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Spatial consequences of defective processing of specific yeast mRNAs revealed by fluorescent in situ hybridization

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ABSTRACT

This work introduces the first use of fluorescent in situ hybridization (FISH) to detect the distribution of specific transcripts in *Saccharomyces cerevisiae*. We have applied this technique to analysis of reporter transcripts from a single, integrated copy, or multicopy plasmids. We have evaluated the effect of splice site deletions or the presence or absence of a terminator/cleavage site and demonstrated that both splicing and polyadenylation affect the export of these transcripts from the nucleus to the cytoplasm. Moreover, we show that the exported pre-mRNAs are substrates for nonsense codon-mediated decay through the *UPF1* pathway. The work presented here demonstrates that the spatial distribution of transcripts will also be an important component of yeast RNA metabolism.

Keywords: cleavage/polyadenylation; nonsense codon-mediated decay; nuclear transport; *S. cerevisiae*

INTRODUCTION

Gene expression requires the ordered events of transcription, RNA processing, export to the cytoplasm, and ultimately the proper translation into the correct protein product. In higher eukaryotic cells, in situ hybridization to specific transcripts has been used to investigate the spatial distribution of these various processes. For example, the sites of transcription and RNA splicing were shown to coincide by in situ hybridization to β-actin pre-mRNA or the adenovirus type 2 major late pre-mRNA (Zhang et al., 1994). These data supported a model of co-transcriptional splicing (reviewed in Rosbash & Singer, 1993).

The molecular genetics, biochemistry, and physiology available in the yeast *Saccharomyces cerevisiae* have provided many insights into the mechanisms of transcription and pre-mRNA splicing; however, very little is known about nucleic acid organization of these two processes in yeast, in part owing to the small size of the nucleus (1.0–2.0 μm in diameter) and lack of a sensitive in situ hybridization assay for specific transcripts. Fluorescent in situ hybridization (FISH) to RNA in *S. cerevisiae* has been limited to the detection of total poly(A)RNA (Amberg et al., 1992; Kadowaki et al., 1992; Forrester et al., 1992).

Under most circumstances, only mature mRNAs are exported from the nucleus to the cytoplasm (for reviews see Maquat, 1991; Nigg et al., 1991; Izaurralde & Mattaj, 1992; Elliott et al., 1994). A previous study in yeast used the translation of a reporter transcript to indirectly evaluate pre-mRNA export to the cytoplasm (Legrain & Rosbash, 1989). Inhibition of the early steps of splicing led to pre-mRNA translation, but transcripts capable of the first catalytic step of splicing, i.e., lariat formation (LI), yielded little translation product. Furthermore, the requirement of pre-mRNA translation restricted the assay to small synthetic introns from which stop codons could be eliminated. These studies could not accurately determine the distribution of the reporter transcripts between the nuclear and cytoplasmic compartments.

To study RNA processing and nuclear export in intact cells of *S. cerevisiae*, we have developed a FISH protocol capable of detecting the spatial distribution of galactose-inducible reporter transcripts. The FISH assay directly and conveniently examines RNA distribution in the nucleus and the cytoplasm. Using this

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assay, we show directly that a defect in the last catalytic step of splicing of a natural intron affects the export of a specific transcript from the nucleus, whereas a defect in the early step, i.e., spliceosome formation, results in export but subjects the transcript to nonsense mediated decay. Furthermore, a cleavage/polyadenylation site is also required for efficient export. The work presented here provides a foundation for the analysis of the spatial distribution of nucleic acids at these various steps of processing when combined with the powerful tools of yeast genetics.

RESULTS

FISH to specific transcripts

Galactose-inducible *lacZ* reporter transcripts were visualized by one-step in situ hybridization using fluorochrome-labeled oligonucleotides complementary to the coding region of *lacZ* (see Fig. 1A for the details of the reporter construct and probes). Galactose-grown cells (Fig. 1B) expressing steady-state levels of *lacZ* transcript exhibited dramatically more signal than that from glucose repressed cells (Fig. 1C). In situ hybridization was also performed on galactose grown cells using oligonucleotides of the identical sequence but synthesized in the opposite orientation to those described in Figure 1. With these probes, no fluorescence was detectable above background (data not shown). We concluded that the FISH protocol detects specific transcripts in *S. cerevisiae*.

