Evidence for the requirement of protein synthesis and protein kinase activity in the temperature regulated stability of a *Tetrahymena* surface protein mRNA

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ABSTRACT

In *Tetrahymena thermophila*, the expression of the temperature-specific surface protein SerH3 is controlled primarily by a temperature-dependent change in the stability of its mRNA. The change in SerH3 mRNA stability occurs very rapidly after a shift in incubation temperature. This change in temperature could affect SerH3 mRNA stability directly by producing structural changes in the mRNA or regulatory factors acting on SerH3 mRNA. Alternatively, the temperature change could act indirectly through a signal transduction pathway leading to *de novo* synthesis of new regulatory factors or modifications of existing regulatory factors. To address these issues, we monitored the effect of temperature on an *in vitro* SerH3 mRNA decay assay and the *in vivo* effects of a variety of inhibitors against protein synthesis and protein kinases on SerH3 mRNA stability. The results of Northern analysis of SerH3 mRNAs in an *in vitro* mRNA decay assay indicate that temperature alone can not change the half-life of this mRNA. Furthermore, slot blot analysis of cytoplasmic RNAs show that protein synthesis and the action of protein kinases are not required for SerH3 mRNA turnover in cells grown at 30°C. In contrast, our results indicate that the rapid decay of the SerH3 mRNA in cells grown at 30°C and shifted to 40°C requires a one time serine/threonine phosphorylation event which occurs at the temperature shift. In addition, the data show that a regulatory protein involved in rapid SerH3 mRNA decay must be newly and continuously synthesized following the temperature shift from 30 to 40°C. These data show the complexity of temperature regulated mRNA decay and indicate that phosphorylation and protein synthesis are major factors in this process.

INTRODUCTION

In both procaryotes and eucaryotes, differential mRNA decay is an important means of regulating gene expression (1). Most

eucaryotic genes which are regulated in this manner share the common characteristic of modulating mRNA stability in response to a specific regulatory signal. To date, little is known about how a regulatory signal triggers specific changes in the half-life of a cytoplasmic mRNA. However, there is mounting evidence that phosphorylation (2-4) and translation are often involved in this process (5).

To better understand the processes involved in controlling mRNA stability in eucaryotes, we have been studying the surface protein genes of the lower eucaryote, *Tetrahymena thermophila*. The expression of these surface protein genes is regulated by temperature. The Serotype H3 (SerH3) protein is expressed at temperatures between 20 and 35°C while the Serotype T (SerT) protein is expressed at temperatures >35°C. It is thought that these proteins evolved to allow this organism to adjust to fluctuations in temperature. SerH3 gene expression during a SerH3 to SerT transition is primarily controlled by a dramatic temperature dependent change in mRNA stability. The half-life of the SerH3 mRNA at 30°C is ~1 h while at 40°C the half-life decreases to 3 min (6). Previously, we have shown that differential SerH3 mRNA stability is a regulated process involving both selective stabilization at 30°C and selective destabilization at 40°C (7). However, there is no evidence indicating how this complicated regulatory mechanism is controlled.

In this communication, temperature, protein synthesis and phosphorylation were examined to determine their role in differential stability of the *Tetrahymena* SerH3 mRNA. To assess these parameters, we monitored *in vitro* mRNA decay rates at different temperatures and *in vivo* SerH3 mRNA decay rates in the presence of two different protein synthesis inhibitors and a variety of protein kinase inhibitors. From these experiments, we found that temperature alone could not change the half-life of the SerH3 mRNA in an *in vitro* mRNA decay assay. However, inhibition of protein synthesis in cells grown at 40°C or in cells grown at 30°C and shifted to 40°C leads to accumulation of the SerH3 mRNA. In addition, inhibition of serine/threonine protein kinases, but not tyrosine protein kinases, leads to stabilization of the SerH3 mRNA during a temperature shift from 30 to 40°C. These results indicate that temperature induced structural changes play little or no part in regulating the rapid decay of SerH3 mRNA at 40°C; that the continued synthesis of a labile

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protein factor is required for rapid decay of this message and that a serine/threonine protein kinase must phosphorylate a protein to trigger the rapid degradation of SerH3 mRNA.

MATERIALS AND METHODS

Cells and culture conditions

Tetrahymena thermophila strain CU427 was grown axenically in enriched proteose peptone (8) at the temperatures indicated in the figure legends. This cell line is homozygous for the SerH3 allele.

