P = 0.0033, *TPO*: P = 0.0048.

backgrounds. This indicated that the lowered transcript levels seen in *Δnup60* cells was not likely due to a defect in synthesis, but rather in post-transcriptional regulation of *ASH1* mRNA. Therefore, the lower mRNA levels were probably caused by degradation of these nuclear mRNAs when they were retained in the nucleus for a prolonged time. Nevertheless, about half of the transcripts were able to leave the nucleus, as they are found to accumulate in the cytoplasm.

Taken together, this analysis showed that *Δnup60* led to a delocalization of active sites of transcription to the nuclear interior, without affecting the transcription frequency. However, the lack of Nup60p-dependent perinuclear localization of the active *ASH1* gene (directly or indirectly) resulted in an mRNP that is not fully export competent and is retained in the nucleus. Evidence suggests that the formation of the locasome, the particle responsible for localizing mRNAs, begins in the nucleus (Long et al. 2001; Shen et al. 2010). To examine whether *NUP60* plays a role in this process, we evaluated *ASH1* mRNA localization upon export to the cytoplasm.

Nup60p facilitates *ASH1* mRNA localization

The different nuclear factors that affect *ASH1* mRNA localization all have been shown to interact directly with the mRNA (e.g., She2p and Puf6p) (Cho et al. 1998; Kruse et al. 2002; Gu et al. 2004). While deletion of She2p completely abrogates *ASH1* mRNA localization, deletion of Puf6p results in a moderate localization defect (Gu et al. 2004). In addition to their presence in the nucleus, these proteins also associate with *ASH1* mRNA during its transport to the bud tip. Nup60p, a structural component of the NPC, is unlikely to be directly associated with localizing *ASH1* mRNA but may be a checkpoint in the pathway. To determine the effect of a *NUP60* deletion on *ASH1* mRNA localization, both wild-type and *Δnup60* strains were analyzed by FISH. Since a *Δnup60* strain showed significant nuclear retention of *ASH1* mRNA, only cytoplasmic mRNA was assessed for the localization phenotype. Wild-type cells showed a tight crescent of mRNA at the bud tip where the majority (85%) of the RNA was anchored (Fig. 5A). When *NUP60* was deleted, a dramatic shift in distribution was observed; the mRNA no longer localized as a crescent but was diffusely distributed either to the bud or throughout the cell (Fig. 5B). This indicated that the deletion of *NUP60* affected the cytoplasmic distribution of *ASH1* mRNA despite not being a physical constituent of the locasome. To determine if this effect was specific only to *ASH1* mRNA localization or generally to other localized RNAs, we scored *IST2* mRNA localization in both wild-type and *Δnup60* strains and found that the ability of *IST2* mRNA to localize was similarly affected (Supplemental Fig. 3).