The signal intensity varied considerably from cell to cell (Fig. 1B). Because the reporter construct is on a multicopy plasmid, we assumed that the observed differences in signal reflected a nonuniform copy number per cell. In order to test this hypothesis, we analyzed a chromosomally integrated *lacZ* cassette under induced or repressed conditions (Fig. 1D,E, respectively). We observed a single distinct nuclear focus in each cell (Fig. 1D: white, represents the overlap between in situ hybridization signal and 4,6-diamidino-2-phenylindole (DAPI)-stained chromatin; blue, DAPI signal elsewhere in the nuclei). The signal was in a subnuclear compartment that does not fill the nucleus, in contrast to experiments using multicopy plasmids (see below). This suggests that the spot of hybridization represented the site of transcription, similar to those seen in

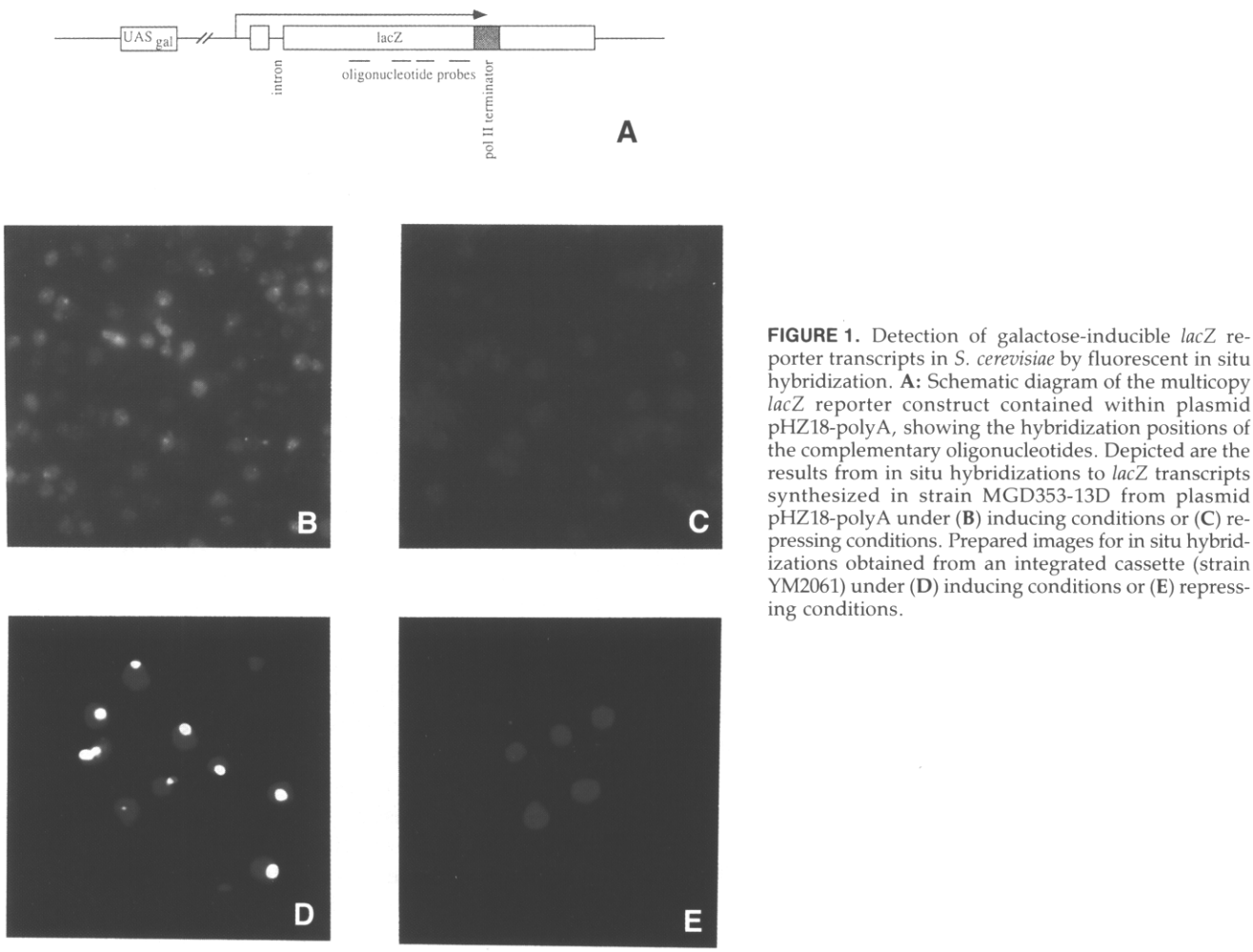

FIGURE 1. Detection of galactose-inducible *lacZ* reporter transcripts in *S. cerevisiae* by fluorescent in situ hybridization. **A:** Schematic diagram of the multicopy *lacZ* reporter construct contained within plasmid pHZ18-polyA, showing the hybridization positions of the complementary oligonucleotides. Depicted are the results from in situ hybridizations to *lacZ* transcripts synthesized in strain MGD353-13D from plasmid pHZ18-polyA under (**B**) inducing conditions or (**C**) repressing conditions. Prepared images for in situ hybridizations obtained from an integrated cassette (strain YM2061) under (**D**) inducing conditions or (**E**) repressing conditions.

mammalian cells (Zhang et al., 1994). The spot was clearly visible within 15 min after galactose induction (data not shown).

Localization of transcripts containing deletions of consensus splice sites

We used the FISH protocol in parallel with biochemical analysis to assess the effects of wild-type and mutated introns on nuclear retention of pre-mRNA. RNA extracted from cells containing a *lacZ* reporter fused to a wild-type intron or to introns with deletions at either the 5′, branch point (BP) or 3′ splice sites was analyzed by primer extension analysis (Fig. 2). The wild-type intron was efficiently processed, whereas all three deletion constructs