

UCLA

UCLA Electronic Theses and Dissertations

Title

Prolonged α -amanitin treatment of cells for studying mutated polymerases causes degradation of DSIF 160 and other proteins

Permalink

<https://escholarship.org/uc/item/6210g73g>

Author

Tsao, David Ching-heng

Publication Date

2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Prolonged α -amanitin treatment of cells for studying mutated polymerases
causes degradation of DSIF 160 and other proteins

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Biochemistry and Molecular Biology

by

David Ching-heng Tsao

2012

© Copyright by

David Ching-heng Tsao

2012

ABSTRACT OF THE THESIS

Prolonged α -amanitin treatment of cells for studying mutated polymerases
causes degradation of DSIF 160 and other proteins

by

David Ching-heng Tsao

Master of Science in Biochemistry and Molecular Biology

University of California, Los Angeles, 2012

Professor Harold G. Martinson, Chair

A useful method for studying the function of the mammalian RNA polymerase II takes advantage of the extreme sensitivity of its largest subunit, Rpb1, to α -amanitin. Mutations of interest are introduced into an α -amanitin-resistant version of Rpb1, which is then expressed ectopically in cells. The phenotypes of these cells are then examined after inhibiting the endogenous wild-type polymerase with α -amanitin. Here, we show that cells that are enabled to grow in α -amanitin by expression of an α -amanitin-resistant Rpb1 exhibit changes in cell physiology that can lead to misleading experimental outcomes. The changes we have characterized include the accelerated degradation of some proteins, such as DSIF160, and the reduced rate of synthesis of others. In one series of experiments, we examined an α -amanitin-resistant construct, with a mutant C-terminal domain (CTD), that was unable to direct poly(A)-dependent transcription termination in cells growing in α -amanitin. The potential interpretation

that the termination defect in this construct is due to the mutation in the CTD was rejected when the construct was found to be termination-competent in cells grown in the absence of α -amanitin. Instead, it appears that certain termination factors become limiting when the cells are grown in α -amanitin, presumably due to the α -amanitin-induced degradation we have characterized and/or to the inadequate transcription of certain genes by the α -amanitin-resistant Rpb1-containing polymerase.

The thesis of David Ching-heng Tsao is approved.

Catherine F. Clarke

Albert J. Courey

Harold G. Martinson, Committee Chair

University of California, Los Angeles

2012

TABLE OF CONTENTS

<u>Section</u>	<u>Page</u>
Abstract	ii
List of Figures	vi
Acknowledgement	vii
Introduction	1
Results	3
Discussion	12
Materials and Methods	15
References	32

LIST OF FIGURES

Figure	Page
1A	16
1B	17
1C	18
1D	19
1E	21
1F	22
2A	23
2B	24
2C	25
2D	26
2E	27
3A	28
3B	29
3C	30
4	31

ACKNOWLEDGEMENTS

This thesis is a format-modified version of the published article of the same title by Tsao DC, Park NJ, Nag A, Martinson HG. 2012. *RNA* **18**:222-9. I would like to thank Dr. Harold Martinson for his mentorship through the years, and teaching the ways of approaching research and giving presentations that continue to benefit me everyday at my job. I thank Noh Jin Park for initiating and doing many of the important experiments for this research project. I also thank former members of the Martinson lab, especially Steven Kim, Amir Kazerouninia, Anita Nag, Frank Rigo, and Ian Orozco, for their friendship and camaraderie. Last but not least I thank my family, especially my parents, and Sally, for their unwavering support.

INTRODUCTION

Genetic analysis of RNA polymerase II (Pol II) function in mammalian cells is facilitated, in the case of its largest subunit Rpb1, by the extreme sensitivity of Rpb1 to inhibition by α -amanitin (α -ama). In such experiments, α -ama-resistant Rpb1 subunits (Rpb1r), bearing mutations of interest, are ectopically expressed in cells treated with α -ama. The α -ama prevents transcription by any Pol II that contains the wild-type Rpb1 so that one can monitor, unambiguously, the activities of the mutant (Bartolomei et al. 1988; Meininghaus et al. 2000; Fong and Bentley 2001; Laurencikienė et al. 2006; Martins et al. 2011; Sims et al. 2011).

One of the most extensively studied regions of Rpb1 is its C-terminal domain (CTD). This is an essential and highly unusual domain, often simply called the Pol II CTD. In vertebrates, the CTD consists of 52 tandemly repeated heptapeptides terminated by an additional 10-amino acid sequence. One of the principal roles of the CTD appears to be the orchestration of diverse interactions with a multitude of proteins that modulate Pol II function and that serve to integrate transcription with numerous downstream processes, such as capping, splicing, and cleavage/polyadenylation of the transcript (Buratowski 2009). The fact that transcription is integrated with downstream events was first demonstrated by the finding that termination of transcription at protein-coding genes in mammalian cells depends on the poly(A) signal, an RNA motif required for 3'-end processing of the transcript (Whitelaw and Proudfoot 1986). Subsequent work has established a strong correlation between the molecular requirements for poly(A)-dependent termination and for cleavage/polyadenylation, both of which rely on the poly(A) signal (Richard and Manley 2009; Kuehner et al. 2011). However, it remains unclear

whether one process actually depends on the other or whether the two processes are driven independently by the same bifunctional apparatus (Qu et al. 2006).

The studies described in the present communication were motivated by previous reports that a particular Rpb1r, whose CTD lacks the final 27 heptads as well as the terminal 10 amino acids, was unimpaired in its ability to support poly(A)-dependent termination (Park et al. 2004), yet was deficient in yielding transcripts that were successfully processed at the 3' end (Fong and Bentley 2001; Fong et al. 2003). One possible interpretation of these results is that poly(A)-dependent termination can proceed independently of poly(A) site cleavage of the transcript. An alternative, but not mutually exclusive, interpretation is that the cleavage and polyadenylation deficiency in the experiments of Fong and colleagues resulted from treating the cells with α -ama. Cells were not treated with α -ama in the termination experiments of Park and colleagues. Instead, nuclei were isolated from healthy cells, and α -ama was used only for the termination assay, carried out on the isolated nuclei by run-on transcription.

To determine whether the treatment of cells with α -ama may give rise to indirect effects relevant to transcription and its downstream events, we investigated the effects of α -ama on transcription termination within cells and its effects on the cellular levels of some representative proteins related to transcription and processing. The results show that, under certain conditions, α -ama treatment of cells leads to the selective degradation of DSIF160 and other proteins and interferes with the ability of even α -ama-resistant polymerases to interact functionally with the poly(A) signal.

RESULTS

An Rpb1r with partially truncated CTD can support transcription termination in healthy cells but not in cells treated with α -amanitin. The rationale for studying CTD mutations by using ectopically expressed Rpb1r as a vehicle is that this allows the inhibition, using α -ama, of any Pol II that contains the co-expressed endogenous wild-type Rpb1. For transcription termination assays, the procedure is simple—nuclei are isolated from cells producing both the ectopically expressed Rpb1r as well as the wild-type Rpb1, and then nuclear run-on transcription is carried out in the presence of α -ama. Only the Rpb1r-containing Pol II (PolIIr) will yield a signal under these conditions, and the ability of the PolIIr to terminate can be inferred from its presence or absence downstream from termination elements. For the assessment of processing, however, the RNA to be characterized must be produced *in vivo*. This requires that the cells be able to survive growth in α -ama long enough for the PolIIr to produce and support the processing of sufficient RNA to be assayed (and also to allow all RNA previously synthesized by the wild-type Pol II to exit the nucleus). This extended growth in α -ama, in turn, requires that the PolIIr (notwithstanding any deficiencies in its mutant CTD) be able to support the transcription and processing required for maintenance of an RNA-processing infrastructure within the cell. Moreover, in the presence of α -ama (and an Rpb1r), synthesis and degradation of the wild-type Rpb1 presumably occurs continuously (Nguyen et al. 1996; Anindya et al. 2007). Therefore, the possibility, in such experiments, that this influence of α -ama on cell physiology may indirectly affect pre-mRNA processing must be considered.

