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Defects in the mRNA export factors Rat7p, Gle1p, Mex67p, and Rat8p cause hyperadenylation during 3′-end formation of nascent transcripts

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ABSTRACT

The biosynthesis and function of eukaryotic mRNAs requires a series of events including nuclear polyadenylation, transport to the cytoplasm, translation, and ultimately mRNA degradation. To identify the interrelationships between these events, we examined the synthesis and fate of mRNAs in several strains defective in mRNA export. Strains carrying lesions in *RAT7*, *GLE1*, *MEX67*, and *RAT8,* produce nascent transcripts carrying poly(A) tails roughly 30 residues longer than the nascent poly(A) tails observed in wild type. In the *rat7-1*, *rat8-2*, and *mex67-5* strains, the hyperadenylated transcripts undergo a novel form of deadenylation to chase into a population with normal poly(A) tail lengths, which cofractionate with polysomes, undergo nonsense-mediated decay, and are degraded by the normal cytoplasmic decay machinery. This suggests a relationship between the mechanism of processing to a normal poly(A) tail length and the ability of these transcripts to proceed in their metabolism. These observations provide further support for the view that mRNA 3′-end formation and mRNA export are mechanistically coupled events.

Keywords: 3′-end formation; mRNA export; mRNP remodeling; polyadenylation

INTRODUCTION

An important feature of eukaryotic gene expression is the compartmentalization of mRNA metabolism. Transcription, pre-mRNA splicing, and 3′-end formation are nuclear events that once completed, yield an mRNA capable of export to the cytoplasm. Transport of mRNA from the nuclear to the cytoplasmic environment signals a transition in the fate of an mRNA, wherein it becomes a template for protein synthesis and ultimately is degraded by the mRNA turnover machinery.

A remarkable aspect of mRNA metabolism is that many events show a mechanistic interdependency. Several observations indicate a requirement for proper 3′-end formation and polyadenylation for efficient mRNA transport to the cytoplasm. For example, recent work using mutations in the *β-globin* gene that result in improper 3′-end formation implicate a coupling between efficient 3′-end formation and the release of nascent transcripts from the site of transcription (Custodio et al., 1999). Indeed, the proper completion of 3′-end formation is required for efficient nuclear export of mammalian mRNAs and it appears that it is the process of polyadenylation, and not merely the presence of a poly(A) tail that is crucial for the generation of an export-competent mRNA (Eckner et al., 1991; Huang & Carmichael, 1996). Two types of observations also indicate a requirement for proper 3′-end formation to promote mRNA export in yeast. First, mRNAs that are lacking a proper 3′-end formation signal accumulate within the nucleus (Long et al., 1995). Second, fluorescent labeling of specific transcripts in living yeast cells demonstrated their nuclear accumulation when assayed in strains defective in 3′ end formation (Brodsky & Silver, 2000). These observations suggest that the transport of the mRNA from the nucleus to the cytoplasm is sensitive to proper 3′ end formation, including polyadenylation.

A fertile area of research for understanding mRNA export and its relationship to other aspects of mRNA metabolism has come from the isolation of numerous mutants that affect mRNA export in *Saccharomyces cerevisiae.* These mRNA export mutants were identified by several criteria, including screening for lesions that result in the nuclear accumulation of poly(A)+ based on in situ hybridization with labeled oligo(dT). These screens led to the identification of ribonucleic acid trafficking (*RAT*), and mRNA transport (*MTR*),

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mRNA export (*MEX*), and GLFG-lethal (*GLE*) genes (Amberg et al., 1992; Kadowaki et al., 1994; Murphy et al., 1996; Segref et al., 1997).

One particular interesting set of gene products affecting mRNA export are Rat7p, Gle1p, Mex67p, and Rat8p. These proteins show extensive genetic and biochemical interaction and localize to varying degrees at the nuclear periphery, in some cases at the cytoplasmic face of the nuclear pore complex (NPC; Gorsch et al., 1995; Kraemer et al., 1995; Del Priore et al., 1996; Murphy & Wente, 1996; Murphy et al., 1996; Segref et al., 1997; Santos-Rosa et al., 1998; Snay-Hodge et al., 1998; Tseng et al., 1998; Hodge et al., 1999; Schmitt et al., 1999; Strahm et al., 1999; Rout et al., 2000; Straber et al., 2000). Rat7p is a nucleoporin that localizes to the cytoplasmic face of the NPC and is also a member of a class of nucleoporins that contain FG-repeat sequences (Kraemer et al., 1995; Gorsch et al., 1995; Rout et al., 2000). These repeat sequences have been suggested to function in the binding of transport receptors (and their cargo) during passage through the nuclear pore channel (for review, see Ohno et al., 1998). Gle1p is a nucleopore-associated protein that localizes asymmetrically to both sides of the NPC, predominantly at the cytoplasmic face (Del Priore et al., 1996; Murphy & Wente, 1996; Strahm et al., 1999; Rout et al., 2000). Overexpression of Gle1p can partially suppress the *rat7-1* temperature-sensitive defects in growth and mRNA export (Del Priore et al., 1996). Mex67p is a shuttling protein, believed to function as a soluble transport receptor for the export of mRNAs (Segref et al., 1997). Mex67p functions as a heterodimer with Mtr2p, and together these proteins bind mRNA. Mex67p/Mtr2p can associate with the FG repeats proteins, including Rat7p (Kadowaki et al., 1994; Santos-Rosa et al., 1998; Straber et al., 2000). The DEAD-box protein, Rat8p is localized largely within the cytoplasm but also shows a localization at the nuclear periphery (Snay-Hodge et al., 1998; Tseng et al., 1998). Its interaction at the nuclear periphery is dependent, in part, upon Rat7p (Hodge et al., 1999; Schmitt et al., 1999). Overexpression of Rat8p can partially suppress the temperature-sensitive growth defects of *rat7-1* (Hodge et al., 1999). Rat8p can be detected in the nucleus in a variety of mutant strains defective in nuclear export, suggesting that it also shuttles between the nucleus and cytoplasm (Hodge et al., 1999). These factors, either as components of the exporting mRNP or components of the NPC, may interact during a final stage of mRNA export, just prior to release of the mRNP into the cytoplasm. This final stage of mRNA export might include the process of mRNP remodeling, wherein nuclear mRNP proteins (e.g., Mex67p) are removed and exchanged for cytoplasmic mRNP proteins. This latter possibility is supported by the fact that Rat8/Dbp5 protein is a member of the DEAD-box family of RNA helicases that utilize the energy of ATP hydrolysis to promote conformational rearrangements in RNA or RNA–protein complexes (Snay-Hodge et al., 1998; Tseng et al., 1998).