accumulated pre-mRNA. The 3′ deletion construct gave rise to substantial levels of lariat intermediate as well as a small amount of mature mRNA, presumably resulting from a cryptic 3′ splice site. These observations were consistent with previous studies in yeast indicating that the 3′ splice site makes an essential contribution to the second step of splicing. Transcripts lacking a 3′ splice site assemble spliceosomes and undergo the first step of splicing efficiently, but arrest at the final joining of the exons. In contrast, deletion of the 5′ splice site and BP sequences inhibits the first catalytic step, due to its effect on the early events of splicing; i.e. spliceosome formation.

The intracellular consequences of these deletions became remarkably evident when these RNAs were visualized by FISH (Fig. 3). The transcripts in the cytoplasm were apparent by the red fluorescence emitted by the conjugated probe. To denote the nucleus, DAPI was used to stain the chromatin. When the signal from the hybridization was in the nucleus, the red and blue signals overlapped and were represented by white. This combination of colors made the compartmentalization of the transcripts visually obvious. This approach revealed that transcripts from the multicopy plasmid containing the wild-type intron construct could be found within the nucleus as well as the cytoplasm (Fig. 3B). However, a dramatically different result occurred with constructs containing a deletion of the 3′ splice site. The nuclear signal was considerably stronger than the wild-type construct and occupied the entire nucleus; however, only a faint cytoplasmic signal was seen. The diffuse nature of the nuclear signal may indicate that the pre-mRNA diffused throughout the nucleoplasm (Fig. 3C compared to Fig. 1D). Cells expressing either the 5′ splice site or BP deletions (Fig. 3D,E, respectively) yielded hybridizations to transcripts that had similar distributions to each other: both constructs gave faint cytoplasmic and nuclear signals. The cytoplasmic signal in both the 5′ and BP deletion constructs was substantially less than the wild-type construct, and the nuclear signal was less intense than that observed for the 3′ deletion construct.