To determine whether the growth of cells in α -ama might affect the ability of a PolIIr to engage in post-transcriptional activities, we set out to assay poly(A)-dependent termination in the

nuclei of Rpb1r-transfected cells that either had or had not been grown for a period of time in α -ama. The reporter construct used for these experiments is shown in Figure 1A. Figure 1B shows a typical termination assay for cells not grown in α -ama but expressing an Rpb1r. Following transfection of both the Rpb1r expression plasmid and the reporter into cells, nuclei were isolated, and termination was assayed by run-on transcription in the presence of α -ama (Nag et al. 2007). Termination of PolIIr transcription downstream from the poly(A) site is indicated by the reduced 174-nt G-less cassette signal compared with that of the 136-nt G-less cassette (Fig. 1B, lane 1). The endogenous α -ama-sensitive Pol II can be seen to behave similarly when assays are conducted in the absence of α -ama on nuclei from cells lacking any transfected Rpb1r (last lane of Fig.5B in Orozco et al. 2002). Figure 1B, lane 2, shows that poly(A)-dependent termination does not occur downstream from a mutant poly(A) signal.

Figure 1B, lane 3, illustrates the use of this assay to characterize a CTD mutation, in this case, the deletion of the entire CTD (Park et al. 2004). When the PolIIr lacks its CTD, the 174-nt cassette signal is not diminished in intensity downstream from a poly(A) signal, showing that the CTD is required for termination. The lack of termination is especially obvious when the reduced efficiency of transcription initiation by the CTD-less PolIIr (Gerber et al. 1995) is taken into account (Fig. 1B; cf. the 136-nt cassette signals in lanes 3 and 4 and those in lanes 1 and 2). Indeed, when compared to the 136-nt cassette signal, the 174-nt cassette signal is actually much stronger than expected from mere lack of termination (cf. lane 3 and lane 2). This is thought to reflect the slowing of transcription (and thus the increase in occupancy of polymerases on the template) that normally precedes termination (Orozco et al. 2002; Nag et al. 2007) but which continues indefinitely when termination is abrogated (Park et al. 2004). A template with a mutant poly(A) signal does not induce slowing of transcription by the CTD-less PolIIr (Fig. 1B, lane 4).

Figure 1C shows the application of the assay illustrated in Figure 1B to the Rpb1r mentioned in the Introduction, which has a partially deleted CTD. Recall that this Rpb1r supports transcription termination in run-on assays carried out on nuclei isolated from healthy cells (Park et al. 2004) but is deficient in supporting the production of processed RNA in cells that had been treated with α -ama (Fong and Bentley 2001; Fong et al. 2003). To determine whether α -ama treatment of cells can compromise the ability of this PolIIr to respond to the poly(A) signal, we carried out poly(A)-dependent termination assays on nuclei isolated from healthy cells or from cells exposed to α -ama for various lengths of time (Fig. 1C). For cells not exposed to α -ama, poly(A)-dependent termination occurred normally for the reporter with a wild-type poly(A) signal (cf. lane 2 and lane 1). However, by the time the cells had been in α -ama for 5.5 h, the termination activity was gone (lane 5, note the increase in post-cassette signal compared with lane 2, despite the decrease in pre-cassette signal owing to reduced overall transcription). Several such experiments are summarized in the 1–25 CTD curve of Figure 1D, which traces the increase in post-cassette/pre-cassette ratio (i.e., the loss in termination activity) as a function of the time spent by the cells in α -ama prior to the termination assay. Additional experiments show that the poly(A) signal-dependent slowing of transcription displayed by the CTD-less PolIIr of Figure 1B, lane 3, was also affected by growing the cells in α -ama, but in this case, the effect was augmented (“No CTD” curve) (Fig. 1D). In contrast, the behavior of two other CTD constructs appeared to be unaffected by growing the cells in α -ama (27–52 CTD and Full CTD curves) (Fig. 1D).

The loss in ability to terminate by the 1–25 CTD PolIIr when cells are treated with α -ama (Fig. 1C,D) is reminiscent of its inability to support the production of processed RNA in cells growing in α -ama (Fong and Bentley 2001; Fong et al. 2003). To check this correlation for the

experiment of Figure 1C, we set aside a portion of each nuclei preparation for a parallel RNase protection assay (Fig. 1E). The results confirmed that the production of 3' processed RNA in cells does, indeed, diminish as a function of time spent by the cells in α -ama. Note that the loss in ability to produce processed RNA in Figure 1E, lane 6, does not necessarily mean that this truncated CTD cannot support processing—just as the failure of poly(A)-dependent termination in lanes 5 and 6 of Figure 1C does not mean that this truncated CTD cannot support poly(A)-dependent termination (which it does in lane 2 of Fig. 1C).

The experiments described so far have involved the SV40 early poly(A) signal. To determine whether treatment of cells with α -ama would compromise the ability of another poly(A) signal to drive termination, we repeated some of the above experiments with the SV40 late poly(A) signal, which differs considerably in sequence from the SV40 early signal and is about five times stronger (Carswell and Alwine 1989; Orozco et al. 2002). Figure 1F shows that, as for the SV40 early poly(A) signal, transcription termination by the 1–25 CTD PolIIr downstream from the SV40 late poly(A) signal, diminishes when the cells are exposed to α -ama. However, the negative effect of α -ama on the SV40 late poly(A) signal (1–25 CTD curve in Fig. 1F) is somewhat less than on the SV40 early signal (1–25 CTD curve in Fig. 1D). This is consistent with the possibility that α -ama decreases the concentration in the cell of one or more factors required for termination and that the stronger SV40 late poly(A) signal is more capable of recruiting these factors at that lower concentration.

Treatment of cells with α -amanitin causes selective protein degradation within the cell. It is well-known that the wild-type α -ama-sensitive Rpb1 is degraded upon treatment of cells with α -ama (Gualis et al. 1979; Nguyen et al. 1996; Anindya et al. 2007). However, the results of Figure 1 suggest that other proteins may be affected as well, even if the wild-type Rpb1

is ectopically replaced with an α -ama-resistant version. To search for other proteins that might be lost upon α -ama treatment, we used Western blots to examine the protein content of cells that had or had not been exposed to α -ama. After blotting for just a few proteins, we observed dramatic losses for two of them, DSIF160 and cyclin F. Figure 2A shows that after 14 hours in α -ama, cells expressing the 1–25 CTD Rpb1r (lanes 1–6) have lost almost their entire inventory of these two proteins.

Initially, we had expected to observe losses of one or more of the well-known cleavage/polyadenylation factors, such as CstF. However, Figure 2A shows that the effect of α -ama on CstF64 levels is relatively minor, especially when compared to the presumptive loading control in this experiment, TBP. To determine whether these reduced levels of CstF might be sufficient to affect transcription termination, we used RNAi. Figure 2B shows that the RNAi-mediated knockdown of CstF to levels that are less than those observed during α -ama treatment of cells has no deleterious effect on transcription termination (the post/pre ratio remains low even after knockdown). A similar experiment in which both CstF64 and CstF77 were knocked down gave the same result (data not shown). In contrast, transcription termination is completely abolished by elimination of the G/U-rich region in the RNA, to which CstF64 binds (Nag et al. 2006). Thus, although CstF64 is presumably required for termination in mammalian cells, as it is in yeast (Birse et al. 1998; Aranda and Proudfoot 2001), the amount of CstF64 that remains after knockdown is still sufficient to fulfill this function. This indicates that the relatively small decrease in CstF caused by α -ama (Fig. 2A) is not, at least by itself, responsible for the α -ama-induced loss in ability of the 1–25 CTD PolIIIr to terminate transcription (Fig. 1C,D). Nevertheless, the loss of cleavage/polyadenylation-related functions upon incubation of cells in

α -ama (Fig. 1C,E) suggests that, directly or indirectly, there is some cleavage/polyadenylation factor(s) that is susceptible to α -ama-induced loss of function.