To reveal the relationships between mRNA export and other aspects of mRNA metabolism, we have used a transcriptional pulse-chase paradigm to examine the structure and fate of newly synthesized mRNAs in strains defective in mRNA export. We report here on one class of mRNA export mutants (*rat7-1*, *rat8-2*, *mex67-5*, and *gle1-4*) that yield transcripts with excessively hyperadenylated poly(A) tails. In the case of *rat 7-1*, *mex67-5*, and *rat8-2* strains, the hyperadenylated transcripts behave like a nascent, nuclear population transiently stalled in their biogenesis. Through an uncharacterized mechanism, these transcripts chase into a population with normal poly(A) tail lengths that can engage the translational machinery and decay via the major cytoplasmic 5′-3′ turnover machinery. These data imply a coupling between the processing of the hyperadenylated transcripts and the ability of these transcripts to proceed in their normal metabolism and further support a mechanistic relationship between the processes of 3′-end formation and mRNA export.

RESULTS

General strategy

To determine how alterations in mRNA export affect the metabolism of yeast mRNAs, we examined mRNA biogenesis and function in several mutants (*gle2*Δ, *rat7-1*, *mex67-5*, *rat8-2*, and *gle1-4*) that show a nuclear accumulation of poly(A)+ signal after a shift to the restrictive temperature (Gorsch et al., 1995; Del Priore et al., 1996; Murphy & Wente, 1996; Murphy et al., 1996; Segref et al., 1997; Snay-Hodge et al., 1998; Tseng et al., 1998). The approach is to use a transcriptional pulse-chase paradigm to induce a pool of newly synthesized transcripts after effecting the loss-of-function phenotype by incubation for 1 h at the restrictive temperature. We utilized the *PGK1pG* and *MFA2pG* reporter transcripts under the control of the *GAL* promoter, which can be rapidly induced and then repressed by carbon source manipulation (Decker & Parker, 1993). This experiment has three important features. First, the examination of newly synthesized transcripts avoids any complications from mRNAs made prior to the shift to restrictive conditions. Second, the chase period allows us to identify and characterize the rates of specific transitions in the metabolism of the mRNAs. Third, because these reporter mRNAs contain a poly(G) tract in their 3′ UTRs that traps intermediates in mRNA degradation by blocking exonuclease progression, we are able to examine the direction and extent of mRNA turnover.

The normal metabolism of the *PGK1pG* mRNA in wild-type cells is shown in Figure 1A. In this and all

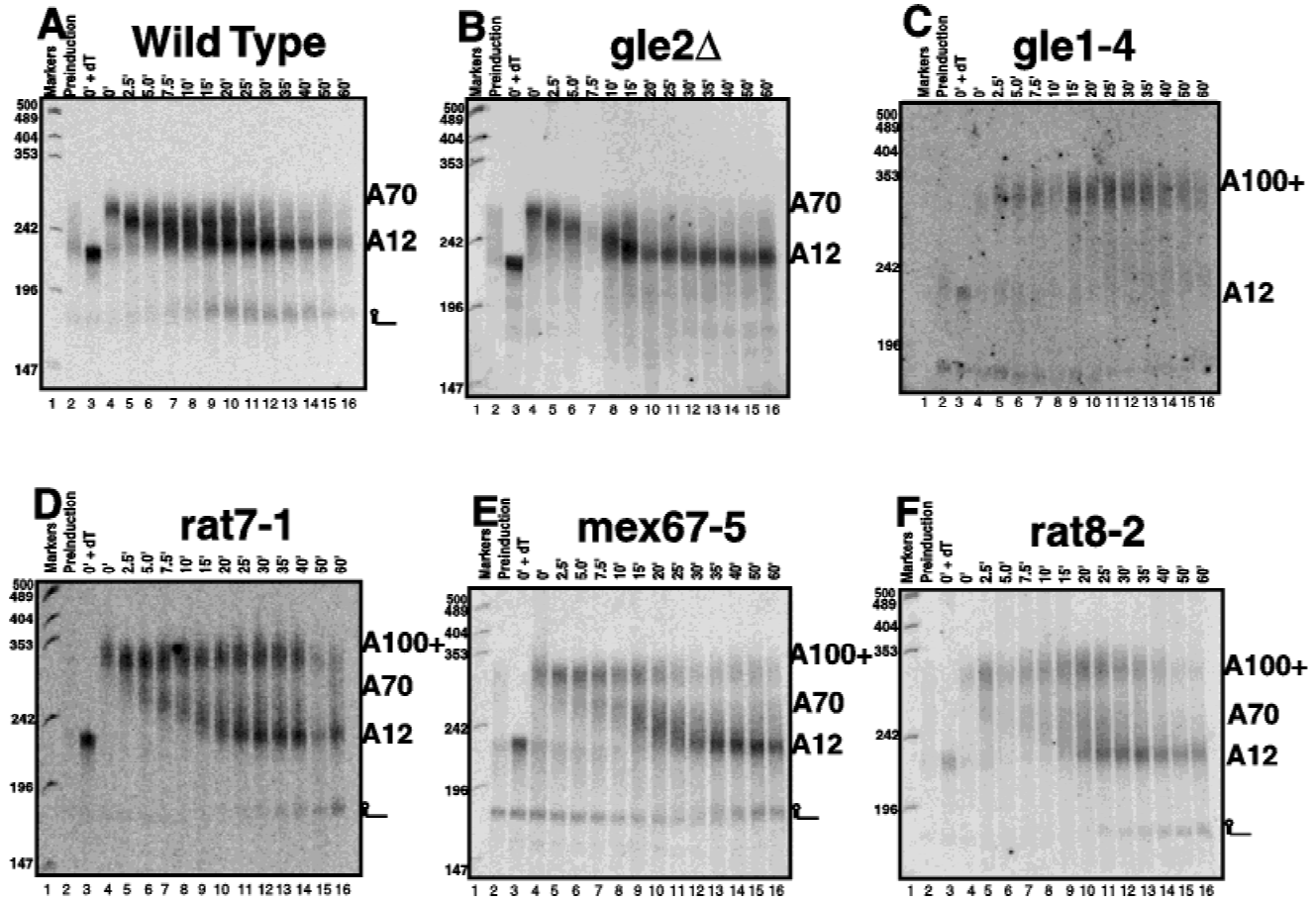

FIGURE 1. Profile of *PGK1pG* mRNA metabolism in wild-type versus mRNA export mutants. Shown are northern blots of *PGK1pG* transcriptional pulse-chase experiments in strains after a 1-h shift to 37 °C. With the exception of **E**, which contains the *PGK1pG* reporter transcript on a plasmid (pRP469; Decker & Parker, 1993), all strains contain the *PGK1pG* reporter construct integrated into the genome (*cup1::LEU2PM*). For each experiment, lane 1 corresponds to molecular weight markers; lane 2 corresponds to the preinduced levels of reporter transcript; lanes 3 and 4 are the zero-minute time point that corresponds to the end of the 5′ galactose induction, just prior to glucose addition, treated with (lane 3) or without (lane 4) oligo dT during the Rnase H treatment (see Materials and Methods). Lanes 5–16 represent selected time points (2.5 to 60 mins) after transcription inhibition as indicated. Estimated poly(A) tail lengths are labeled and the position of the 5′-3′ trimmed poly(G) decay intermediate is indicated on the sides of each panel. **A:** Wild type (yRP841); **B:** *gle2*Δ strain (yRP1581); **C:** *gle1-4* (yRP1580); **D:** *rat7-1* (yRP1579); **E:** *mex67-5* (yRP1577 +pRP469); and **F:** *rat8-2* (yRP1582).