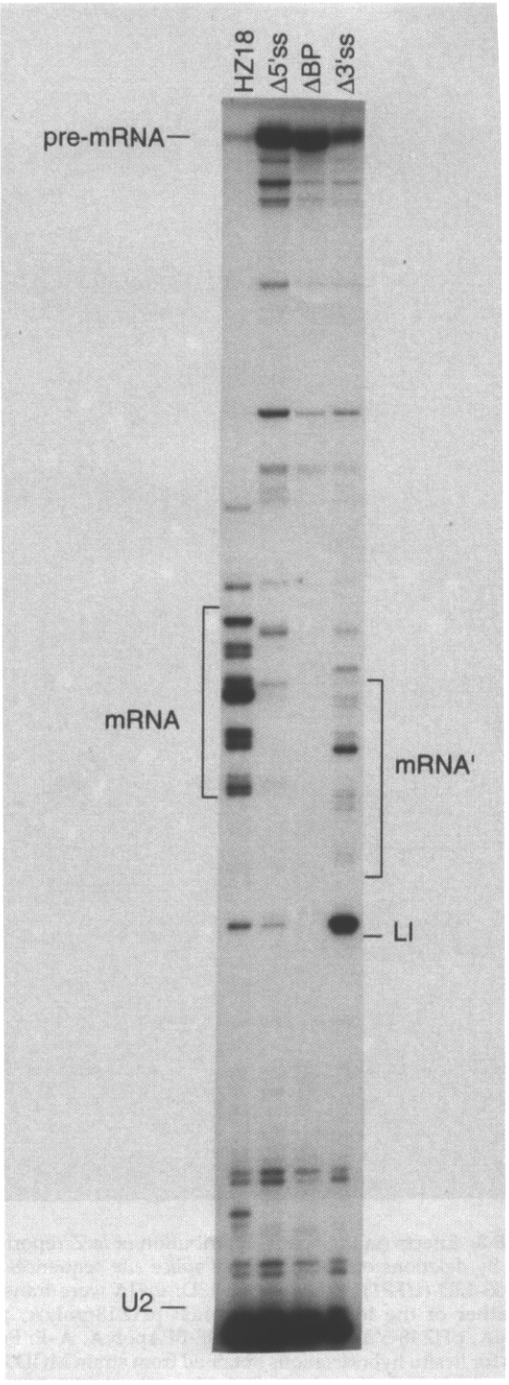

FIGURE 2. Primer extension results showing the species of *lacZ* transcripts produced in strain MGD353-13D from plasmids: pHZ18-polyA (wt), pHZ18-5′Δ polyA, pHZ18-BPΔ polyA, and pHZ18-3′Δ polyA.

Cytoplasmic pre-mRNA levels are regulated by nonsense codon-mediated decay

The nuclear signal from the 3′ deletion construct demonstrated directly that spliceosome assembly resulted in nuclear pre-mRNA retention. However, the absence of an intense cytoplasmic signal for the 5′ and BP deletion pre-mRNAs contrasted with previously published results (Legrain & Rosbash, 1989). In this work,