Tetrahymena RNA isolation, Northern blot and slot blot analysis

Total cytoplasmic RNA was isolated from *Tetrahymena* cells by the methods of Calzone *et al.* (9). Total cytoplasmic RNA was subjected to electrophoresis in 1.2% agarose-formaldehyde gels, was transferred to nitrocellulose and was hybridized with ³²P-labeled probes according to the methods of Bannon *et al.* (10). Slot blots of cytoplasmic RNA were prepared according to procedures described by Calzone *et al.* (9). In all cases, a 27 nucleotide (nt) poly dT probe was used to assess that equal amounts of poly A⁺ RNA were loaded from each sample. This control hybridization experiment was accomplished by removing the previously hybridized SerH3 or ribosomal protein gene probe and re-hybridizing with the ³²P-end-labelled poly dT probe.

Probes

The SerH3 gene probe used in this study was pH2.2, a 2220 bp genomic fragment containing the entire SerH3 gene cloned into the *Hind*III site of pUC19 (11). The ribosomal protein L32 gene probe was λTt20. λTt20 is a 300 bp cDNA clone which identifies a 600 nt mRNA [6; Love and Bannon, unpublished data]. The 27 nt poly dT probe used to detect the general mRNA population was made on an ABI model 391 DNA synthesizer according to manufacturer's instructions. The pG2.2-90 plasmid contains DNA sequences corresponding to the full length SerH3 mRNA cloned into the *Hind*III and *Xba*I sites of pGEM4Z. This plasmid was used as a template for *in vitro* transcription reactions and produces the entire SerH3 transcript with an additional 7 nt and 5 nt of vector DNA at the 5' and 3' ends, respectively.

In vitro synthesis of ³²P-labelled SerH3 mRNA

The pG2.2-90 plasmid was linearized by the restriction enzyme *Bam*HI at the multiple cloning site of the vector 3' of the cloned SerH3 fragment. Transcription reactions were carried out for 1 h at 37°C in a mixture of 1 µg of the linearized DNA template, 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 20 U of RNasin, 0.5 mM each of ATP, CTP, GTP, UTP, 25 µCi each of [α-³²P]UTP and ATP, 20 U of T7 RNA polymerase in a final volume of 20 µl. The reaction was stopped by addition of RNase free DNase at a concentration of 1 U/µg of template DNA for 15 min at 37°C. The mRNA was then purified by phenol-chloroform extraction and ethanol precipitation.

Hybridizations

The poly dT probe was labelled using polynucleotide kinase and [γ-³²P]ATP (3000 Ci/mMol) by an end-labelling reaction (12).

All other probes were labelled with [α-³²P]dCTP (3000 Ci/mMol) by the random primer method (13,14) to a specific activity of 10⁸ c.p.m./µg of DNA. Probes pH2.2 and λTt20 were hybridized to filters at 65°C and the poly dT probe was hybridized at 40°C in 5 × SSPE (5 mM disodium EDTA, 40 mM NaOH, 50 mM NaH₂PO₄ and 0.9 M NaCl), bovine serum albumin (1 mg/ml), 1 × SPED (0.1% Ficoll, 0.1% PVP 360, 0.1% bovine serum albumin, 6 mM SDS, 2 mM disodium pyrophosphate, 2 mM tetrahydro-EDTA). After incubation for at least 12 h, the filters were washed in four changes of room temperature 2 × SSPE-0.1% SDS. A final wash was done at the hybridization temperature in the same solution. Filters were air dried and exposed to Kodak XAR-5 X-ray film.

mRNA half-life determinations

For half-life measurements, actinomycin D was used at a final concentration of 25 µg/ml to block transcription. At this concentration, there was a >95% inhibition of ³H-uridine incorporation into trichloroacetic acid precipitable material. *Tetrahymena* cells were grown under the conditions described in the figure legends and aliquots were removed at various times and used to isolate total cytoplasmic RNA. Three µg of RNA from each sample were then applied to a nitrocellulose membrane and hybridized with the SerH3 gene probe or the ribosomal protein gene probe followed by hybridization with the poly dT probe. Relative amounts of SerH3 mRNAs and total mRNAs in each sample were quantified by densitometric scans of slot blot autoradiograms. Relative amounts of SerH3 mRNA were then corrected for unequal loading of total mRNA.