subsequent transcriptional pulse-chase experiments, transcription was induced for 5 min. The newly synthesized *PGK1pG* reporter transcripts have a poly(A) tail of ~70 residues (Fig. 1A, compare lanes 3 and 4). This population of transcripts then undergoes deadenylation over the next 10–15 min to an oligo(A) form (Fig. 1A, lanes 7–16) with approximately 10–15 residual adenosines. The oligo(A) population is then decapped by the Dcp1p decapping enzyme and degraded 5′ to 3′ by the exoribonuclease encoded by the *XRN1* gene (Hsu & Stevens, 1993; Muhlrad et al., 1995; Beelman et al., 1996). This 5′-to-3′ degradation leads to the production of a 5′-to-3′ trimmed decay intermediate (first appearing at ~7.5 mins; Fig. 1A, lane 7) extending from the poly(G) tract to the 3′ end of the mRNA (Decker & Parker, 1993).

Lesions affecting mRNA export show a diversity of alterations in mRNA metabolism

The investigation of lesions affecting mRNA export revealed three different types of phenotypes with regard to the metabolism of the mRNA. The first type of phenotype is observed in the *gle2*Δ strain. Gle2p is a NPC-associated protein that localizes symmetrically to both nuclear and cytoplasmic faces of the NPC and plays a structural role in nucleopore and/or nuclear envelope biogenesis (Murphy et al., 1996; Bailer et al., 1998; Rout et al., 2000). As assessed by oligo d(T) staining for nuclear poly(A)+ RNA, *gle2*Δ cells exhibit a defect in mRNA export. Although modest in comparison to other mRNA export mutants, a perinuclear accumulation of poly(A)+ is detected in the *gle2*Δ strains at the

permissive temperature, and this accumulation is enhanced after a 30-min incubation at the restrictive temperature (Murphy et al., 1996; Bailer et al., 1998). Surprisingly, deletion of *GLE2* has little effect on the synthesis and metabolism of *PGK1pG* transcripts. For example, transcripts synthesized in this strain carry normal poly(A) tails of approximately 70 residues that deadenylate at a rate similar to that seen in wild-type cells (Fig. 1B). This might reflect that mRNA export is relatively efficient in the *gle2*Δ strain under the experimental conditions we used. Interestingly, however, the oligo(A) population of *gle2*Δ transcripts tend to persist, whereas the 5′-to-3′ trimmed fragment is only slowly produced, suggesting that decapping and/or 5′-to-3′ exonucleolytic decay may be delayed in this background. This delay is also seen in the examination of the *MFA2pG* transcript (data not shown).

A different mRNA phenotype is observed in the *gle1-4* mutant strain. A transcriptional pulse-chase of *PGK1pG* in this mutant strain yields two distinct phenotypes (Fig. 1C). First, the *PGK1pG* mRNA carries a hyperadenylated poly(A) tail. RNAse H treatment of the zero minute time in the presence of oligo dT shows the relative length of poly(A) tail addition of approximately 120 residues (Fig. 1C, compare lanes 3 and 4). Second, the hyperadenylated population of transcripts persist over the course of the experiment, with only very low levels of decay intermediates ever produced.

A third class of mRNA phenotype is observed in the *rat7-1*, *mex67-5*, *rat8-2* strains. In this class, we observe newly synthesized *PGK1pG* transcripts with hyperadenylated tails of ~120 residues, similar to the length of poly(A) tails observed in the *gle1-4* strain (Fig. 1D,E,F). However, in these mutants, we observe that shortly after the synthesis of the hyperadenylated population of transcripts, a discrete population with poly(A) tails of ~70 residues appears (Fig. 1D, lanes 5–7). This poly(A) tail length is characteristic of *PGK1pG* transcripts synthesized in wild-type strains (Fig. 1A). In the *rat7-1*, *mex67-5*, and *rat8-2* strains, the normal poly(A) tail population consistently appears after the production of the hyperadenylated species, indicative of a precursor/product relationship between the two. Notably, in this class of mutants, the normal poly(A) tail population produced from the hyperadenylated population deadenylates to an oligo(A) population, similar to the deadenylation seen in wild-type strains (Fig. 1A, lanes 6–11). Shortly following the appearance of the oligo(A) species is the appearance of the 5′-to-3′ trimmed poly(G) fragment (Fig. 1A, lanes 13–16). Together these results show that the hyperadenylated transcripts produced in *rat7-1*, *rat8-2*, and *mex67-5* strains can proceed, through an uncharacterized transition, to a normal poly(A) tail population whose decay at least superficially appears normal (see below).

The extensive (120+) hyperadenylation of mRNAs is not observed in these export mutants at the permissive temperature of 24 °C. However, in the *rat8-2* strain grown at 24 °C, a partial increase of approximately 15 residues is observed (data not shown). This result is consistent with the observed changes in poly(A)+ length distribution on steady-state RNA previously seen in other alleles of *rat8-2* at the permissive and restrictive temperatures (*dbp5-1*, and *dbp5-3*; Tseng et al., 1998).

In the *rat7-1* strain, the hyperadenylation is extremely rapid, appearing on newly synthesized transcripts within 2.5 min after a shift to 37 °C (Fig. 2A; data not shown). The increase in poly(A) tail length is also observed on a second reporter transcript, *MFA2pG* (Fig. 2B), indicating that this phenotype occurs on multiple, if not all, transcripts produced in a *rat7-1* strain after a shift to the restrictive temperature. The hyperadenylation of *rat7-1* transcripts is more extensive than that observed in mutants defective for the Pab1p-associated poly(A) nuclease (PAN). This nuclease, consisting of Pan2p and Pan3p, is proposed to function in an initial nuclear trimming of the nascent poly(A) tail (Boeck et al., 1996; Brown et al., 1996; Brown & Sachs, 1998). In *pan2*Δ strains, the *PGK1pG* poly(A) tail is extended ~10–15 nt beyond that observed in wild-type cells and is