Nup60p is responsible for localizing Nup2p as well as Mlp1/2p to the nuclear periphery. Likewise, *Δmlp1* cells show nuclear retention of improperly spliced transcripts.
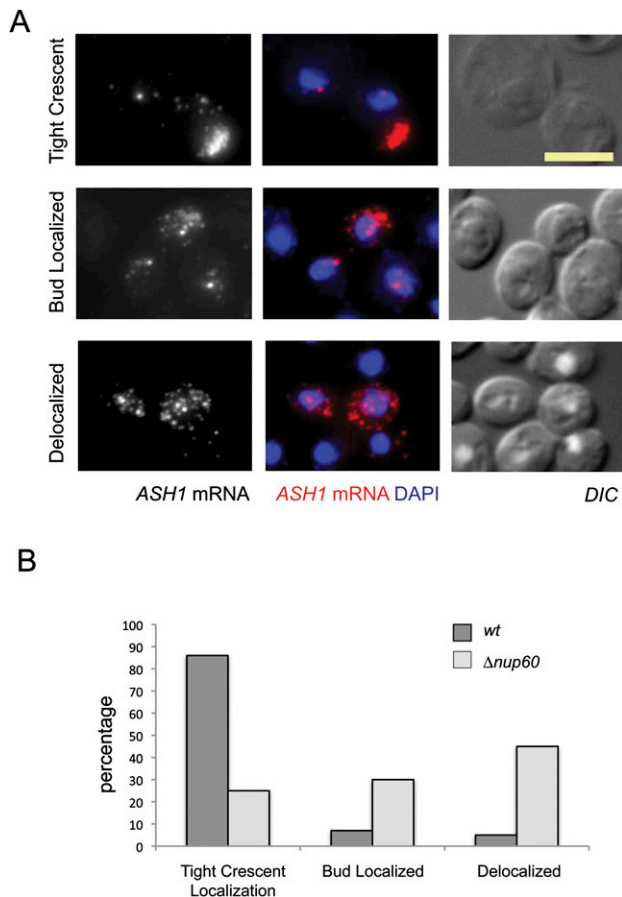
FIGURE 5. Deletion of *NUP60* affects *ASH1* mRNA localization. (*A*) Examples of the localization phenotypes scored by in situ hybridizations of *ASH1* mRNA. "Tight crescent" shows the majority of *ASH1* tightly localized in a crescent shape to the bud tip (*top*). "Bud localized" shows the majority of *ASH1* mRNA contained within the bud, but no strong enrichment at the bud tip (*middle*). "Delocalized" phenotype shows *ASH1* mRNA distributed throughout the mother and the daughter cells (*bottom*). *ASH1* transcripts that were present in the nucleus (as determined by DAPI staining) were not scored for localization. (*B*) Histogram showing the change in localization of *ASH1* in Δ*nup60* vs. wild-type strains (*n* = 51 wild type, *n* = 75 *nup60*).

To test if the mRNA retention phenotype was a secondary effect of Nup2p or Mlp1/2p release from the nuclear pore, we performed FISH on Δ*nup2*, Δ*mlp1*, Δ*mlp2*, and Δ*mlp1/ mlp2* cells and determined the retention and localization defect. Neither the *MLP* deletion strains nor Δ*nup2* showed a nuclear accumulation of *ASH1* mRNA. Nor was its localization affected, indicating that the observed phenotype was not due to the delocalization of those proteins from the nuclear periphery, but rather was a direct consequence of *NUP60* deletion (data not shown).

Nuclear retention is correlated with transcription site localization in the absence of *NUP60*

The effects of the deletion of *NUP60* on the perinuclear localization of the *ASH1* site of transcription, nuclear

accumulation, and cytoplasmic localization of the *ASH1* mRNA were correlated simultaneously within single cells. Δ*nup60* cells that did not accumulate *ASH1* mRNA in the nucleus showed a random distribution of their transcription sites within the nucleus. However, in cells that retained *ASH1* mRNA in the nucleus, gene loci frequently appeared in the interior, suggesting a correlation between the two phenotypes (Supplemental Fig. 4). Yet the percentage of cells localizing *ASH1* mRNA did not show any correlation with the nuclear position of transcription. The reason for this might be that transcription and export occur within seconds, whereas localized mRNA in the bud tip was transcribed long before the events in the nucleus were scored and the position of transcription may have changed. A correlation for contemporaneous events is more probable than for those separated significantly in time.

Distribution of Ash1p is altered upon deletion of *NUP60*

ASH1 mRNA localization is not completely abrogated in Δ*nup60* cells, as 25% of the cells properly localized *ASH1* mRNA, suggesting that the localization machinery per se is intact and functional. Localization is a means for the asymmetric delivery of Ash1p only to the bud nucleus. For this to occur, *ASH1* mRNA is translationally regulated during transport to the bud tip (Chartrand et al. 2002; Gu et al. 2004; Paquin et al. 2007; Deng et al. 2008). To assess whether the *NUP60* deletion had an effect on the localization of Ash1p, we performed immunofluorescence (IF) against C-terminal myc-tagged Ash1p in both wild-type and Δ*nup60* strains (Fig. 6A). In wild-type cells, 85% (±4.13) of budding cells that were expressing Ash1p showed asymmetric Ash1p distribution, but in Δ*nup60* cells this decreased to 58% (±16.99) (Fig. 6B). For an additional confirmation of the protein localization defect the number of single cells that express Ash1p is a measure of the asymmetry that occurred previous to mitosis. In wild-type cells, 7.6% (±0.53) of the total cell population showed Ash1p expression in single cells. Deletion of *NUP60* increased this to 20.7% (±7.16) (Fig. 6C). These results confirm that the distribution of Ash1p is dependent upon proper functioning of Nup60p. The Ash1p levels in both wild-type and Δ*nup60* cells were quantified by Western blot (not shown). Despite the changes in *ASH1* mRNA levels and in Ash1p distribution, the total cellular level of Ash1p remained unchanged between the two strains. Since the total amount of *ASH1* mRNA decreased and protein levels were constant, an additional level of regulation may exist to compensate (Gu et al. 2004).