Cytoplasmic extract preparation and *in vitro* mRNA decay assay

Cytoplasmic extract for mRNA decay assays were prepared as described by McMillan *et al.* (7). *In vitro* mRNA decay reactions consisted of 50 ng of *in vitro* transcribed, ³²P-labelled, SerH3 mRNA incubated with crude cytoplasmic extract containing 50 µg of protein and 45 U of RNasin. Reactions took place in a final volume of 50 µl in degradation buffer [20 mM Tris-HCl (pH 8), 5 mM KCl, 0.15 mM NaCl] at the temperatures noted in the figure legends. Final reaction mixtures contained 20 mM Tris (pH 8), 5 mM KCl, 0.15 mM NaCl, 1 mM KAc, 2.5 mM MgCl₂, 2.5 µM aurintricarboxylic acid, 0.1 mM DTT, and 0.025% NP-40. Reactions were stopped by addition of 100 µl of a solution containing 2 mM EDTA, 20 mM Tris-HCl (pH 8), and 0.5% SDS. RNAs from each reaction were phenol-chloroform extracted and ethanol precipitated and then analyzed on agarose-formaldehyde gels. SerH3 mRNA was visualized by exposing the gel to Kodak XAR-5 X-ray film.

Protein kinase activator and inhibitor experiments

A variety of kinase inhibitors and one kinase activator were tested for their ability to alter SerH3 mRNA stability or steady state level. A number of different concentrations and pre-incubation times were assayed for each chemical. In each case, the concentration and pre-incubation time noted below were optimal in terms of effects on SerH3 mRNA stability or steady state levels and for overall cell survival. *Tetrahymena* cells were grown overnight at 30 or 40°C to a concentration of 200 000 cells/ml. Cells were then pre-incubated with Phorbol 12-myristate 13

Figure 1. Cytoplasmic levels of SerH3 mRNA rapidly change when *Tetrahymena* cells are subjected to temperature shifts between 30 and 40°C. Cells were either grown at 30°C and then shifted to 40°C (30–40°C) or grown at 40°C and then shifted to 30°C (40–30°C). Total cytoplasmic RNA was isolated at 0, 5, 10, 30 and 60 min after the temperature shift. Five µg of RNA isolated from each time point were subjected to electrophoresis on denaturing agarose gels, blotted to nitrocellulose and hybridized with the SerH3 gene clone (A) or the *Tetrahymena* ribosomal L32 protein gene clone (B).

acetate (PMA; 200 nM), calphostin C (400 nM), or herbimycin A (875 nM) for 2 h or with staurosporine (50 nM) for 75 min. At the end of this pre-incubation period, cells grown at 40°C were shifted to 30°C while cells grown at 30°C were either maintained at 30°C or shifted to 40°C. RNA isolation and SerH3 mRNA half-life determinations from cells treated in this manner were performed as described above.

Protein synthesis inhibitor experiments

Cycloheximide and puromycin were used at final concentrations of 10 and 250 µg/ml, respectively. At these concentrations, there was >95% inhibition of ³H-amino acid mix incorporation into trichloroacetic acid precipitable material over the time course of the experiments. *Tetrahymena* cells were grown under the conditions described in the figure legends and aliquots were removed at various times and used for RNA isolation as described above.

RESULTS

Cytoplasmic levels of SerH3 mRNA rapidly change when *Tetrahymena* cells are subjected to temperature shifts between 30 and 40°C (Fig. 1A). In contrast, the steady state levels of a *Tetrahymena* ribosomal protein mRNA are unaffected by the temperature change (Fig. 1B). Previous results have shown that the alterations in steady-state levels are due to de-stabilization of the SerH3 mRNA following the shift to 40°C and to stabilization of the mRNA following the shift to 30°C (6,7). The rapid and specific change in SerH3 mRNA stability could be explained by a temperature induced change in SerH3 mRNA secondary structure which makes this mRNA more or less accessible to ribonucleases, or a structural change in one or more ribonucleases which alters enzyme activity.