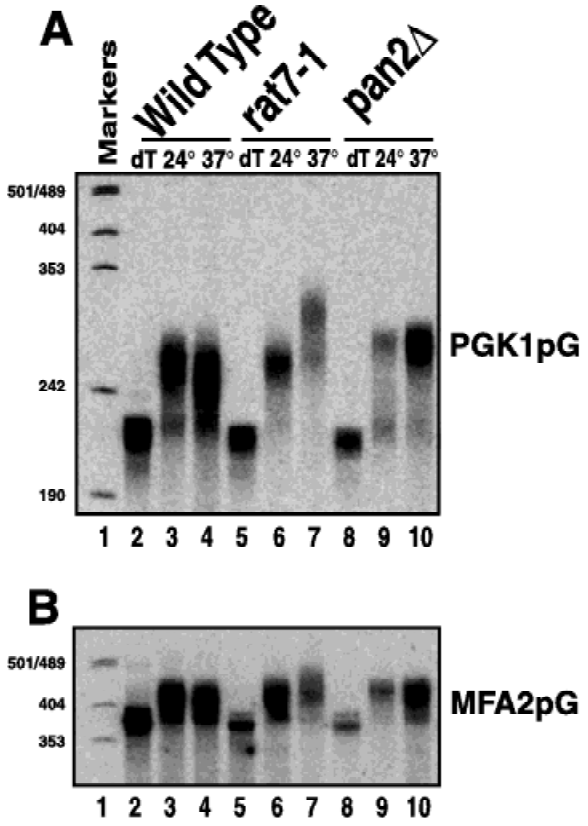

FIGURE 2. Comparison of poly(A) tail lengths in wild-type *rat7-1* and *pan2*Δ strains. **A:** Northern blot of a pulse of *PGK1pG* mRNA synthesized in parallel cultures of wild-type (yRP841); *rat7-1* (yRP1579); or *pan2*Δ (yRP1578) grown at 24 °C without or with a shift to 37 °C for 1 h. Lanes 2, 5, and 8 are the 24 °C samples treated with oligo dT during the RNase H reactions. **B:** Same blot probed for *MFA2pG* mRNA.

believed to represent the nascent length of poly(A) tail added during 3′-end formation (Brown & Sachs, 1998). Interestingly, the slight increase in poly(A) tail length observed in the *rat8-2* strain at 24 °C is similar to the *pan2*Δ length. Together, these observations imply that the hyperadenylation seen in the *rat7-1*, *rat8-2*, *mex67-5*, and *gle1-4* strains at 37 °C is distinct from the modestly extended poly(A) tails seen in a *pan2*Δ strain.

Partial suppression of the rat7-1 mRNA hyperadenylation phenotype by overexpression of Rat8p

The temperature sensitivity of the *rat7-1* strain can be partially suppressed by overexpression of Rat8p (Hodge et al., 1999) To address whether or not suppression would extend to the hyperadenylation mRNA phenotype, we assayed the poly(A) tail length on the PGK1pG transcript in the *rat7-1* strains overexpressing Rat8p. An examination of the poly(A) tail distribution in *rat7-1* strains carrying the *RAT8* gene on a high copy plasmid after 10 mins of transcription revealed a shift in the relative distribution of hyperadenylated and normal poly(A) tail lengths toward the normal (A70) tail length (Fig. 3A). For example, quantitation of the abundance of hyperadenylated and normal poly(A) tail populations showed that in the control *rat7-1* strain transformed with an empty vector, the normal poly(A) tail population represented roughly 49% of the total. In contrast, in the *rat7-1* strain overexpressing Rat8p, the level of normal poly(A) tail population increased to ~74%. Overexpression of Gle1p can also suppress the *rat7-1* temperature-sensitive growth defect, albeit less effectively than overexpression of Rat8p (Del Priore et al., 1996; Hodge et al., 1999). Similarly, only a modest increase in the level of normal poly(A) tail population (~55%) was observed in this strain (Fig. 3A). In control experiments, overexpression of Rat8p or Gle1p in a wild-type strain had no effect on poly(A) tail metabolism (data not shown).

There are two possible interpretations to this result. First, overexpression of Rat8p (and to a lesser extent, Gle1p) in the *rat7-1* background might allow the synthesis of a normal poly(A) tail length population, in addition to the aberrant hyperadenylated population. Alternatively, overexpression of Rat8p could speed the transition between the hyperadenylated *rat7-1* transcripts and the transcripts carrying normal poly(A) tail lengths. We cannot rigorously distinguish between these two possibilities at this time.

rat7-1 transcripts can engage the translation machinery

The normal poly(A) tail population that is produced from the hyperadenylated populations in the *rat7-1*, *rat8-2*, and *mex67-5* strains appears to behave as mRNA from wild-type cells. This suggests that this population has entered the normal pathway of mRNA export, eventual

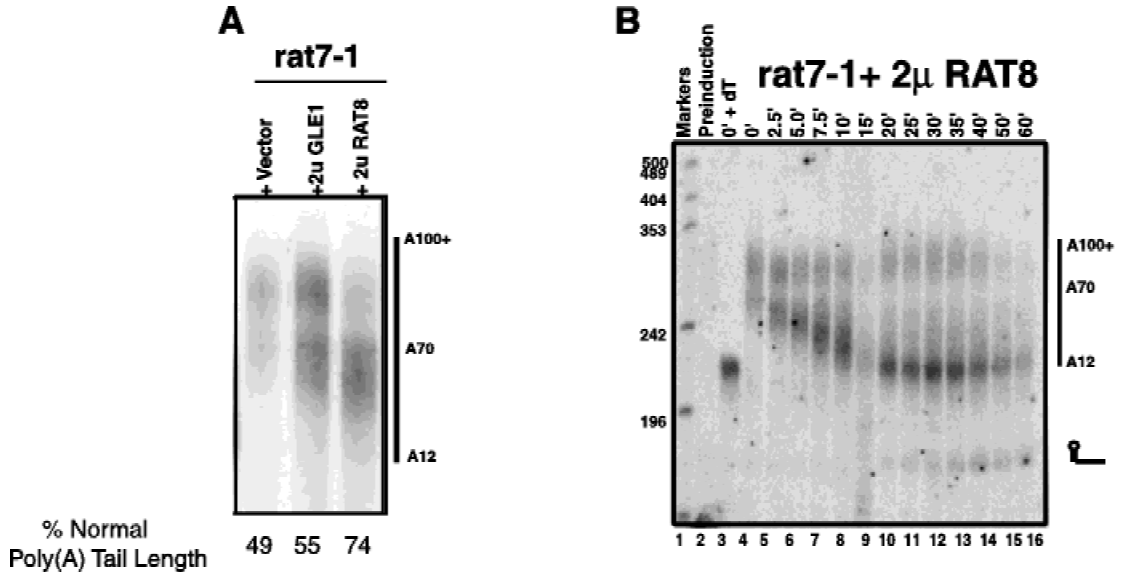

FIGURE 3. Overexpression of Rat8p and Gle1p can suppress the hyperadenylation phenotype of *rat7-1* transcripts. **A:** Northern analysis of the distribution of poly(A) tails on the PGK1pG reporter construct in the *rat7-1* strain (yRP1579) transformed with 2μ plasmid alone (vector), 2μ plasmid carrying the *GLE1* gene (2μ GLE1; gift of S. Wente) or a 2μ plasmid carrying the RAT8 gene (2μ RAT8; gift of C. Cole). Cells were grown in minimal media containing 2% raffinose at 24 °C. After a 1-h incubation at 37 °C, the transcript was induced by the addition of galactose to 2% for 10 min and then the cells were harvested and total RNA prepared. Shown below each lane is the percentage of total that the normal poly(A) tail population represents as determined by quantitation of the blot. **B:** A transcriptional pulse-chase experiment of the *rat7-1* strain carrying the RAT8 overexpression plasmid.

translation, and cytoplasmic decay. To examine if these mRNAs were capable of associating with the translational machinery, two experiments were done using the *rat7-1* strain as an example of this phenotypic class. In the first experiment, we addressed the extent of ribosome association of *rat7-1* transcripts by probing polysome gradient fractions of *rat7-1* extracts to determine where within the gradient the induced *PGK1pG* transcripts fractionate. To facilitate this analysis, the strains were grown in a neutral carbon source to an appropriate density, at which time they were shifted to 37 °C to effect the loss-of-function phenotype. After a 25-min incubation at 37 °C, transcription of the *GAL-UAS-PGK1pG* reporter gene was induced for a period of 35 min. At the end of this induction period, the distribution of *PGK1pG* transcripts observed in the *rat7-1* strain include those with hyperadenylated poly(A) tails, those that have transitioned to a normal poly(A) tail, as well as those that have undergone subsequent deadenylation, which produces a distribution of poly(A) tail lengths from approximately 70 to 10 residues.