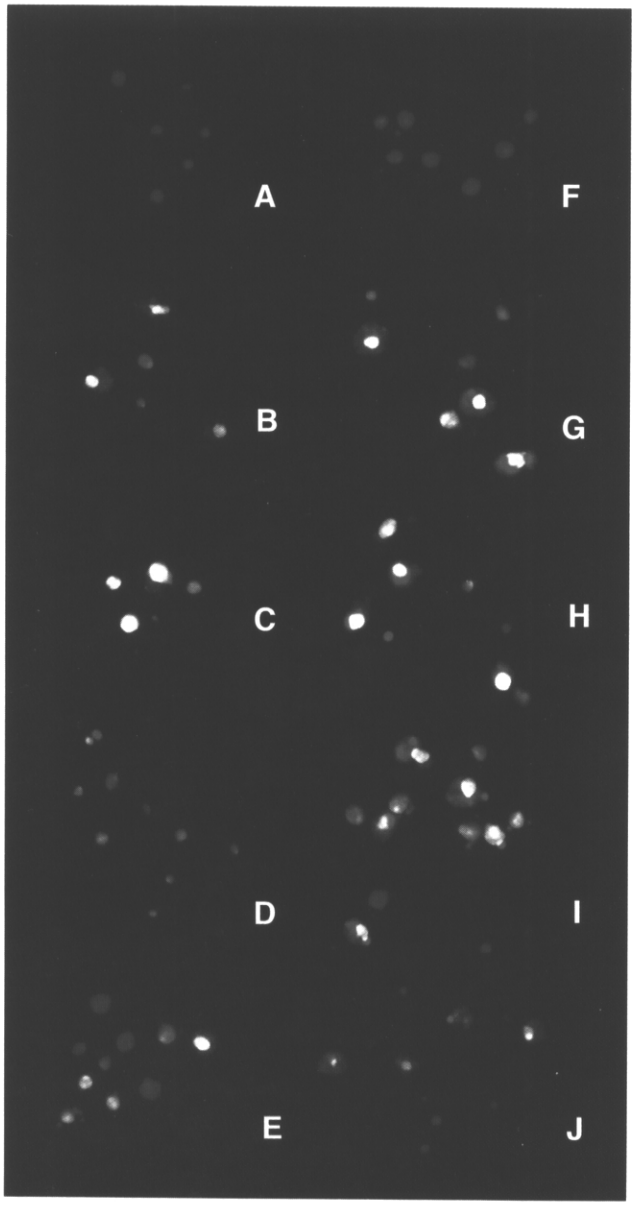

FIGURE 3. Effects on the spatial distribution of *lacZ* reporter transcripts by deletions of the consensus splice site sequences. Strain MGD353-13D (*UFP1*) and MGD353-13D::*upf1Δ* were transformed with either of the following plasmids: pHZ18-polyA, pHZ18-3′ΔpolyA, pHZ18-5′ΔpolyA, or pHZ18-BPΔpolyA. **A–E:** Prepared images for in situ hybridizations obtained from strain MGD353-13D. **F–J:** Prepared images from in situ hybridizations obtained from strain MGD353-13D::*upf1*. Results for steady state galactose induced cultures for plasmids pHZ18-polyA (B,G), pHZ18-3′ΔpolyA (C,H), pHZ18-5′ΔpolyA (D,I), and pHZ18-BPΔpolyA (E,J) are shown. Glucose grown negative controls for plasmid pHZ18-polyA (A,F) are shown.

cytoplasmic β-galactosidase activity was used to assay efficient nuclear export of *lacZ* pre-mRNA. The pre-mRNA that was exported contained an open reading frame with small synthetic introns. Therefore, absence of splicing could result in a protein with β-galactosidase activity. In contrast, because our work does not rely on translation, the pre-mRNAs in our experiments contained natural introns, which were significantly larger and had numerous translational termination codons in all reading frames. We hypothesized, therefore, that they could be substrates for the nonsense codon-mediated decay pathway (Peltz & Jacobson, 1993). To investigate the effect of this pathway on the intracellular distribution of the 5′ and BP deletion transcripts, we examined a yeast strain disrupted for *UPF1*, necessary for nonsense codon-mediated decay (Leeds et al., 1991). We observed that the cytoplasmic levels of the 5′ and BP deletion transcripts were dramatically increased by the genetic abrogation of nonsense codon-mediated decay (compare Fig. 3D to 3I, and Fig. 3E to 3J). We also detected a slight increase in nuclear signal (compare Fig. 3D to 3I). This may reflect a possible stabilization of nuclear RNA (Belgrader et al., 1993, 1994; Cheng & Maquat, 1993) or may be a secondary consequence of the *upf1* mutation. In addition, we detected modestly increased cytoplasmic signal from the 3′ deletion construct (compare Fig. 3C to 3H). This reflects either pre-mRNA escaping nuclear retention or mature mRNA (containing nonsense codons) resulting from a cryptic splicing event (Fig. 2). We observed insubstantial differences in the intracellular steady-state levels of RNA resulting from the wild-type intron (compare Fig. 3B to 3G).

An RNA polymerase II terminator/polyadenylation signal affects the distribution of RNA

The cytoplasmic stability (Sachs, 1993) and nuclear export of transcripts synthesized by RNA polymerase II rely on 3′ cleavage/polyadenylation events (Eckner et al., 1991). To address this effect in yeast, we analyzed the intracellular distribution of *lacZ* transcripts in the absence of an RNA polymerase II terminator/polyadenylation signal. The original *GAL1-lacZ* plasmids (pHZ18) (Teem & Rosbash, 1983) were constructed without an RNA polymerase II terminator/polyadenylation sequence in close proximity to the 3′ end of the *lacZ* coding region. Consequently, *lacZ* transcripts terminate at the subsequent termination sequences, either cryptic or located at the end of the adjacent gene, *URA3*. The plasmids described in Figures 1–3 contained the terminator from the yeast *ADHI* gene cloned into the 3′ coding portion of *lacZ* (Fig. 1A). In the absence of the terminator, the nuclear signal was far more intense than that observed in its presence (compare Fig. 4B to 4C). The majority of signal was due to mature (spliced) mRNA because little signal was observed using intron-specific probes (not shown).