The effect of temperature on SerH3 mRNA stability was assessed by an *in vitro* mRNA decay assay which has been shown to mimic the differential SerH3 mRNA decay rates observed *in vivo* (7; Fig. 2). Crude cytoplasmic extracts were prepared from *Tetrahymena* cells grown at 30°C (Fig. 2A and B), when the SerH3 mRNA is stable, or at 40°C (Fig. 2C and D), when the SerH3 mRNA is unstable. *In vitro* transcribed, ³²P-labeled SerH3 mRNA was then added and the extract from cells grown at 30°C

Figure 2. The stability of the SerH3 mRNA *in vitro* is affected by components in the extracts rather than the incubation temperature of the assay. Cytoplasmic extracts were isolated from cell lines grown at 30 (30 Ext; A and B) or 40°C (40 Ext; C and D). *In vitro* mRNA decay assays containing 50 µg of cytoplasmic extract and 50 ng of ³²P-labeled SerH3 mRNA were incubated at 30 (30 Inc; A and D) or 40°C (40 Inc; B and C) and samples were taken at 0, 5, 10, 30, 60 and 90 min. RNAs from each time point were purified by phenol-chloroform extraction and then analyzed on agarose-formaldehyde gels. SerH3 mRNAs were visualized by exposing gels to Kodak XAR-5 X-ray film (lower panel). Radioactive signals were quantitated by densitometric scanning and then plotted as a percentage of mRNA remaining compared to time 0 (upper panel).

was incubated at 40°C (Fig. 2B) and the extract prepared from cells grown at 40°C was incubated at 30°C (Fig. 2D). Samples were taken at the time points indicated in Figure 2 and the RNA from each time point was isolated by phenol-chloroform extraction and ethanol precipitation followed by formaldehyde-agarose gel electrophoresis. These experiments showed that the SerH3 mRNA is more stable in extracts isolated from cells grown at 30°C than it is in extracts isolated from cells grown at 40°C irrespective of the assay temperature (compare Fig. 2A with 2B, C and D). These results indicate that temperature alone can not alter SerH3 mRNA half-life and that differential mRNA stability is probably accomplished via the *de novo* synthesis of regulatory molecules or the post-translational modification of existing factors.

In order to determine if *de novo* synthesis of regulatory molecules was required for temperature regulated mRNA stability, the half-life of cytoplasmic SerH3 mRNAs were compared in the presence or absence of the translation inhibitors cycloheximide or puromycin at 30°C and during a temperature shift from 30 to 40°C. Wild-type cells were grown overnight at the permissive temperature (30°C). Cycloheximide and actinomycin D were then added and cultures were either maintained at 30°C or shifted to 40°C. Total cytoplasmic RNA was isolated at various times after the addition of the inhibitors and equivalent

Figure 3. Rapid decay of the SerH3 mRNA following a temperature shift from 30 to 40°C requires protein synthesis. Cells were grown for 14 h at 30°C and then cycloheximide (10 µg/ml) and actinomycin D (25 µg/ml) were added to cultures that were maintained at 30°C or shifted to 40°C. Total cytoplasmic RNA was isolated at 0, 30, 60 and 90 min (30°C) or 0, 5 and 10 min (40°C) following addition of either actinomycin D alone (squares, -) or actinomycin D and cycloheximide (circles, +). (A) Three µg of RNA from each time point were applied to nitrocellulose and hybridized with the SerH3 gene clone. Hybridization signals were quantitated by densitometric scanning and then plotted as a percentage of mRNA remaining compared to time 0. Each point represents the average of three experiments. Bars represent standard error of the mean. (B) The same experiment as in panel A except that the ribosomal protein gene clone was used as a probe and signals were not quantitated.

amounts of RNA were applied to nitrocellulose and probed with the SerH3 gene clone or the ribosomal protein gene clone (Tt20). As shown in Figure 3A, the SerH3 mRNA has a significantly longer half-life after a temperature shift from 30 to 40°C in the presence of the protein synthesis inhibitor. In contrast, the stability of this message is unaffected in cells grown at 30°C. This protein synthesis inhibitor also has no effect on the stability of the ribosomal protein mRNA at either temperature over the time course of these experiments (Fig. 3B). Similar results are obtained when puromycin (250 µg/ml) is used instead of cycloheximide (data not shown). These results indicate that protein synthesis is required for the rapid de-stabilization of the SerH3 mRNA after a temperature shift from 30 to 40°C.