In the wild-type strain, the bulk of the *PGK1pG* full-length transcript colocalizes with the polysome fraction (Fig. 4A, lanes 12–19). An important result is that in the *rat7-1* strain, a significant percentage of transcripts cofractionate with polysomes (Fig. 4B, lanes 11–19), implying that these transcripts have exported the nucleus and are actively translating. Although some hyperadenylated transcripts are detected in the polysome fractions, the bulk of the population of *rat7-1* transcripts that cofractionate with ribosomes have deadenylated to a normal poly(A) tail distribution. This indicates that *rat7-1* transcripts can eventually associate with the translation machinery, and that deadenylation to a normal poly(A) tail might occur as a result of the onset of translation. Importantly, a significant pool of hyperadenylated transcripts localizes to the free mRNP pool (Fig. 4B, lanes 4–8). This pool most likely represents a stalled, nuclear population of transcripts.

A second experiment to determine if these mRNAs can be translated was to determine their susceptibility to mRNA surveillance. Transcripts containing premature termination codons are rapidly degraded via a mechanism that is tightly coupled to translation termination (for reviews, see Czaplinski et al., 1999; Hilleren & Parker, 1999). The susceptibility to mRNA surveillance thus provides an assay for whether transcripts can engage the translational machinery. Given this, we examined the turnover of a nonsense-containing transcript, *PGK1(N103)pG* (Muhlrad & Parker, 1994), in the wild-type and *rat7-1* strains.

The nonsense *PGK1(N103)pG* decays rapidly in both the wild-type and *rat7-1* strains (Fig. 4C,D; compare to normal *PGK1pG* transcript in Fig. 1A,D). As expected, the nonsense transcript synthesized in the *rat7-1* strain carries a hyperadenylated poly(A) tail. Notably, however, any normal poly(A) tail length population usually produced from the hyperadenylated population is absent in the *rat7-1* strain (Fig. 4D). This suggests that any hyperadenylated nonsense-containing transcripts that transition to a normal poly(A) tail length are immediately subject to nonsense-mediated degradation. Because this decay mechanism is intimately coupled to translation termination, this result argues that *rat7-1* transcripts can engage the translational machinery. Alternatively, these results can also be interpreted to indicate that mRNA surveillance can occur on the hyperadenylated mRNAs prior to their processing to a normal poly(A) tail length. Thus, although we cannot rigorously order the entry into translation relative to the transition to a normal poly(A) tail length, these results clearly argue that these mRNAs can enter translation prior to, during, or after the transition to a normal poly(A) tail length. Interestingly, the disappearance of the hyperadenylated pool of nonsense transcripts is delayed in the *rat7-1* strain compared to the nonsense transcripts synthesized in the wild-type strain. Because nonsense transcripts decay in a translation-coupled mechanism, this result argues that there is a rate-limiting step in the metabolism of these mRNAs prior to their entry into translation.

Components of the major 5′-3′ cytoplasmic mRNA turnover machinery are required for the degradation of the *rat7-1* transcripts

In a final set of experiments to address the fate of the *rat7-1* transcripts, we determined that their decay was dependent upon Dcp1p and Xrn1p, components of the cytoplasmic mRNA degradation machinery. Dcp1p is a component of the decapping enzyme that removes the 7-methyl guanosine cap from the 5′ end of the transcript. This exposes the body of the transcript to a rapid 5′-3′ exonuclease activity carried out by Xrn1p (Hsu & Stevens, 1993; Muhlrad et al., 1995; Beelman et al., 1996). In both the *dcp1*Δ *rat7-1* and *xrn1*Δ *rat7-1* strain, deadenylated mRNA accumulates and the 5′ trimmed poly(G) fragment is not produced (Fig. 5), whereas mutation in the *RAT1* gene, which encodes a nuclear 5′-to-3′ exonuclease (Amberg et al., 1992; Johnson, 1997) did not inhibit the production of decay intermediates of the *rat7-1* transcripts (data not shown). In that the 5′-3′ turnover machinery functions in the cytoplasm, this data further supports the interpretation that these mRNAs are reaching the cytoplasm and undergoing normal mRNA metabolism.

DISCUSSION

Lesions in nucleoporin and nucleoporin-associating proteins can affect the extent of polyadenylation on nascent transcripts

Our observations indicate that one class of mutants that exhibit a particularly rapid and potent inhibition of