To learn more about the α -ama effect, we decided to focus on DSIF160. Figure 2C shows that both high and low concentrations of α -ama induce loss of DSIF160. Figure 2D, lanes 6 and 8, show that the 27–52 CTD and Full CTD Rpb1r constructs, though able to rescue transcription termination (Fig. 1D) and pre-mRNA processing (Fong and Bentley 2001; Fong et al. 2003) from the effects of α -ama, cannot efficiently rescue DSIF160 from α -ama-induced degradation. Thus, the effects of α -ama are complex, with different functions being affected in different ways. This conclusion is reinforced by the results of Figure 2E, which show again, using a different Rpb1r construct, that α -ama causes DSIF160 to be lost under conditions where the ability of transcription to terminate is rescued.

The loss of DSIF160 in the presence of α -ama could reflect impaired DSIF160 synthesis coupled with a short half-life, or it could reflect accelerated degradation. Figure 3A shows that blocking protein synthesis with cycloheximide (Hinkson and Elias 2011) does not result in rapid loss of DSIF160. Indeed, the half-life of DSIF160 appears to be comparable to that of CstF64 and HDAC-1 (chosen arbitrarily for comparison) and much greater than that of cyclin F, a protein of known short half-life (Fung et al. 2002). This suggests that the α -ama-induced loss of DSIF160 reflects accelerated DSIF160 degradation. To test this possibility, we treated cells with α -ama in the presence or absence of MG132, a proteasome inhibitor (Lee and Goldberg 1998). Figure 3B shows that MG132 rescued DSIF160 from α -ama-induced loss (cf. lanes 3 and 6 with lanes 2 and 5, respectively). Cyclin F was also rescued from α -ama-induced loss by MG132, albeit in a form with reduced mobility on the gel (Cope and Deshaies [2006] have also observed a slow form of cyclin F in MG132). In contrast, the amount of CstF64 in α -ama-treated cells was

not increased at all by the presence of MG132 (cf. lanes 3 and 6 with lanes 2 and 5, respectively). The effect of MG132 on the amount of HDAC-1 in α -ama-treated cells was similarly small.

We wondered whether the addition of MG132 would rescue the ability of the 1–25 CTD Rpb1r to support transcription termination in α -ama-treated cells. Recall that this particular Rpb1r is unable to support transcription termination downstream from the SV40 early poly(A) signal when ectopically expressed in cells that are treated with α -ama (Fig. 1C,D). Figure 3C shows that MG132 partially restores the ability to terminate under these circumstances, suggesting that some, but not all, of the functional deficit caused by α -ama is due to protein degradation in the cells.

It is interesting that the ectopic expression of certain Rpb1r constructs can fully rescue transcription termination in α -ama-treated cells (Figs. 1D, 2E) despite the continued degradation of DSIF160 (Fig. 2D,E). This suggests that it is not the ability of MG132 to rescue DSIF160 from degradation (Fig. 3B) that partially restores transcription termination to α -ama-treated cells (Fig. 3C). Thus, we can identify at least three groups of proteins whose presence or function is impacted by the presence of α -ama in cells—DSIF160, cyclin F, and, presumably, other proteins that are degraded but not required for transcription termination; unknown proteins, required for termination, that can be rescued by MG132 in cells expressing the 1–25 CTD Rpb1r (Fig. 3C); and other unknown proteins important for termination that cannot be rescued by MG132 (Fig. 3C).

Inadequate Rpb1r expression in α -ama-treated cells allows transcription initiation but not termination. We have seen (Fig. 1C) that the 1–25 CTD Rpb1r is able to support transcription termination within cells that also express normal levels of the wild-type Rpb1 (i.e., in cells not exposed to α -ama) but not in cells that depend exclusively on the 1–25 Rpb1r for Pol

II activity (i.e., in cells growing in α -ama). In contrast, the 27–52 CTD, the Full CTD, and the 1–22 CTD Rpb1r constructs can support transcription termination even when they are the sole source of Rpb1 activity (Figs. 1D, 2E). This suggests that, although these three constructs cannot prevent the α -ama-induced degradation of certain proteins such as DSIF160 (Figs. 2D,E, 3B), they, nevertheless, do support the production of sufficient levels of other (as yet unidentified) proteins required for transcription termination. A significant distinction between the 1–25 CTD Rpb1r and the other Rpb1r constructs is that the 1–25 Rpb1r is expressed poorly, possibly reflecting a destabilizing effect of that particular CTD mutation on the Rpb1 (Chapman et al. 2005), but the others are expressed well, in transfection experiments (Park et al. 2004; Rosonina & Blencowe 2004). Therefore, we wondered whether the ability of the 27–52 CTD, the Full CTD, and the 1–22 CTD Rpb1r constructs to support termination, when they are the sole source of Rpb1 activity in the cell, might be related to the robustness of their expression following transfection.

To determine whether the Rpb1r expression level would affect the efficiency of transcription termination, we carried out the experiment of Figure 4. Lanes 1 and 2 show that, when the 1–25 CTD and 1–22 CTD Rpb1r constructs were each co-expressed with the endogenous wild-type Rpb1 (i.e., cells grown in the absence of α -ama), they supported transcription termination downstream from the SV40 early poly(A) signal with similar efficiencies, in agreement with Figures 1D and 2E. Also in agreement with the previous figures, when expression of the endogenous Rpb1 was prevented with α -ama, termination by the 1–25 Rpb1r (lane 3), but not by the 1–22 Rpb1r (lane 4), was inhibited. However, when the amount of transfecting 1–22 Rpb1r expression plasmid was gradually reduced (lanes 5–8), the efficiency of termination was similarly diminished, ultimately approaching the level of the poorly expressed

1–25 Rpb1r (lane 3). The simplest interpretation of these results is that inadequate amounts of one or more short-lived proteins, required for termination, are produced when very small amounts of expression plasmid are transfected. Note, however, that even when there is insufficient Rpb1r to produce essential proteins for transcription termination, the factors required for transcription itself (i.e., the polymerase and its initiation and elongation factors) remain at sufficient levels to produce the non-terminating read-through transcription that is detected in our assays (e.g., Fig. 1C, lanes 5 and 6).

DISCUSSION

Mammalian Pol II Rpb1 function can be studied by use of elegant genetic experiments in which the endogenous polymerase is inactivated with α -ama while a mutant Rpb1 is provided ectopically in an α -ama-resistant form. However, this approach usually requires growing cells in the presence of α -ama, which raises the possibility that off-target or indirect effects of α -ama on cell physiology may influence phenotype in ways that need to be distinguished from any direct effects that the Rpb1 mutation may have on function. For example, the 1–25 CTD Rpb1r used in Figure 1C can initiate and elongate transcription in cells treated with α -ama but exhibits a deficit in termination of transcription (lanes 5 and 6). Yet, this defect is not a direct consequence of the CTD mutation on termination, because the 1–25 CTD Rpb1r can support transcription termination just fine within cells not growing in α -ama (Fig. 1C, lane 2). Instead, the termination defect is an indirect consequence of the fact that cells growing in α -ama are forced to rely on the poorly expressed 1–25 CTD Rpb1r polymerase for all of their Pol II transcription, which apparently results in the insufficient production of unidentified short half-life proteins required for termination (Fig. 4). Consistent with this possibility, inhibiting protein degradation while cells are grown in α -ama partially restores transcription termination by the 1–25 CTD construct (Fig. 3C).