DISCUSSION

In this study we introduce a protocol that detects specific yeast transcripts using fluorescent in situ hybridization. This yeast FISH protocol will provide a rapid

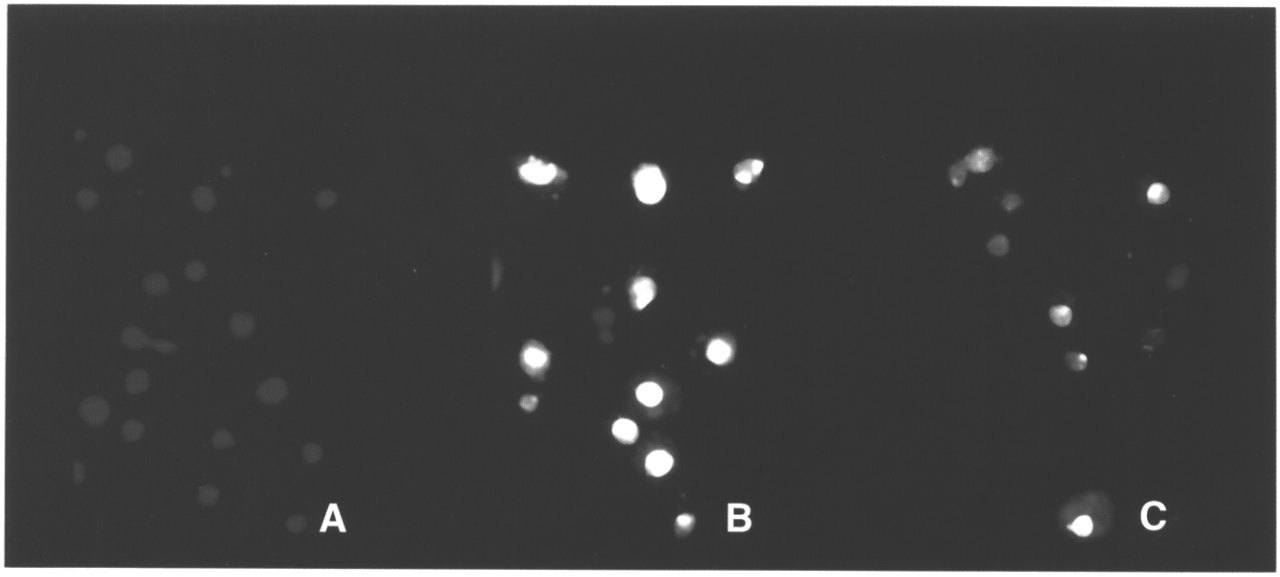

FIGURE 4. Effect of an RNA polymerase II terminator/polyadenylation site on the intracellular distribution of *lacZ* reporter transcripts. Depicted are prepared images from in situ hybridizations. Strain MGD353-13D was transformed with either plasmid pHZ18 or pHZ18-polyA. **A:** Glucose-grown negative control for pHZ18. **B:** Galactose-induced steady-state signal obtained from plasmid pHZ18 (no terminator). **C:** Galactose-induced steady-state signal obtained from plasmid pHZ18-polyA (strong terminator). One of the oligonucleotides described in the Materials and methods and indicated by the # should hybridize only to transcripts synthesized from the construct without the terminator and not to transcripts synthesized from the construct with the terminator.

and unequivocal method for investigating many aspects of RNA processing and transport. In addition to providing specificity for screening and further analysis of mutants with spatial defects, it should also allow the introduction of a temporal component into these studies (galactose induction) as well as the effects of sequence alterations on both RNA processing and nuclear export.

This approach provides an unequivocal determination of nuclear and cytoplasmic compartments, obviating the need for cell fractionation. Our results for the intracellular distribution of the various transcripts containing splice-site deletions provide a more direct, convenient, and accurate assessment of nuclear retention of transcripts and their export to the cytoplasm than has been possible previously. Using probes specific to the coding region of the *lacZ* reporter transcript, the wild-type RNA was seen to be efficiently processed and exported to the cytoplasm. The in situ hybridization experiments performed with the three deletion constructs visualized mainly unprocessed transcripts as determined by primer extension. The 5′ and BP deletion constructs yielded pre-mRNAs, which were competent for nuclear export. They were also degraded by the nonsense codon-mediated decay pathway because of the presence of termination codons in all three reading frames. This result indicated that any splicing factors that interact with either of these deletion constructs were not sufficient to sequester these pre-mRNAs in the nucleus. In contrast, transcripts synthesized from the 3′ splice site deletion construct were largely retained within the nucleus, presumably due to efficient spliceosome assembly. Nonetheless, a small amount of pre-mRNA transcribed from this construct escaped nuclear retention and was exported to the cytoplasm. This pre-mRNA was also a substrate for nonsense-mediated decay.