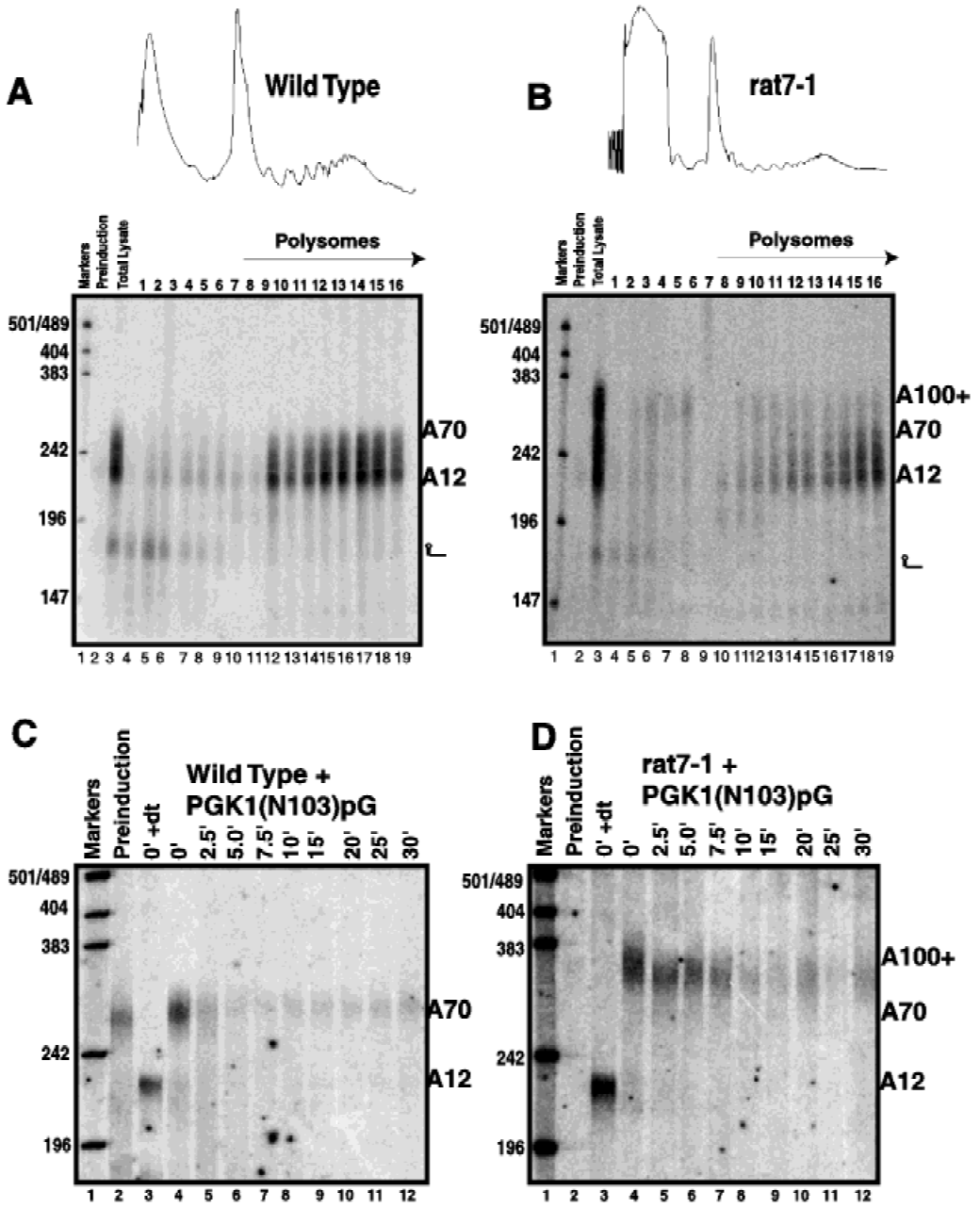

FIGURE 4. The *rat7-1* transcripts can engage the translation machinery. **A,B:** Cofractionation of galactose-induced *PGK1pG* transcripts with polysomes in wild-type (yRP840) and *rat7-1* (yRP1579) strains. Shown are the A_{254} traces made during fractionation of the sucrose gradients. Below the traces are the corresponding northern blots of the fractions from each gradient probed for *PGK1pG*. In each northern blot, lane 1 represents molecular weight markers; lane 2 represents total RNA harvested from aliquots of the cultures just prior to transcriptional induction; lane 3 represents total RNA harvested from the extract just prior to fractionation onto the gradient; lanes 4–19 represent total RNA harvested from the 16 fractions of the gradient. The distribution of poly(A) tail lengths are indicated, as is the position of the 5′-to-3′ trimmed poly(G) fragment. **C,D:** Nonsense transcripts synthesized in the *rat7-1* strain are rapidly degraded once they transition to a normal poly(A) tail length. Transcriptional pulse-chase reactions of the nonsense *PGK1(N103)pG* reporter construct (pRP555; Muhlrad & Parker, 1994) in wild-type (yRP683) and *rat7-1* (yRP1573) strains.

export (*rat7-1*, *mex67-5*, *rat8-2*, and *gle1-4*) share a common phenotype of extreme hyperadenylation of newly synthesized transcripts. This work extends previous observations that the steady-state poly(A) tail distribution as assessed by pCp labeling of the entire population is increased in some mRNA export mutants (e.g., Piper & Aamand, 1989; Forrester et al., 1992; Singleton et al., 1995; Tseng et al., 1998). Interestingly, hyperadenylation is not observed in *gle2*Δ strains. This result might indicate that Gle2p does dramatically affect mRNA export. This interpretation is supported by a side-by-side comparison that showed that the level of

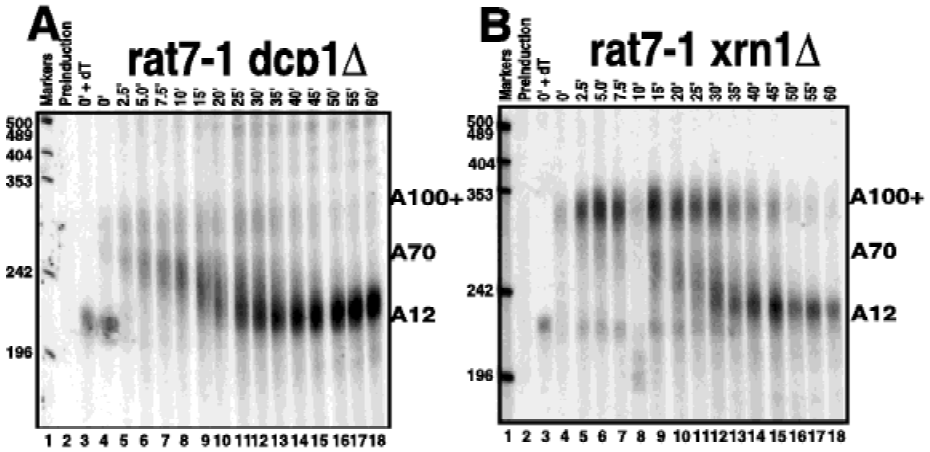

FIGURE 5. Dcp1p and Xrn1p are required for the progression of the *rat7-1* transcripts into decay intermediates. Northern blots of *PGK1pG* transcriptional pulse-chase reactions in *rat7-1* strains also carrying mutations in *DCP1* (**A**; yRP1583) or *XRN1* (**B**; yRP1584). In **A**, the nascent length of the poly(A) tails appears to be shorter in the *rat7-1 dcp1*Δ strain, but this is due to incomplete RNAse H-directed cleavage of the full-length *PGK1pG* transcripts.

poly(A)+ staining is significantly less robust in *gle2*Δ than in *mex67-5* cells (Bailer et al., 1998). Alternatively, different export mutants might differentially affect the level of adenylation. In that nuclear poly(A)+ staining has traditionally been the primary tool used to investigate mRNA export defects, some mutations whose primary defect is hyperadenylation might be characterized as mRNA export mutants.

This raises the interesting question as to how defects in mRNA export, including defects in nucleoporins (Rat7p), which are presumably physically removed from the site of poly(A) tail addition, lead to the appearance of hyperadenylated transcripts. Hyperadenylation might represent the nascent length of poly(A) tails synthesized on normal transcripts, or be due to a longer dwell time in the nucleus, and only detected in these mutants because of a slowed rate in nuclear export. Although we cannot formally rule out these possibilities, two observations make them unlikely. First, we have been unable to detect the hyperadenylated species in the wild-type strain even after extremely long exposures, although we are able to detect a population of transcripts with poly(A) tails that coincide with the length observed in the *pan2*Δ mutant (see Fig. 2). Second, we did not detect any hyperadenylated species in the *gle2*Δ strains. Although the mRNA export defect may be minor in *gle2*Δ cells, they do accumulate low levels of perinuclear poly(A)+ RNA at both the permissive and restrictive temperatures (Murphy et al., 1996; Bailer et al., 1998). If detection of the hyperadenylated species is a consequence of delayed export, then we would have expected to detect a minor level of hyperadenylated transcripts in the *gle2*Δ strain.