Growing cells in α -ama also leads directly to protein degradation, even with Rpb1r constructs that can support unimpaired transcription termination in the α -ama-grown cells (e.g., Fig. 2D, lanes 6 and 8; Fig. 2E). The phenomenon of protein degradation in α -ama-treated cells is well-known. Blocking transcription with α -ama leads to stalling of transcription complexes and wholesale degradation of the large subunit of the endogenous polymerase (Nguyen et al.

1996; Anindya et al. 2007). Moreover, this process is presumably ongoing in Rpb1r-rescued cells growing in the presence of α -ama because the α -ama does not block synthesis of the wild-type Rpb1. It is possible that these widespread upheavals in the cell have significant ripple effects on other processes. Ironically, despite the multiple effects of α -ama documented here, degradation of Rpb1 in the presence of α -ama is actually quite specific—it appears to be a consequence of the blockage of transcription per se (Anindya et al. 2007) and it is not perceptibly accompanied by the degradation of certain other subunits of the polymerase itself (Nguyen et al. 1996). It is, perhaps, for this reason that the possibility of wide-ranging indirect effects of α -ama on cell physiology has not previously received close scrutiny in experiments involving the use of α -ama.

When we began these experiments, we searched for effects on the major cleavage and polyadenylation proteins because these are known to be involved in poly(A)-dependent termination. Although we included DSIF160 and cyclin F only as controls, it is they, not the polyadenylation proteins, that are lost when cells are exposed to α -ama (Fig. 2). The loss of cyclin F in α -ama grown cells (Fig. 2A) may be related to its short half-life (Fig. 3A), but we note that this loss is not rescued by the 1–22 CTD Rpb1r (Fig. 3B, lane 5), which rescues completely the ability to terminate transcription (Fig. 2E). In contrast to cyclin F, DSIF160 does not have a particularly short half-life (Fig. 3A), so its loss is unambiguously due to α -ama-induced degradation, consistent with the observation that loss of DSIF160 is prevented by blocking protein degradation with MG132 (Fig. 3B). Regarding the 3'-end processing proteins, there does appear to be some loss of CPSF after long times in α -ama (Fig. 2C), but the rate of CPSF loss is much lower than the rate at which the ability to terminate transcription is lost (Fig. 1C,D). Similarly, although there is often some loss of CstF when cells are treated with α -ama (Figs. 2A,E, 3B), greater losses due to RNAi have no effect on termination (Fig. 2B).

We have not found conditions for which an ectopically expressed Rpb1r could entirely eliminate the physiological perturbations of growing cells in α -ama. Even expressing an Rpb1r with a completely intact CTD did not rescue DSIF160 from degradation (Fig. 2D, lane 8), despite the fact that this Rpb1r can fully support efficient transcription termination (Fig. 1D). It is possible that certain Rpb1-interacting proteins, of which DSIF160 (Yamaguchi et al. 1999) and Rpb2 (Guialis et al. 1979) would be examples, are at risk for continuous co-degradation (Papavassiliou et al. 1992) in α -ama-grown cells, along with the continuously synthesized and degraded endogenous Rpb1. And this may be what is responsible for the month-long crisis that long-term cell cultures go through (despite stably expressing a fully intact Rpb1r) as they adapt to growth in α -ama (Meininghaus et al. 2000). Finally, we note that co-degradation of Rpb1-interacting elongation factors would be of particular relevance to experiments that examine parameters and processes related to transcription elongation in cells growing in α -ama.

MATERIALS AND METHODS

All Rpb1r expression plasmids were provided by David Bentley (Fong and Bentley 2001), except for the 1–22 CTD Rpb1r, which was from Ben Blencowe (Rosonina and Blencowe 2004). Run-on transcription was carried out on COS cell nuclei that were lysed in high salt and heparin to dissociate most proteins from the DNA and its engaged polymerases (Park et al. 2004; Nag et al. 2007). Addition of α -ama to the run-on reaction (0.3 $\mu\text{g}/\text{mL}$ final concentration) ensured that only the ectopically expressed Rpb1r were detected. The distance transcribed during run-on is short so the amount of [^{32}P] RNA produced is essentially proportional to the number of polymerases engaged on the template. Selected regions of the template code for G-less cassettes. When isolated by T1 RNase digestion, the intensity of [^{32}P] label in the cassettes of various lengths is proportional to the number of polymerases within the cassette boundaries on the template. When living cells were treated with α -ama, higher concentrations were used than for run-on transcription to ensure rapid entry into the cell (Nguyen et al. 1996). For experiments presented here, two different concentrations (2.5 or 20 $\mu\text{g}/\text{mL}$) were used, which we have found to be essentially interchangeable.

Western blotting was carried out according to the BM Chemi-luminescence Western Blotting Kit (Roche) protocol, on nuclei prepared as previously described (Park et al. 2004). Nitrocellulose membranes were used for protein transfer and, for stripping, the Restore Plus Western Blot stripping buffer (Thermo) was used. Anti-B10 was from Chemicon; anti-DSIF160 and anti-symplekin were from BD Signal Transduction; and anti-TBP (N12), anti-cyclin F (C20), and anti-HDAC-1 (H51) were from Santa Cruz Biotechnology. Anti-CPSF73 and anti-CstF64 (3A7) were gifts from David Bentley and Clint MacDonald, respectively.

FIGURE 1A

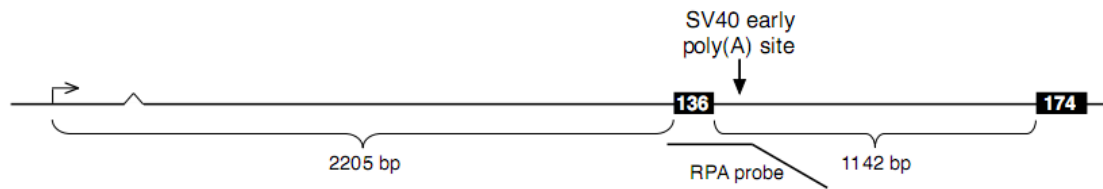


Figure 1. An α -amanitin-resistant Pol II lacking half its CTD supports poly(A) dependent termination in healthy cells but not in cells challenged with a-amanitin. **(A)** Reporter plasmid for the SV40 early poly(A) signal. This is a drawing to scale of construct number 6 in Figure 5A of Orozco et al. (2002) and is the same construct as used for Figure 1C in Park et al. (2004). The transcribed RNA contains two G-less cassettes, 136 and 174 nt in length, at the positions shown.

FIGURE 1B

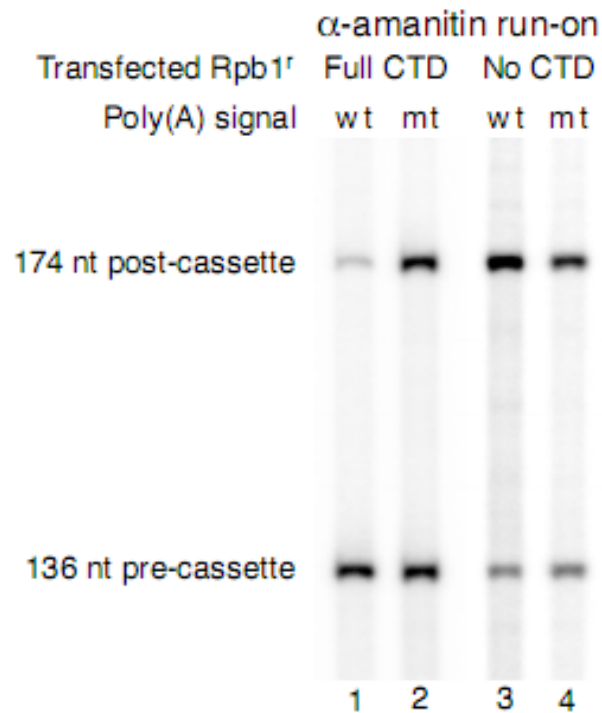


Figure 1B. The PolIIr G-less cassette assay (Park et al. 2004), showing termination for an Rpb1r with intact CTD and pausing for an Rpb1r with no CTD. The reporter shown in part A was used and had either a wild-type poly(A) signal, or one bearing a mutant poly(A) signal hexamer (Orozco et al. 2002).