DISCUSSION

Nuclear export of mRNAs has been studied predominantly using only poly(A)$^+$ as a marker. However, when the export of specific mRNAs has been examined, it has been shown
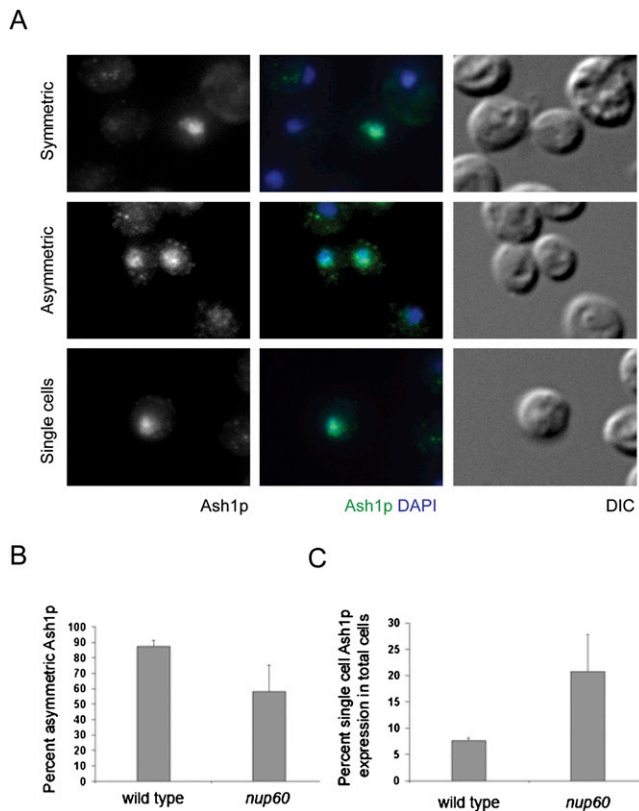
FIGURE 6. *NUP60* deletion decreases the asymmetric localization of Ash1p. (*A*) Examples of the localization phenotypes scored by immunofluorescence of Ash1p-myc in wild-type and Δ*nup60* cells. When Ash1p is exclusively localized in the bud nucleus it was considered asymmetric localization (*top*). Ash1p in both the mother and daughter nuclei was considered symmetric localization (*middle*). Ash1p is also found in the nuclei of single cells (*bottom*). Nuclei were stained with DAPI. (*B*) The effect of deleting *NUP60* on the asymmetric distribution of Ash1p in budding yeast: percent of budding cells. $P = 0.011$ (*C*) The effect of deleting *NUP60* on single cells: percent of single cells expressing Ash1p. $P = 0.036$ ($n = 191$ wild type, $n = 287$ Δ*nup60*).