An alternate explanation for the hyperadenylation of nascent mRNAs in these export mutants is that these lesions primarily lead to a failure to remove nuclear mRNP (shuttling) proteins that may have functioned in earlier steps during the biogenesis of the mRNA. This view is supported by several observations. First, Rat7p, Mex67p, Gle1p, and Rat8p all show localization at the cytoplasmic face of the NPC, and have been proposed to play a role in the remodeling of the mRNP during export (Gorsch et al., 1995; Del Priore et al., 1996; Murphy & Wente, 1996; Segref et al., 1997; Snay-Hodge et al., 1998; Tseng et al., 1998; Hodge et al., 1999; Schmitt et al., 1999; Strahm et al., 1999; Rout et al., 2000; Straber et al., 2000). Second, both Mex67p and Rat8p are shuttling proteins, suggesting they may play an additional role in the nucleus (Santos-Rosa et al., 1998; Hodge et al., 1999). In this light, both Mex67p and Rat8p have been found to interact either directly or indirectly with the yeast protein, Yra1p (Schmitt et al., 1999; Straber & Hurt, 2000; Stutz et al., 2000). The mammalian homolog of this protein, Aly1p, was recently shown to facilitate the preferential export of mRNPs having had the history of pre-mRNA splicing (Zhou et al., 2000). Third, one effect of the *mex67-5* mutation at the nonpermissive temperature is its own mislocalization from the nuclear periphery into dotlike particles dispersed throughout the cytoplasm (Segref et al., 1997). This might indicate that at the restrictive temperature, mex67-5p is inappropriately retained on the exported mRNP.

Hyperadenylation is linked to the delayed metabolism of nuclear transcripts

Our observations suggest a simple interpretation that the presence of a hyperadenylated poly(A) tail blocks the export of mRNA out of the nucleus. This view is supported by the observation that *rat7-1* hyperadenylated mRNAs that are able to be processed to a normal

poly(A) tail length can undergo normal mRNA metabolism by the criteria that they can be translated, undergo normal cytoplasmic deadenylation, decapping, and 5′-to-3′ degradation.

An alternate view is that hyperadenylation impacts an earlier step in mRNA biogenesis, for example, release of the transcript from the site of transcription. This view is supported by the recent observation by Custodio et al. (1999) that *β-globin* transcripts with a mutant poly(A) cleavage signal are retained at the site of transcription. Moreover, Rosbash and colleagues have recently found that the yeast *SSA4* and *HSP104* heat shock mRNAs are hyperadenylated in the *rat7-1*, *mex67-5*, and *rat8-2* strains, and that these transcripts accumulate at or near the site of transcription (T. Heick-Jensen & M. Rosbash, pers. comm.). In this case, hyperadenylation may signal an abnormality in 3′-end formation that inhibits the release of nascent transcripts from the site of transcription. Results from our transcription pulse-chase experiments would indicate that the hyperadenylated transcripts are released, albeit slowly, from their stall at the transcription site in *rat7-1*, *mex67-5* and *rat8-2* cells. The transition to a normal poly(A) tail population that we observe in transcripts from these strains might occur during or after export to the cytoplasm and engagement of the translation machinery.