Because the majority of UPF1 protein is found in the cytoplasm and nonsense-containing mRNAs are associated with polyribosomes, UPF1 function is most likely exerted in the cytoplasm (Leeds et al., 1991; He et al., 1993; Atkin et al., 1995). This is consistent with our results showing an increase in cytoplasmic signal for the splice site deletion pre-mRNAs. However, a nuclear function for UPF1 cannot be discounted. Recent studies in mammals have suggested that nonsense-mediated decay may be initiated by translation while the nonsense-mediated decay substrates are being exported from the nucleus (Belgrader et al., 1993, 1994; Cheng & Maquat, 1993). Alternatively, UPF1 might identify nonsense-containing mRNAs in the nucleus and chaperone these transcripts to the site of degradation.

Our results suggest that proper termination and perhaps polyadenylation are important for nuclear export of RNA polymerase II-derived transcripts. The nuclear accumulation of transcripts resulting from a weak terminator could result from an increase in the number of nascent transcripts on the multicopy plasmid and/or inefficient nuclear export of released transcripts. If released transcripts were retained in the nucleus, correct

3′ end formation may be important for efficient nuclear export in *S. cerevisiae*. Correct 3′ end processing of the non-polyadenylated histone mRNAs has been shown to be important for nuclear export (Eckner et al., 1991). Another possibility is that these transcripts might be retained in the nucleus due to interactions with unidentified nuclear components (Boelens et al., 1995). If so, increased expression of the reporter transcripts may saturate nuclear retention factors and force an increase in nuclear export. FISH could also be used to screen yeast mutants deficient in 3′ end formation to address the relationship between proper 3′ end formation and nuclear export. Technological developments currently underway using electron microscopy (Bassell et al., 1994) should lead to higher resolution analysis of some of the subcellular distributions described in this work.

MATERIALS AND METHODS

Yeast strains, media, and microbiological techniques

Table 1 lists the yeast strains used in this study. Cells were grown in either synthetic media minus uracil (for selection of the plasmid) or rich media (YEP) (Sherman et al., 1986). Carbon sources in the media were either 2% glucose or 3% galactose/1% sucrose. Yeast plasmid transformations (Ito et al., 1983) and gene disruption (Rothstein, 1983) were performed as described.

Plasmid constructions

The following plasmids were used in this study: pHZ18, pHZ18-polyA, pHZ18-5′Δ polyA, pHZ18-BPΔ polyA, and pHZ18-3′Δ polyA. Plasmid pHZ18 (Teem & Rosbash, 1983) is a multicopy yeast plasmid containing a galactose-inducible *lacZ* construct with exon 1, intron 1, and part of exon 2 from the yeast *RP51A* gene inserted into the 5′ end of *lacZ*. Plasmid pHZ18-polyA was constructed from plasmid pHZ18 (Teem & Rosbash, 1983) by insertion of a PCR product corresponding to the 3′ end of the *ADH2* gene cloned as a *Hinc* II–*Sph* I fragment in to the *Sac* I site of pHZ18 (Teem & Rosbash, 1983). The oligonucleotides used for PCR were 5′-AAAAAAAAGAGCTCAGATCTGGTACCCGGGGATCCTCTAGA-3′ and 5′-AAAAAAAAAAGCTTGCATGCGAAGGAAAATG-3′.

Plasmids pHZ18-5′Δ polyA, pHZ18-BPΔ polyA, and pHZ18-3′Δ polyA were constructed from pHZ18-polyA by site directed mutagenesis using the oligonucleotides:

5′-GACTAGCAATAACAAAATGGACGTCTAATATGGACTAAAGGAGGC-3′,

5′-GTGTTTTTGATATCAGTAGACGTCCAAGTTGAATTGCATTTA-3′,

5′-TATTGCTTTTCGTCATTTGACGTCGGTAGAGTTAGAACCAAG-3′,

for the 5′, BP, and 3′ splice site deletions, respectively. In each case, 6 nt corresponding to the consensus splice site were removed and replaced with an *Aat* II restriction site (underlined).

UPF1 gene disruption

Strain MGD353-13D::*upf1*Δ was constructed from strain MGD353-13D (Seraphin et al., 1988). The *UPF1* gene (Leeds et al., 1992) and flanking sequences were subcloned into the polylinker of plasmid PTZ19R as a *Bam*H I–*Eco*R I fragment isolated from plasmid YCpPL63 (Leeds et al., 1992). An internal *Bgl* II fragment was removed from the subcloned *UPF1* construct and replaced with a *Bam*H I–*Bgl* II fragment containing *hisG/URA3/hisG* (Alani et al., 1987). This plasmid was digested with *Bam*H I and *Eco*R I and used to disrupt the *UPF1* locus by gene transformation (Rothstein, 1983). URA$^+$ transformants were selected, and candidate colonies for the *upf1* deletion without the *URA3* marker by recombination between the *hisG* repeats were identified by growing the cells on media containing 5-fluorooritic acid (Boeke et al., 1987). Deletion of *upf1* in the candidate colonies was confirmed by Southern blotting (Southern, 1975).