Experiments were next performed to determine if continued protein synthesis was required to maintain SerH3 mRNA instability at 40°C and if inhibition of translation could prevent SerH3 mRNA accumulation following a temperature shift from 40 to 30°C. Wild-type cells were grown overnight at 40°C to a concentration of 200 000 cells/ml. Cycloheximide was added and cells were maintained at 40°C for 75 min and then shifted to 30°C for 1 h. Total cytoplasmic RNA was isolated at the time points indicated in Figure 4 and equivalent amounts of RNA were applied to nitrocellulose and hybridized with the SerH3 gene probe. In the presence of cycloheximide there is a significant increase in the steady state levels of SerH3 mRNA. This is in contrast to untreated cells grown at 40°C where the SerH3

Figure 4. The SerH3 mRNA accumulates to low levels in the cytoplasm of cells grown at 40°C when protein synthesis is inhibited. Cells were grown at 40°C for 14 h and then either treated with cycloheximide (bottom row, panel A) or left untreated (top row, panel A). Cultures were maintained at 40°C and total cytoplasmic RNA was isolated at 30, 60 and 75 min after the addition of the inhibitor. Cells were then shifted to 30°C and total cytoplasmic RNA was isolated at 10, 30 and 60 min after the temperature shift. Three µg of RNA isolated from each time point were applied to nitrocellulose and hybridized with the SerH3 gene clone. Hybridization signals were quantitated by densitometric scanning and then plotted as relative amounts of SerH3 mRNA present at each time point (B). Unshaded—untreated; Shaded—10 µg/ml cycloheximide.

mRNA never accumulates to detectable levels (Fig. 4A). Furthermore, cycloheximide fails to prevent accumulation of the SerH3 mRNA following a temperature shift from 40 to 30°C, although the steady-state levels of this message do not reach the level of the control. Taken together these results show that on-going protein synthesis is required for the rapid decay of the SerH3 mRNA observed in cells grown at 40°C. Thus, it is likely that *de novo* synthesis of a regulatory protein(s) is required for SerH3 mRNA decay at 40°C.

As discussed above, SerH3 mRNA stability may also be controlled by the post-translational modification of regulatory factors. Phosphorylation is a post-translational modification which has been implicated in many eucaryotic regulatory pathways. Cellular phosphorylation states are maintained by two general groups of regulatory molecules; protein kinases and protein phosphatases (15). Several protein kinases and phosphatases have been isolated from protozoa and their enzymatic activities are similar to their mammalian counterparts (16–20). To examine the possibility that SerH3 mRNA stability is regulated via a change in the cellular phosphorylation state, we began our investigation by testing a variety of protein kinase inhibitors and activators for their ability to affect SerH3 mRNA half-life. It should be noted that specific inhibitors, such as those used in this study, can affect cellular functions other than the ones specified, and the results must be interpreted with this in mind.

Tetrahymena cells were grown at 30°C to a concentration of 200 000 cells/ml. Cells were then treated with staurosporine, PMA, calphostin C or herbimycin A as indicated in Materials and

Figure 5. The general protein kinase inhibitor staurosporine has a dramatic effect on SerH3 mRNA stability in cells shifted from 30 to 40°C. *Tetrahymena* cells were grown for 14 h at 30°C. At time 0, actinomycin D was added (25 µg/ml) and cells were either maintained at 30°C or shifted to 40°C. Total cytoplasmic RNA was isolated at 0, 5, 10, 30 and 60 min and 2 µg of RNA were applied to nitrocellulose and hybridized with the SerH3 gene clone. The blot was stripped and rehybridized with ³²P-labelled oligo-dT to assess the amount of poly A⁺ mRNA loaded for each sample (data not shown). In each case the quantities of poly A⁺ RNA in each well were equivalent. Cells were either untreated (A) or pre-treated with 50 nM staurosporine (B), 200 nM PMA (C), 400 nM calphostin C (D), 875 nM herbimycin A (E).