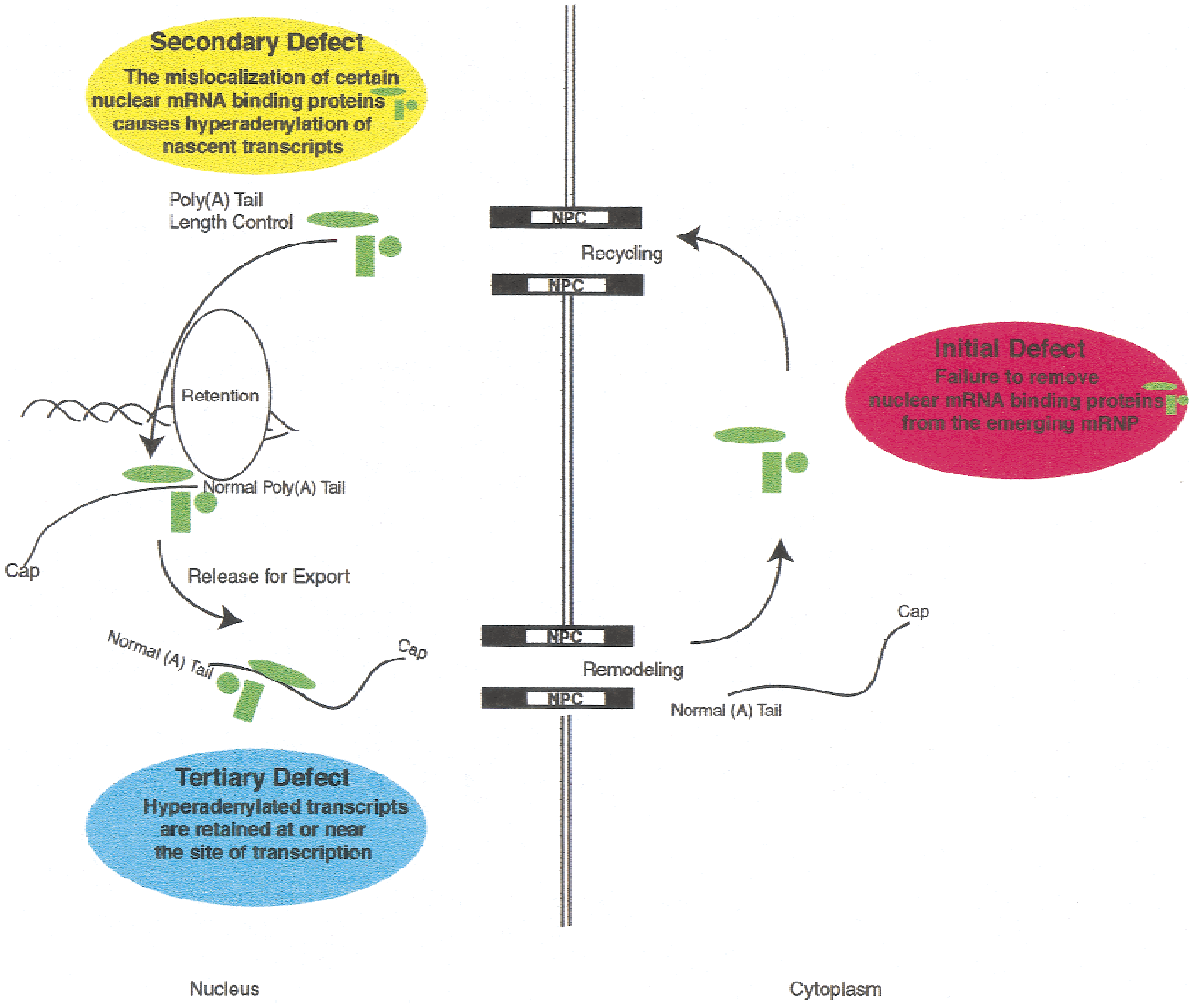

FIGURE 6. Hypothesis for how lesions in Rat7p, Gle1p, Rat8p, and Mex67p lead to sequential defects in mRNA metabolism that result in hyperadenylation and nuclear accumulation of mRNA. In this view, the primary defect of lesions in *RAT7*, *GLE1*, *MEX67*, and *RAT8* is the failure to remodel or remove nuclear mRNA-binding proteins from the transcript as it emerges into the cytoplasm through the nucleopore complex (NPC). Ordinarily, these nuclear mRNA-binding proteins would recycle back into the nucleus, but they are mislocalized into the cytoplasm. Some of these factors are important for poly(A) tail length control, and their sequestration in the cytoplasm leads to the secondary defect of aberrant hyperadenylation of nascent transcripts. The aberrant hyperadenylation is somehow sensed, which leads to the tertiary defect of retention of these transcripts at the site of transcription.

These results imply that a sensing system exists within the cell to inhibit mRNAs with inappropriate 3′ ends from being exported. In principle, such a system could function in two general manners. First, the formation of a proper 3′ end could allow the nascent mRNA to associate with specific factors that mediate transport out of the nucleus. Alternatively, the formation of a proper 3′ end might relieve a retention system that maintains the nascent transcript in the nucleus. The biological function of such a system would be to ensure that any mRNAs that are transported to the cytoplasm have a proper mRNP organization.

Taken together, these data suggest the following possible hypothesis (diagramed in Fig. 6) to describe how lesions in Rat7p, Mex67p, Gle1p, and Rat8p lead to a defect in mRNA export. In this hypothesis, the primary defect of lesions in these factors lies in their failure to appropriately remodel the mRNP after transit through the pore. This failure leads to a mislocalization or cytoplasmic sequestration of specific shuttling factors that also play a role in promoting proper poly(A) tail metabolism. The reduced nuclear levels of these factors lead to inappropriate (hyperadenylated) poly(A) tail length. An important aspect of this model is that hyperadenylated transcripts are retained in the nucleus, perhaps near the site of transcription by an unknown mechanism that senses inappropriate mRNP composition or architecture. In this view, the rapid and robust poly(A) accumulation observed in these strains by oligo d(T) staining is likely a consequence of the excessive adenylation and transcript retention.

MATERIALS AND METHODS

Yeast strains and genetic manipulations

The relevant genotypes of the strains used in this study are listed in Table 1. All DNA manipulations, yeast transformations, matings, sporulation, and tetrad dissections were performed using standard protocols and techniques (Guthrie & Fink, 1991). In that some strains were not absolutely isogenic, transcriptional pulse-chase reactions were analyzed from at least two individual strains of each genotype and yielded mRNA phenotypes consistent with the data presented. Introduction of the reporter construct bearing a nonsense mutation was done by transformation of strains yRP683 and yRP1573 with the plasmid pRP555 that contains the *PGK1pG* with a nonsense codon, *PGK1(N103)pG* reporter construct under the control of the *GAL UAS* (Muhlrad & Parker, 1994). *GLE2* and *RAT8* overexpression plasmids were kind gifts of S. Wente and C. Cole, respectively, and were used to transform the *rat7-1* strain yRP1579 using standard procedures.

Transcriptional pulse chase

Transcriptional pulse-chase experiments were performed essentially as previously described (Decker & Parker, 1993). In all cases, the cultures were shifted to 37 °C for 1 h to effect the loss-of-function phenotype prior to transcriptional induction.

RNA analysis

Total RNA extractions, RNase H manipulations, denaturing polyacrylamide electrophoresis, electrophoretic transfer, and northern blotting were carried out as previously described (Caponigro et al., 1993; Decker & Parker, 1993).

Polysome analysis

Polysome extracts were made according to the method of Atkin et al. (1995) with the following modifications. Cultures of 500 mL were grown at 24 °C in standard YEP media containing 2% raffinose to an A_{600} of ~0.3, at which time they were transferred to 37 °C for 25 min. Prewarmed (37 °C) galactose was then added to the culture to a final concentration of 2% and transcription was allowed to proceed for 35 mins, at

TABLE 1. Yeast strains used in this study.

Strain	Genotype	Source
YRP683	*MATa leu2-3,112 lys2-201 his4-539 trp1-1 ura3-52*	Roy Parker
YRP840	*MATa cup1::LEU2PM leu2-3,112 his4-539 trp1-1 ura3-52*	Roy Parker
YRP841	*MAT α cup1::LEU2PM leu2-3,112 lys2-201 trp1-1 ura3-52*	Roy Parker
YRP1069	*MAT α dcp1::URA3 ura3-52 leu2-3,112 lys2-201 trp1-1 cup1::LEU2PM*	Roy Parker
YRP1199	*MAT α xrn1::URA3 his4-539 leu2-3,112 trp1-1 ura3-52*	Roy Parker
yRP1573	*MAT α rat7-1 his3 Δ 200 ura3-52 leu2 Δ 1*	Charles Cole
yRP1574	*MATa rat8-2 ura3-52 trp Δ 63 leu2 Δ 1*	Charles Cole
yRP1575	*MAT α gle1-4 ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100*	Susan Wente
yRP1576	*MATa gle2::HIS3 ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	Susan Wente
yRP1577	*MATa mex67::HIS3 ade2 his3 leu2 trp1 ura3 [PUN100-LEU2-mex67-5]*	Ed Hurt
yRP1578	*MAT α pan2::URA3 cup1::LEU2PM leu2-3,112 trp1-1 lys2-201ura3-52*	Roy Parker
yRP1579	*MATa rat7-1 cup1::LEU2PM his3 Δ 200 his4-539 leu2 Δ 1 or leu2-3,112 ura3-52*	This study
yRP1580	*MATa gle1-4 cup1::LEU2PM ura3-1 or ura3-52 trp1-1 his3-11,15 and/or his4-539*	This study
yRP1581	*MATa gle2::HIS3 cup1::LEU2PM his3-11,15 or HIS3 ura3-1 or ura3-52 leu2-3,112 trp1-1*	This study
yRP1582	*MATa rat8-2 cup1::LEU2PM leu2-3,112 or leu2 Δ 1 lys2-201 trp1-1 or Δ 63 ura3-52*	This study
yRP1583	*MATa rat7-1 dcp1::URA3 leu2-3,112 or leu2 Δ 1 lys-201 trp1-1 ura3-52*	This study
yRP1584	*MATa rat7-1 xrn1::URA3 cup1::LEU2PM lys2-201 trp1-1 leu2 Δ 1 or leu2-3,112 his4-539 HIS3 or his3 Δ 200*	This study

which time dextrose was added to a final concentration of 4%. During extract preparation, cyclohexamide was omitted.

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