that there is both diversity and specificity in the export mechanism (Brodsky and Silver 2000). However not all poly(A)$^+$ RNA is mRNA; therefore, any global study on poly(A)$^+$ RNA is not necessarily representative of single transcripts. Here we have employed high resolution imaging combined with quantification to explore the RNA export function of nonessential nucleoporins and nuclear pore associated proteins. We have described the selective nuclear retention of a specific set of transcripts upon deletion of a nucleoporin. These genes have previously been identified to code for transcripts that localize to the bud tip of dividing yeast cells dependent on She2p (Long et al. 1997; Takizawa et al. 1997; Shepard et al. 2003). However, although She2p first associates with *ASH1* mRNA in the nucleus (Shen et al. 2009), She2p is not a requirement for export. *ASH1* mRNA is not retained in the nucleus in a Δ*she2* strain and a Δ*she2/nup60* double deletion still

shows the nuclear retention phenotype seen in Δ*nup60* (data not shown). This suggests that the Nup60p acts early on in the localization process, independently of She2p. Nuclear retention of RNA does not prove that Nup60p is directly involved in the export process. In fact, it is unlikely that Nup60p serves as a general RNA export factor because Δ*nup60* cells show no growth defect. This suggests that that Nup60p has a specific role in the regulation of either the export of localizing transcripts or the nuclear steps in mRNP formation of the locasome.

The previously identified functions of Nup60p are diverse: interacting with karyopherin β (Kap95p) and anchoring the mobile Nup2p, as well as the myosin-like proteins (*MLP1* and *MLP2*) and the sumoylating proteins (*ULP1* and *ULP2*), to the nuclear periphery. Previous studies have also shown that deletion of *NUP60* can retain poly(A)$^+$ RNA in the nucleus and in the retention of improperly spliced transcripts in the nucleus. This is supported by the identification of Nup60p as an essential component of a perinuclear mRNA surveillance/quality-control complex (Skružný et al. 2009) that is functionally linked to the endoribonuclease Swt1p. Our results showing an increase in intron-containing transcripts upon deletion of *NUP60* support these findings. Here we show additional roles for *NUP60*: the selective retention of transcripts in the nucleus that ultimately localize in the cytoplasm and the impairment of this localization. Importantly, this phenotype is specific for Δ*nup60* and not a secondary effect of their ability to tether MLPs or Nup2p to the nuclear periphery as the phenotype is not observed in Δ*nup2*, Δ*mlp1*, Δ*mlp2*, and Δ*mlp1/mlp2* strains. Further analysis of the effect of Nup60p-interacting proteins on the localization of *ASH1* mRNA will help elucidate a mechanism of how a nucleoporin specifically acts to affect the process of mRNA localization.

Previous studies (Zhao et al. 2004; Dilworth et al. 2005; Schmid et al. 2006; Taddei et al. 2006) indicate that the peripheral position of the transcription site led to greater transcriptional activity. While our results reflect a similar decrease in transcript levels concomitant with transcription site internalization, we do not see a decrease in transcriptional activation (the number of transcripts at the site). Since there are no differences in the amount of *ASH1* nascent RNAs between wild-type and Δ*nup60* strains, the differences seen in the abundance of *ASH1* mRNA as well as the effect on localization are likely post-transcriptional.

A subset of the mRNAs reaches the cytoplasm and is properly localized. The fate of the nuclear-retained transcripts is not known. Possibly they were degraded by a nuclear quality control mechanism (Houseley et al. 2006). However, the overall Ash1p level does not change. Wild-type *ASH1* mRNA requires localization for translation to be initiated (Paquin et al. 2007), and in mutations that affect both *ASH1* mRNA localization and translation (e.g., Δ*puf6*, Δ*loc1*) we see increased amounts of Ash1p. A decrease in total cellular mRNA without changes in cellular protein

levels indicates an increase in translation relative to wild type, which would be consistent with a model where Nup60p facilitates the assembly of locasome components onto the *ASH1* mRNA prior to export.

We can only speculate why Nup60p is needed for proper export of these mRNAs and their localization in the cytoplasm. Since localization is not completely abrogated upon *NUP60* deletion, the core localization machinery appears functional. It is possible that Nup60p acts as a checkpoint for the export of localized mRNAs, facilitating the export of transcripts that have bound the appropriate nuclear factors, e.g., the translational regulator Puf6p. Without Nup60p, export is slowed and mRNAs are retained in the nucleus.