FIGURE 1C

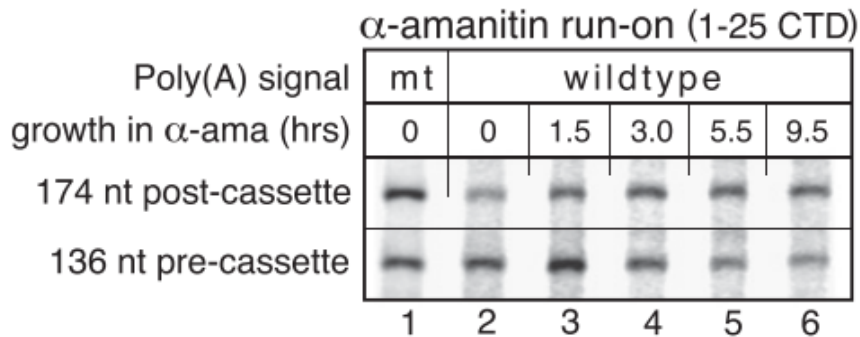


Figure 1C. An Rpb1r with only the first 25 heptads of its CTD supports normal poly(A)-dependent termination in healthy but not in α -ama-treated cells. Cells growing in 100-mm plates were transfected with three plasmids, the reporter, the 1–25 CTD Rpb1r expression plasmid, and a plasmid bearing the RNA polymerase III transcription unit for the U6 RNA. The latter was included as a transfection control for use when the recovery of processed RNA from the nuclei was assayed (see below). Cells were harvested, and nuclei were isolated 2½ days after transfection. At the indicated number of hours before harvest, α -ama was added to the cultures (20 μ g/mL final concentration), and growth was continued until the cells were collected. For each plate of cells harvested, 20% was used for this run-on assay, and 80% was used for the assay of part E.

FIGURE 1D

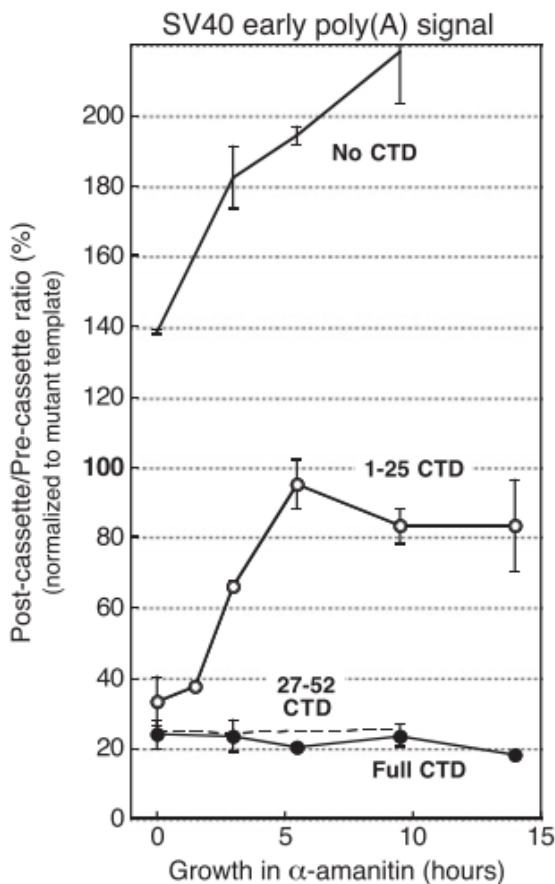


Figure 1D. Quantitative summary of the ability of various Rpb1r CTD variants to support coupling of poly(A) signal function to transcription in cells grown for various lengths of time in α -ama. Procedures were as for part C. The values plotted correspond to the post-cassette/pre-cassette ratio for each lane (expressed as a percent), normalized to the equivalent ratio for a mutant reporter run in parallel (Orozco et al. 2002). The No CTD curve presents the average and range for two independent experiments ($n = 2$). For the ascending time points of the 1–25 CTD curve, $n = 10, 1, 2, 2, 6,$ and $8,$ where the error shown is the range when $n = 2,$ and the standard deviation when $n > 2.$ The error is too small to be visible for the 3-h point. The 27–52 CTD curve

is based on a single experiment with time points at 0, 3, 5.5, and 9.5 h. For the Full CTD curve, n
= 4, 2, 2, 3, and 1.

FIGURE 1E

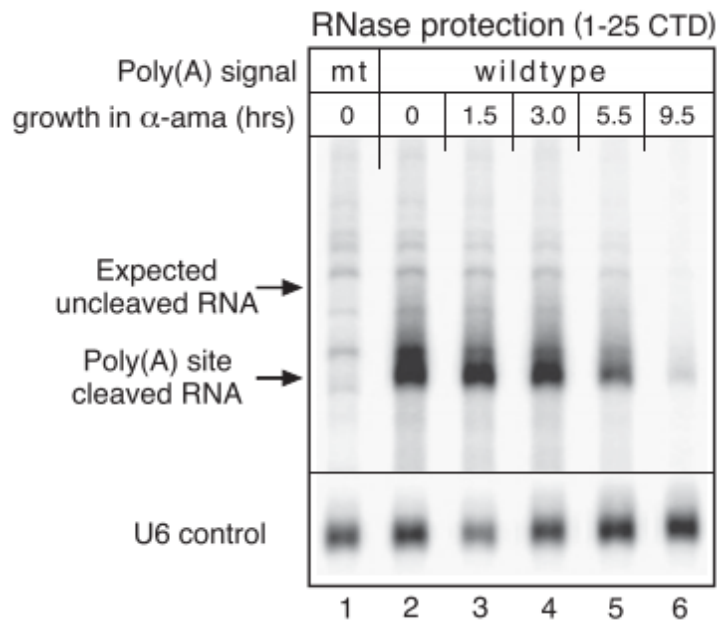


Figure 1E. An Rpb1r with only the first 25 heptads of its CTD does not support production of 3'-end processed RNA in the presence of α -ama. The RNase protection was carried out on RNA isolated from nuclei that were part of the same batch as used for the corresponding time points in part C.

FIGURE 1F

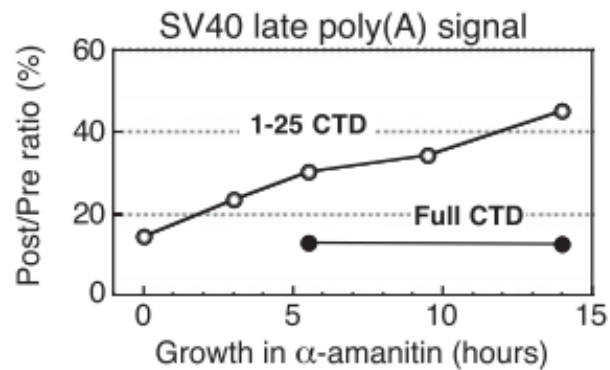


Figure 1F. Growth of cells in α -ama also affects termination efficiency for a strong poly(A) signal. Each curve is a single experiment, carried out as for part D. The reporter used (see Fig. 1A in Orozco et al. 2002) was similar to the one shown in part A, with the principal difference being the presence of the strong SV40 late poly(A) signal in place of the weaker SV40 early poly(A) signal. The ordinate is the same as for part D and is called simply the “Post/Pre ratio” for convenience.