Methods. At time zero, actinomycin D was added and cells were either shifted to 40°C or maintained at 30°C. Total cytoplasmic RNA was isolated at the time points indicated in Figure 5 and 3 µg of total cytoplasmic RNA isolated at each time point were then applied to a nitrocellulose membrane and hybridized with the cloned SerH3 gene or the ribosomal protein gene. Staurosporine is a potent general inhibitor of protein serine/threonine and tyrosine kinases (21) while herbimycin A specifically inhibits protein tyrosine kinases (22). Calphostin C is a specific inhibitor of protein kinase C (21) while the phorbol ester, PMA is an activator of protein kinase C (23). As can be seen in Figure 5, the protein tyrosine kinase inhibitor, herbimycin A, as well as the protein kinase C activator and inhibitor have little effect on SerH3 mRNA half-life in *Tetrahymena* cells grown at 30°C or shifted to 40°C. However, the general protein kinase inhibitor, staurosporine, has a dramatic effect on SerH3 mRNA half-life in cells shifted to 40°C. Under these conditions the half-life of the SerH3 mRNA increases from ~3 min in control cells (6) to ~18 min in staurosporine treated cells (Fig. 6A). In contrast, the stability of the SerH3 mRNA in cells grown at 30°C and of a *Tetrahymena* ribosomal protein mRNA (Fig. 6B) are unaffected by staurosporine treatment.

Since the initial rapid decay of the SerH3 mRNA in cells shifted from 30 to 40°C required kinase activity, we wondered if there was a continuous requirement for this activity and whether kinases were required for SerH3 mRNA accumulation. In order to address these questions, cells were grown at 40°C for at least 12 h, staurosporine (50 nM) was then added, and samples were taken for RNA isolation at various times after addition of the kinase inhibitor. The cells were then shifted to 30°C and samples were taken for up to 60 min after the temperature shift. Total cytoplasmic RNA was isolated from each sample and used for slot blot analysis. Figure 7 shows that inhibition of kinase activity with staurosporine had little effect on SerH3 mRNA steady state levels at 40°C or following a temperature shift from 40 to 30°C. Similar results are obtained when experiments are performed in

Figure 6. Staurosporine increases the half-life of the SerH3 mRNA in cells shifted from 30 to 40°C. Cells were grown either in the presence (circles, +) or absence (squares, -) of staurosporine as described in Figure 5 followed by a temperature shift from 30 to 40°C. (A) Samples were taken for RNA isolation and slot blots were hybridized with the SerH3 gene clone as previously described. Hybridization signals were quantitated by densitometric scanning and then plotted as a percentage of mRNA remaining compared to time 0. Each point represents the average of three experiments. Bars represent standard error of the mean. (B) The same experiment as in panel A except that the ribosomal protein gene clone was used as a probe and signals were not quantitated.

the presence of calphostin C, herbimycin A or PMA (data not shown). These results are consistent with the idea that the initial rapid decay of the SerH3 mRNA in cells shifted from 30 to 40°C requires phosphorylation. The tyrosine kinase inhibitor, herbimycin A, has no effect on SerH3 destabilization, therefore, the regulatory signal is most likely mediated through the activity of a serine/threonine protein kinase. In addition, it appears that kinase activity is not required for the continued rapid decay of the SerH3 mRNA observed in *Tetrahymena* cells incubated at 40°C, or for the accumulation of SerH3 mRNA in cells shifted from 40 to 30°C.

DISCUSSION

The *Tetrahymena* SerH3 surface protein gene is a constitutively transcribed gene whose expression is primarily controlled by differential mRNA stability (6,24). The SerH3 gene appears to be unique among other *Tetrahymena* genes studied thus far which are controlled primarily at the transcriptional level (24). While this mode of regulation may be unique in *Tetrahymena*, in both prokaryotes and eukaryotes there are now many examples of genes whose expression is controlled by differential mRNA stability (25). Many of these genes require protein synthesis for differential mRNA stability but only a few reports have implicated the action of kinases or phosphatases in this process (2-4).

The results presented in Figures 3 and 4 indicate that protein synthesis is required for the rapid destabilization of the SerH3 mRNA at 40°C. The relationship between translation and mRNA

Figure 7. Inhibition of serine/threonine kinase activity by staurosporine has little effect on SerH3 mRNA steady state levels in cells grown at 40°C or following a temperature shift from 40 to 30°C. Cells were grown at 40°C for 14 h. Staurosporine was then added, cells were maintained at 40°C for 75 min and a cell sample was removed for total cytoplasmic RNA isolation (time 0). Cells were then shifted to 30°C and total cytoplasmic RNA was isolated at 0, 5, 10, 30 and 60 min following the temperature shift. Two µg of RNA from each time point were applied to nitrocellulose and hybridized with the SerH3 gene clone. The blot was stripped and rehybridized with ³²P-oligo dT as described in the legend for Figure 4. (A) untreated; (B) staurosporine treated cells.