We propose a model that incorporates our observations. Nup60p tethers the *ASH1* gene to the nuclear periphery and may facilitate the interaction of *ASH1* mRNA with the nuclear locasome components that are responsible for tagging it as a localized mRNP. Without Nup60p, the probability of this interaction is decreased and export is inefficient. If exported, *ASH1* mRNA not contained within a properly assembled mRNP will be normally translated and degraded. This is reflected in the incomplete localization of *ASH1* mRNA seen in *Δnup60* cells. *ASH1* mRNA that is properly loaded with the nuclear *trans*-acting factors will be translationally inhibited for its transport to the bud tip where the RNP will be regulated in a wild-type manner, resulting in a variable amount of properly localized *ASH1* mRNA.

The idea of the nuclear pore as a static, uniform gate controlling the exchange of large molecules between the nucleus and cytoplasm is rapidly evolving toward a more dynamic, functional process, where there is diversity in composition and function of either the pores themselves or of the proteins associated with them (Tran and Wente 2006). Our results demonstrate that Nup60p plays a functional role in both the nuclear and cytoplasmic fate of localizing transcripts in *S. cerevisiae*. Future work will focus on the mechanism controlling the nuclear retained transcripts (export vs. degradation) as well as its effect on the localization of these transcripts.

MATERIALS AND METHODS

Media and growth conditions

Yeast was grown in either YPD or selective dropout (SD) media and grown at 30°C. Heat shock of *mex67-5* was performed at 37°C for 20 min.

Plasmids and yeast strains

Deletion strains from the *S. cerevisiae* deletion collection all have parental genotype: Mat A BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0. Genes were deleted using homologous recombination using a KAN marker, K699 (*MATa, ade2-1, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1*, GAL, psi+) (Jansen et al. 1996). *Mex67-5*

(Mata.ade2.his3.leu2.trp1.ura3.MEX67::his (don100-leu-mex67-5) was obtained from C. Cole (Dartmouth College).

Fluorescence in situ hybridization

Probe labeling and in situ hybridizations were performed as previously described (Long et al. 1997; Zenklusen et al. 2008; Zenklusen and Singer 2010). For dT in situs the final formamide concentration used was changed to 15%. All others were performed with 40% formamide. FISH probe sequences are shown in Supplemental Table 1.

Fluorescence microscopy

Imaging was performed on an Olympus BX61 wide-field epi-fluorescence microscope using an Olympus 100X, 1.35NA objective with HC DIC optics. Images were acquired using a Photometrics CoolSNAP HQ CCD with 100W mercury arc lamp or EXFO X-Cite 120 light source using Chroma filters for DAPI (model 11004), Cy3 (model SP102), Cy3.5 (model SP103), and Cy5 (model 41008) . IPLab software versions 3 and 4 (BD Biosciences) was used for image acquisition and analysis. Three-dimensional image data were acquired using a z step size of 200 nm.

Image analysis

Measuring nuclear retention of RNA

Image acquisition, single mRNA detection and segmentation of nuclear and cellular boundaries were done as described previously (Zenklusen et al. 2008; Zenklusen and Singer 2010). A minimum of 100 cells were analyzed for each strain and probe set. Typically multiple fields containing 30–60 cells per field were analyzed. Each field was used as a sample set and little variation between the fields were observed. Three-dimensional (3D) data sets were acquired and the 3D data was transformed into a two-dimensional image for each channel (RNA and nuclear) using a maximum intensity projection. To determine the poly(A)$^+$ retention phenotype, fluorescence intensity of the poly(A)$^+$ signal in the nuclear and cytoplasmic binary masks were measured using custom-made software.

Scoring intra-nuclear localization

Three-dimensional image data of RNA and DAPI stained nuclei were acquired using a wide-field microscope as described. 31-z planes (200 nm step size) were acquired for every field of cells analyzed. The location of the transcription site was manually scored as being peripheral, subperipheral, or internal relative to the DAPI signal using published scoring criteria (Thomsen et al. 2003).

Statistical analysis

Statistical analysis was performed using Excel (Microsoft) and Prism (GraphPad). All errors presented in this manuscript are standard deviations. All *P*-values presented in this manuscript are the result of student *t*-tests.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at http://www.rnajournal.org.

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