In situ hybridization

Cells were grown to mid-log phase and immediately fixed in 1/10 volume of fresh 40% formaldehyde and washed in Buffer B as described previously (Forrester et al., 1992). Spheroplasts were generated by incubating 10^8 yeast cells in 1 mL of Buffer B containing 0.1 mg/mL oxalyticase (Enzogenetics), 28.6 mM β-mercaptoethanol, 60 μg/mL phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 20 mM vanadyl ribonucleoside complex (VRC) (Gibco BRL), 120 units/mL RNase inhibitor (Boehringer Mannheim) for 8–10 min at 30 °C. Spheroplasts were washed and adhered to coverslips as previously described (Forrester et al., 1992). Following the last wash with ice cold Buffer B, the coverslips were stored in 70% ethanol at −20 °C.

Each coverslip for in situ hybridization was removed from 70% ethanol and rehydrated in 5 mL of 2× SSC for 5 min at room temperature and subsequently incubated in 2×

TABLE 1. Yeast strains used in this study.

Strain	Genotype	Source
MGD353-13D	*MAT**a** leu2-3,112 ura3-52 trp1-289 arg4 ade2*	Seraphin et al., 1988
MGD353-13D::*upf1*Δ	*MAT**a** leu2-3,112 ura3-52 trp1-289 arg4 ade2 upf1*	This study
YM2061	*MAT**a** lys2-801 met ura3-52 his2-200 LEU2::pRY181 (GAL1/lacZ)*	Flick and Johnston, 1990

SSC/40% formamide for 5 min at room temperature. Coverslips were inverted onto 20 μL of 40% formamide/2× SSC/2.5 mg/mL bovine serum albumin (BSA)/10 mM VRC containing a total of 100 ng of fluorescently labeled oligonucleotides, 100 μg of sonicated salmon sperm DNA, and 100 μg of *E. coli* tRNA. Sequences of the complementary oligonucleotides used for in situ hybridization to *lacZ* transcripts are as follows:

5′CAGCGCCCGT*TGCACCACAGAT*GAAACGCCGA GT*TAACGCCAT*CAAAAATT*T3′,

5′AT*TTCGGCGCT*CCACAGTTTCGGGT*TTTCGACG TT*CAGACGTAGTGT*GACT3′,

5′AT*TGGCACCAT*GCCGTGGGTTT*CAATATTGGCT T*CATCCACCACAT*ACAGT3′,

5′GGCT*GCGGTAGTT*CAGGCAGT*TCAATCAACT*G TTTACCTTGT*GGAGCGA3′#, and

5′CCGT*AAGCCGACCACGGGT*TGCCGTTTT*CATC ATATTT*AATCAGCGACT*G3′.

Oligonucleotides were directly labeled with CY3 fluorochrome at amino-modified thymidine residues (Zhang et al., 1994) indicated by the asterisk (*). Hybridizations were performed at 37 °C for at least 3 h. Following hybridization, each coverslip was washed twice at 37 °C for 15 min in 40% formamide/2× SSC, twice at room temperature in 1× SSC for 15 min, and once in 1× phosphate-buffered saline for 15 min. Coverslips were mounted in phenylenediamine containing DAPI as described previously (Forrester et al., 1992).

Images were captured using a cooled charge-coupled device camera, digitized, and analyzed (Carrington et al., 1990; Elliott et al., 1992; Taneja et al., 1992). Images were corrected for dark field current and background. Images were not subjected to the image restoration algorithm; hence, signal in the images represents in-focus and out-of-focus light. In the images, red color represents hybridization signal, and blue color represents the position of the nucleus determined by DAPI staining. Red and blue pseudo-colored images were superimposed. White indicates overlap between red and blue colors.

RNA fractionation and analysis

RNA extractions and primer extensions were carried out according to methods published previously (Pikielny & Rosbash, 1985) using an oligonucleotide (5′-CGCTTGACGGTCT TGGTTC-3′) complementary to exon 2 of the *RP51A* gene.

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