FIGURE 2A

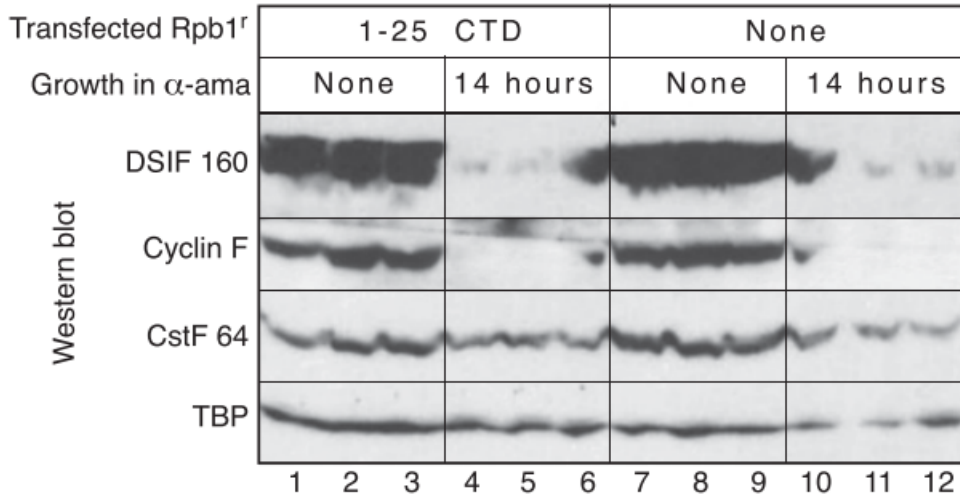


Figure 2. Treatment of cells with α -amanitin causes selective protein loss within the cell. **(A)** DSIF160 and cyclin F are lost when cells expressing the 1–25 CTD Rpb1r are incubated in α -ama. Nuclei were prepared in triplicate from cell cultures essentially equivalent to those used for the zero- and 14-h time points of the 1–25 CTD curve of Figure 1D. Nuclear proteins were blotted for DSIF160, CstF64, TBP and, after stripping, cyclin F. Note that there is considerable spillover from lanes 7 and 9 into lanes 6 and 10, which is most noticeable for DSIF and cyclin F.

FIGURE 2B

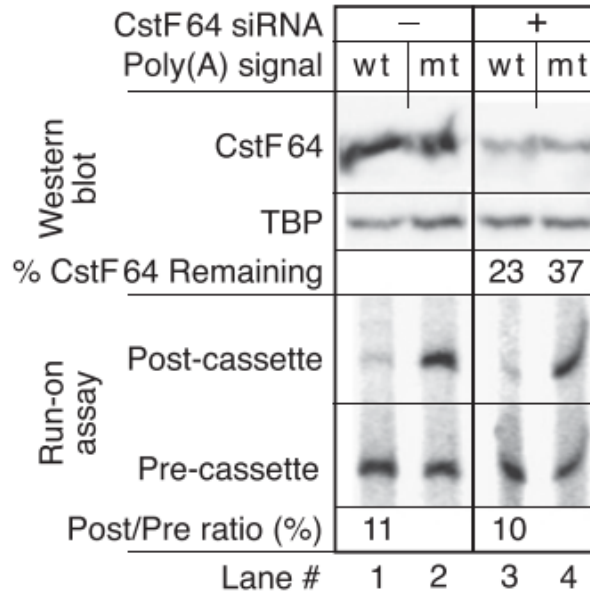


Figure 2B. Moderate RNAi-induced knockdown of CstF64 has no effect on transcription termination. Transfection of 293T cells with siRNA (Dharmacon SMARTpool M-011245-00) was carried out based on the procedure of Wagner and Garcia-Blanco (2002), with the siRNA transfections on days 2, 4, and 6 (all using Lipofectamine 2000; Invitrogen) and the reporter transfection on day 5 (using Fugene 6; Roche). The “% CstF64 remaining” in lanes 3 and 4 was calculated relative to the mock transfections of lanes 1 and 2, after normalizing to the amount of TBP present. The “Post/Pre ratio” was calculated as for Figure 1D,F.

FIGURE 2C

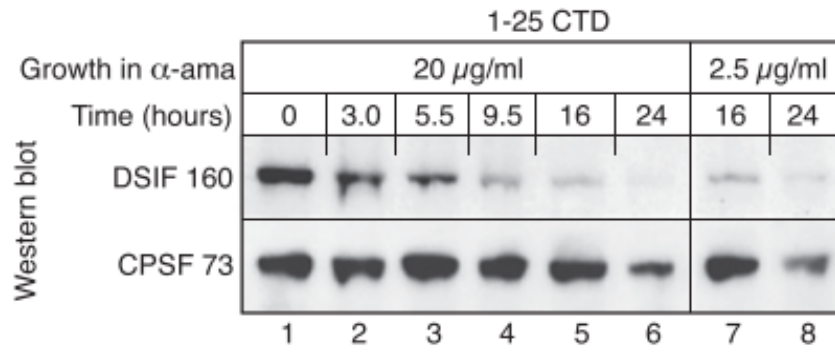


Figure 2C. DSIF160 is subject to α -ama-induced loss at both high and low concentrations of α -ama.

FIGURE 2D

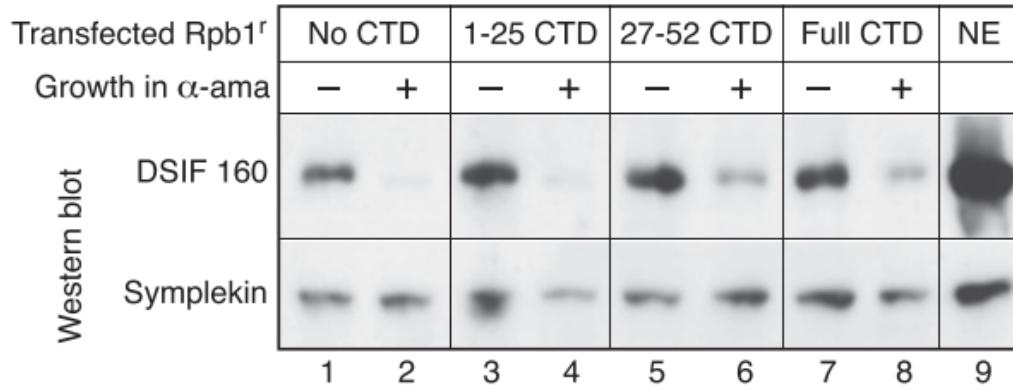


Figure 2D. α -ama causes loss of DSIF160 regardless of whether there is also an effect on RNA processing or transcription termination. Cells for the “+” lanes were grown in 2.5 μ g/mL of α -ama for 24 h.