decay is still open to interpretation, but it is clear that the rapid degradation of many unstable mRNAs requires ongoing protein synthesis (5). In some instances translation initiation is important to the mRNA decay process. For example, the GM-CSF mRNA is stabilized when it is fused to a mutated form of the adenovirus tripartite leader that inhibits translation (26). In other instances there appears to be a requirement for a highly labile degradation factor that rapidly loses activity when translation is inhibited (27). In yeast, cycloheximide slows decay of the *MFA2* mRNA by inhibiting the decapping reaction in the degradation pathway. Whether this reaction is inhibited in cycloheximide treated cells because the decapping enzyme is extremely labile or the inhibitor indirectly leads to transcripts accumulating as an mRNP form that is particularly resistant to decapping remains to be determined (28). In the case of SerH3, two pieces of evidence suggest the synthesis of a labile protein rather than the act of translation is important for SerH3 mRNA turnover. First, both puromycin and cycloheximide prevent rapid destabilization of the SerH3 mRNA following a temperature shift from 30 to 40°C. Puromycin inhibits translation by preventing mRNA loading onto polysomes. Cycloheximide, on the other hand, inhibits peptidyl transferase and thus peptide bond formation. The mRNA however, remains polysome bound. Therefore, if the SerH3 mRNA is degraded by nucleases that are ribosome associated, it most likely would not be stabilized following a temperature shift from 30 to 40°C in the presence of cycloheximide. In addition, McMillan *et al.* (7) have shown that *in vitro* transcribed SerH3 mRNA is rapidly degraded in the presence of extract isolated from *Tetrahymena* cells grown at 40°C. Since it is unlikely that this mRNA is loaded onto ribosomes under these conditions (29), the message would not be in close association with ribosome-bound degradative machinery. Furthermore, addition of cycloheximide to this *in vitro* degradation system has no effect on the turnover of the SerH3 mRNA (McMillan and Bannon, unpublished data). Taken together, these data suggest that a regulatory protein involved in SerH3 mRNA decay must be newly synthesized following a temperature shift from 30 to 40°C. In addition, this labile regulatory factor must be continually synthesized at 40°C to maintain SerH3 mRNA instability.

Our data indicate that a serine/threonine protein kinase activity is required for the rapid decay of the SerH3 mRNA normally observed when *Tetrahymena* cells are shifted from 30 to 40°C. The involvement of phosphoproteins in controlling mRNA

stability is not without precedent. Staurosporine and calphostin C destabilize the interleukin-1 mRNA indicating that protein kinase C is involved in regulating the half-life of this message (2). In addition, the adenosine-uridine binding factor protein (AUBF) which binds to the destabilizing AUUUA motif found in the glucose transporter mRNA is a phosphoprotein whose activity is tightly coupled to its phosphorylation state (4). In the case of SerH3, staurosporine inhibits the rapid decay of this mRNA in cells shifted from 30 to 40°C but has little or no effect on steady state levels or half-life of the SerH3 mRNA in cells grown at 30°C, 40°C or shifted from 40 to 30°C (compare Figs 6 and 7). Furthermore, addition of staurosporine to this *in vitro* degradation system has no effect on the turnover of the SerH3 mRNA (McMillan and Bannon, unpublished data). These results indicate that the phosphorylation required for rapid decay of the SerH3 mRNA is a one time event which occurs at the temperature shift. The fact that kinase activity is not a continuous requirement for rapid decay at 40°C implies that either: (i) the phosphoprotein is an extremely stable molecule that acts continuously at temperatures greater than 35°C or, (ii) it acts once at a committed step of the destabilization pathway that is initiated by a 30 to 40°C temperature shift. The latter explanation seems more likely since even an extremely stable phosphoprotein would be diluted from the cytoplasm by continuous cell division.

In summary, our results show that the temperature regulated stability of the SerH3 mRNA is a complex event requiring on-going protein synthesis and protein kinase activities. A possible model that would explain our data would include the following aspects. A rise in temperature triggers a serine/threonine protein kinase to phosphorylate a protein involved very early in the SerH3 mRNA degradation pathway. This pathway ultimately culminates in the translation of a labile factor involved in SerH3 mRNA decay. While the complete SerH3 mRNA degradation pathway is yet to be elucidated, these results, coupled with an *in vitro* mRNA decay system (7), make this an attractive model for the study of general mechanisms involved in regulating mRNA stability.

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