FIGURE 2E

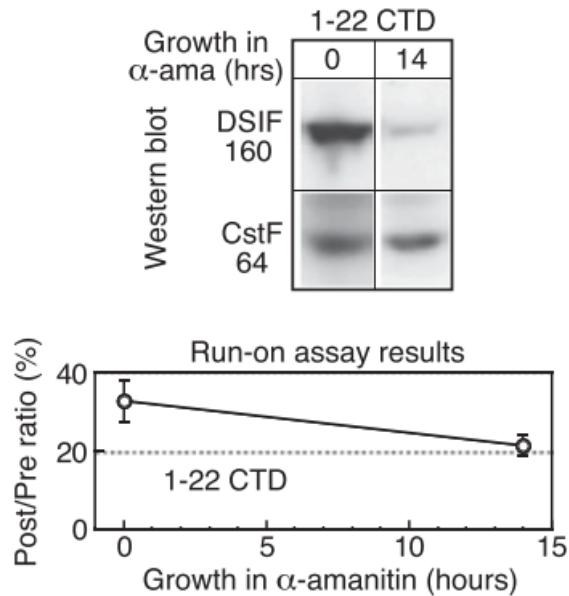


Figure 2E. Confirmation, using the 1–22 CTD Rpb1r expression construct (Rosonina and Blencowe 2004), that DSIF160 is more susceptible to α -ama-induced loss (Western blot) than are termination factors (Run-on assay). The α -ama, dissolved in DMSO, was added to a concentration of 20 μ g/mL for the 14-h time point, and DMSO alone was added for the no α -ama control. The run-on assay panel presents the average and standard deviation for three independent experiments conducted in 6-well plates. The Western blot shows the results of a single scaled-up experiment using 60-mm plates.

FIGURE 3A

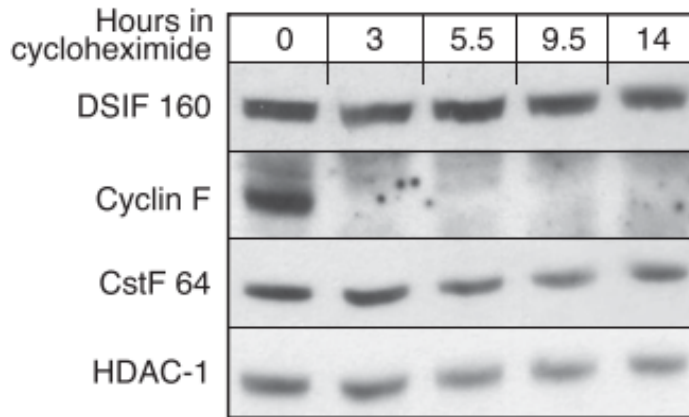


Figure 3. α -amanitin induces protein degradation. (A) DSIF160 does not normally have a short half-life. Untransfected cells were cultured for three days in 35-mm plates, and then nuclei were isolated and assayed by Western blotting. At the indicated number of hours before harvest, cycloheximide was added to a final concentration of 20 $\mu\text{g}/\text{mL}$.

FIGURE 3B

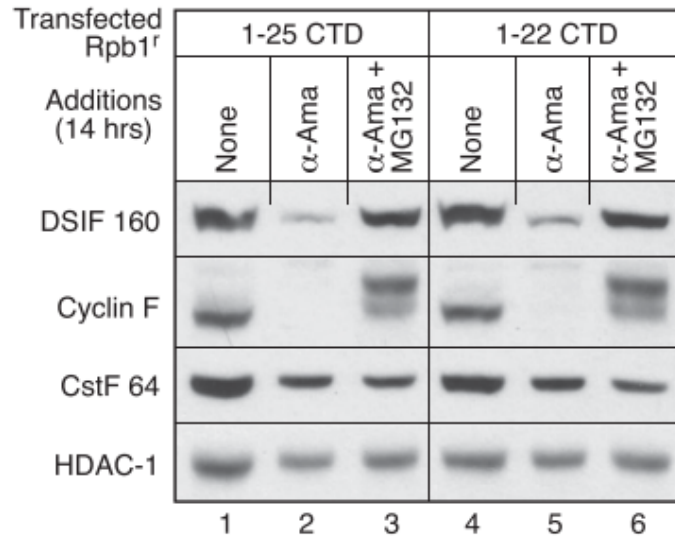


Figure 3B. Inhibition of protein degradation prevents the α -ama-induced loss of DSIF160. The data were obtained essentially as described for the Western blot of Figure 2D. For lanes 3 and 6, MG132, at a final concentration of 10 μ M, was included with the α -ama.

FIGURE 3C

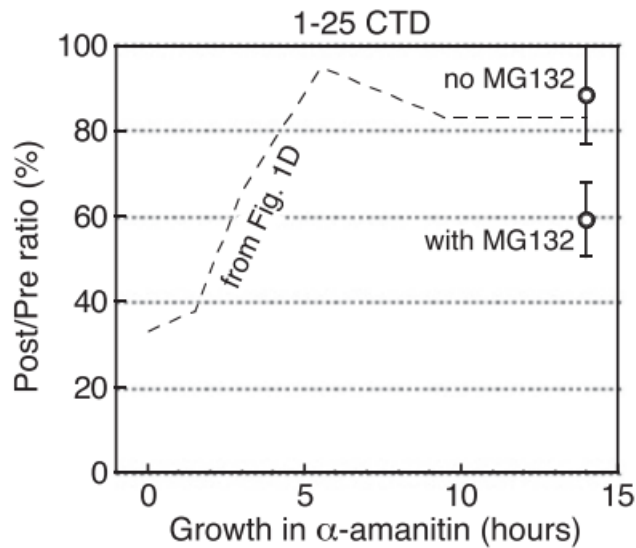


Figure 3C. Inhibition of protein degradation partially rescues α -ama-inhibited transcription termination. Cells treated with 20 $\mu\text{g}/\text{mL}$ of α -ama for 14 h were grown in parallel with cells treated simultaneously with α -ama and 10 μM MG132, and assayed for termination in a manner similar to that described for Figure 1C. The results shown are the average and standard deviation from five independent experiments. Shown for comparison is the 1–25 CTD curve from Figure 1D. In fact, the “no MG132” value comes from a subset of the 14-h time point samples of the 1–25 CTD curve.

FIGURE 4

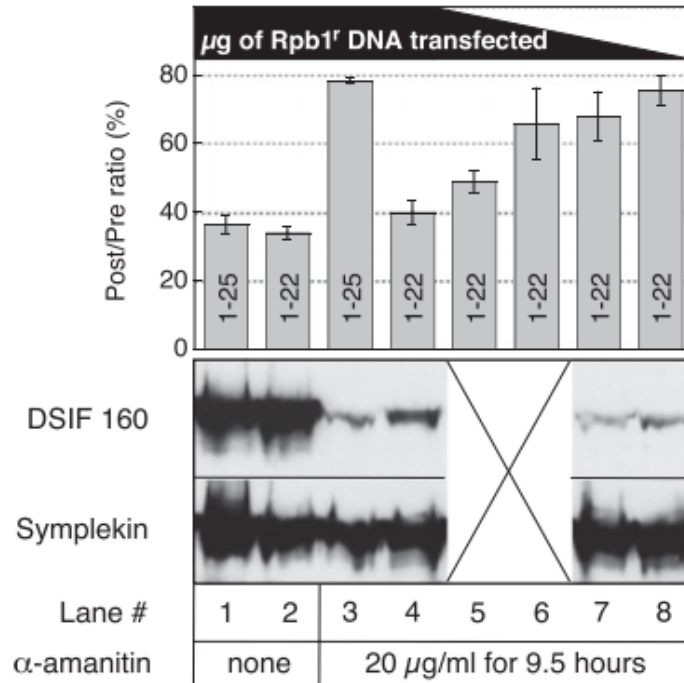


Figure 4. Maintenance of adequate levels of some termination factors depends on the expression of sufficient Rpb1. Lanes 1–4 show a typical experiment in which cells in 35-mm plates were transfected with 0.1 µg of either the 1–25 CTD or the 1–22 CTD Rpb1r construct. After growing without or with α -ama for 9.5 h, each sample was split for run-on and Western blot assays. Lanes 5–8 show the results of forcing the cells to survive on progressively less transfected 1–22 Rpb1r, in which the amount of 1–22 Rpb1r DNA transfected was 4%, 2%, 1%, and 0.5% as much as that for lanes 1–4. No Western blot was carried out for lanes 5 and 6.

REFERENCES

1. Anindya, R., Aygun, O. & Svejstrup, J.Q. 2007. Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1. *Mol Cell* 28: 386-397.
2. Aranda, A. & Proudfoot, N. 2001. Transcriptional termination factors for RNA polymerase II in yeast. *Mol Cell* 7: 1003-1011. Bartolomei, M.S., Halden, N.F., Cullen, C.R. & Corden, J.L. 1988. Genetic analysis of the repetitive carboxyl-terminal domain of the largest subunit of mouse RNA polymerase II. *Mol Cell Biol* 8: 330-339.
3. Birse, C.E., Minvielle-Sebastia, L., Lee, B.A., Keller, W. & Proudfoot, N.J. 1998. Coupling termination of transcription to messenger RNA maturation in yeast. *Science* 280: 298-301.
4. Buratowski, S. 2009. Progression through the RNA polymerase II CTD cycle. *Mol Cell* 36: 541-546.
5. Carswell, S. & Alwine, J.C. 1989. Efficiency of utilization of the simian virus 40 late polyadenylation site: effects of upstream sequences. *Mol Cell Biol* 9: 4248-4258.
6. Chapman, R.D., Conrad, M. & Eick, D. 2005. Role of the mammalian RNA polymerase II C-terminal domain (CTD) nonconsensus repeats in CTD stability and cell proliferation. *Mol Cell Biol* 25: 7665-7674.
7. Cope, G.A. & Deshaies, R.J. 2006. Targeted silencing of Jab1/Csn5 in human cells downregulates SCF activity through reduction of F-box protein levels. *BMC Biochem* 7: 1.

8. Fong, N. & Bentley, D.L. 2001. Capping, splicing, and 3' processing are independently stimulated by RNA polymerase II: different functions for different segments of the CTD. *Genes Dev* 15: 1783-1795.
9. Fong, N., Bird, G., Vigneron, M. & Bentley, D.L. 2003. A 10 residue motif at the C-terminus of the RNA pol II CTD is required for transcription, splicing and 3' end processing. *EMBO J* 22: 4274-4282.
10. Fung, T.K., Siu, W.Y., Yam, C.H., Lau, A. & Poon, R.Y. 2002. Cyclin F is degraded during G2-M by mechanisms fundamentally different from other cyclins. *J Biol Chem* 277: 35140-35149.
11. Gerber, H.P., Hagmann, M., Seipel, K., Georgiev, O., West, M.A., Litingtung, Y., Schaffner, W. & Corden, J.L. 1995. RNA polymerase II C-terminal domain required for enhancer-driven transcription. *Nature* 374: 660-662.
12. Guialis, A., Morrison, K.E. & Ingles, C.J. 1979. Regulated synthesis of RNA polymerase II polypeptides in Chinese hamster ovary cell lines. *J Biol Chem* 254: 4171-4176.
13. Hinkson, I.V. & Elias, J.E. 2011. The dynamic state of protein turnover: It's about time. *Trends Cell Biol* 21: 293-303.
14. Kuehner, J.N., Pearson, E.L. & Moore, C. 2011. Unravelling the means to an end: RNA polymerase II transcription termination. *Nat Rev Mol Cell Biol* 12: 283-294.
15. Laurencikiene, J., Kallman, A.M., Fong, N., Bentley, D.L. & Ohman, M. 2006. RNA editing and alternative splicing: the importance of co-transcriptional coordination. *EMBO Rep* 7: 303-307.

16. Lee, D.H. & Goldberg, A.L. 1998. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 8: 397-403.
17. Martins, S.B., Rino, J., Carvalho, T., Carvalho, C., Yoshida, M., Klose, J.M., de Almeida, S.F. & Carmo-Fonseca, M. 2011. Spliceosome assembly is coupled to RNA polymerase II dynamics at the 3' end of human genes. *Nat Struct Mol Biol* 18: 1115-1123.
18. Meininghaus, M., Chapman, R.D., Horndasch, M. & Eick, D. 2000. Conditional expression of RNA polymerase II in mammalian cells: Deletion of the carboxyl-terminal domain of the large subunit affects early steps in transcription. *J Biol Chem* 275: 24375-24382.
19. Nag, A., Narsinh, K., Kazerouninia, A. & Martinson, H.G. 2006. The conserved AAUAAA hexamer of the poly(A) signal can act alone to trigger a stable decrease in RNA polymerase II transcription velocity. *RNA* 12: 1534-1544.
20. Nag, A., Narsinh, K. & Martinson, H.G. 2007. The poly(A)-dependent transcriptional pause is mediated by CPSF acting on the body of the polymerase. *Nat Struct Mol Biol* 14: 662-669.
21. Nguyen, V.T., Giannoni, F., Dubois, M.F., Seo, S.J., Vigneron, M., Kedinger, C. & Bensaude, O. 1996. In vivo degradation of RNA polymerase II largest subunit triggered by alpha-amanitin. *Nucleic Acids Res* 24: 2924-2929.
22. Orozco, I.J., Kim, S.J. & Martinson, H.G. 2002. The poly(A) signal, without the assistance of any downstream element, directs RNA polymerase II to pause in vivo and then to release stochastically from the template. *J Biol Chem* 277: 42899-42911.

23. Papavassiliou, A.G., Treier, M., Chavrier, C. & Bohmann, D. 1992. Targeted degradation of c-Fos, but not v-Fos, by a phosphorylation-dependent signal on c-Jun. *Science* 258: 1941-1944.
24. Park, N.J., Tsao, D.C. & Martinson, H.G. 2004. The Two Steps of Poly(A)-Dependent Termination, Pausing and Release, Can Be Uncoupled by Truncation of the RNA Polymerase II CTD. *Mol Cell Biol* 24: 4092-4103.
25. Qu, X., Perez-Canadillas, J.M., Agrawal, S., De Baecke, J., Cheng, H., Varani, G. & Moore, C. 2006. The C-terminal domains of vertebrate CstF-64 and its yeast orthologue Rna15 form a new structure critical for mRNA 3'-end processing. *J Biol Chem*. 282: 2101–2115.
26. Richard, P. & Manley, J.L. 2009. Transcription termination by nuclear RNA polymerases. *Genes Dev* 23: 1247-1269.
27. Rosonina, E. & Blencowe, B.J. 2004. Analysis of the requirement for RNA polymerase II CTD heptapeptide repeats in pre-mRNA splicing and 3'-end cleavage. *RNA* 10: 581-589.
28. Sims, R.J., 3rd, Rojas, L.A., Beck, D., Bonasio, R., Schuller, R., Drury, W.J., 3rd, Eick, D. & Reinberg, D. 2011. The C-terminal domain of RNA polymerase II is modified by site-specific methylation. *Science* 332: 99-103.
29. Wagner, E.J. & Garcia-Blanco, M.A. 2002. RNAi-mediated PTB depletion leads to enhanced exon definition. *Mol Cell* 10: 943-949.
30. Whitelaw, E. & Proudfoot, N. 1986. α -thalassaemia caused by a poly(A) site mutation reveals that transcriptional termination is linked to 3' end processing in the human $\alpha 2$ globin gene. *EMBO J* 5: 2915-2922.

31. Yamaguchi, Y., Wada, T., Watanabe, D., Takagi, T., Hasegawa, J. & Handa, H. 1999. Structure and function of the human transcription elongation factor DSIF. *J Biol Chem* 274: 8085-8092.