STRUCTURAL DETERMINANTS OF mRNA TURNOVER IN YEAST

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ABSTRACT

STRUCTURAL DETERMINANTS OF mRNA STABILITY IN YEAST

Large differences exist in the decay rates of individual mRNAs yet the molecular basis for such differences is substantially unknown. We have developed a procedure for the measurement of individual mRNAs in the yeast Saccharomyces cerevisiae which utilizes northern or dot blotting to quantitate the levels of individual mRNAs after thermal inactivation of RNA polymerase II in an rpb1-1 temperature-sensitive mutant strain (RY260). To assess the reliability of half-life measurements obtained in this manner, we have compared the results of this procedure to results obtained by three other procedures (pulse-chase analysis, approach to steady-state labeling, and inhibition of transcription with thiolutin) and also evaluated whether heat-shock alters mRNA decay rates. We find that: i) for most mRNAs, all four procedures yield comparable relative decay rates and ii) there are no significant differences in the mRNA decay rates measured in heat-shocked or non-heat-shocked cells. Of the 20 mRNAs studied, 11, including those encoded by HIS3, STE2, STE3, and MATα1, were unstable (t1/2 < 7 min) and 4, including those encoded by ACT1 and PGK1, were stable (t1/2 > 25 min). We have begun to assess the basis and significance of such differences in the decay rates of these two classes of mRNA. The following parameters have been analyzed to determine their role in mRNA decay: i) mRNA size; ii) poly(A) tail metabolism; iii) translational status; iv) relative content of rare codons; and v) structures and sequences within the 3'-untranslated region (UTR).
To identify the structural determinants responsible for the rapid decay of the unstable HIS3 and STE2 mRNAs, recombinants of their respective genes were constructed and transformed into strain RY260 on centromere-containing vectors, and the half-lives of the resulting chimeric mRNAs were measured in vivo. Chimeric genes were constructed in which the 3'-UTR of ACT1 was replaced with the corresponding region of the unstable HIS3 or STE2 mRNAs. The decay rate of the ACT1-5'-HIS3-3' mRNA was very similar to that of the stable endogenous ACT1 mRNA, implying that the 3'-end of HIS3 is not sufficient to transfer the instability phenotype of the HIS3 mRNA. The HIS3-5'-ACT1-3' mRNA from the reciprocal construct was unstable, suggesting that HIS3 instability determinants are located within its 5'-UTR or coding sequence. A 411 nucleotide (nt) deletion within the HIS3 coding region (with either the HIS3 or ACT1 3'-UTR) was stabilized 3-fold suggesting this region is necessary for the rapid decay of HIS3 mRNA. Insertion of these 411 nts in-frame into the entire ACT1 gene had no significant effect on the stability of the hybrid mRNA implying that these HIS3 sequences are not sufficient to function on their own and that they may have to interact with HIS3 5'-sequences. The ACT1-5'-STE2-3' hybrid mRNA decayed with an intermediate half-life of 12 min. Furthermore, an 82% deletion of the STE2 coding region increased the half-life by nearly 2-fold. Both results suggest that instability determinants of STE2 mRNA are not restricted to the 3'-UTR. Our overall conclusion is that mRNA stability is not dictated by simple, transferable elements (sequences or structures), but may involve interactions between multiple determinants in the mRNA.
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CHAPTER I

INTRODUCTION
Chapter I of this thesis is a review of the literature on mRNA turnover in eukaryotic cells, and is divided into two main sections. The first section focuses on higher eukaryotes and includes a summary of the non-specific and specific structural determinants involved in the decay of individual mRNAs and a description of the environmental and developmental factors which regulate specific mRNA decay rates. The second section is a summary of what is known about mRNA decay in the budding yeast, Saccharomyces cerevisiae. I have included only a minimum of information on mRNA decay in prokaryotes since this field has been reviewed recently (Belasco and Higgins, 1988).

A. METHODOLOGY FOR THE ANALYSIS OF mRNA DECAY RATES

Methodologies for measuring mRNA half-lives include those which monitor changes in the labeling kinetics of mRNA (approach to steady-state labeling and pulse-chase procedures) and those in which the biosynthesis of mRNA is inhibited by various drugs. The approach to steady-state labeling procedure is based on the following theory: incorporation of label (usually $[^{3}H]$-uridine) into either the mRNA population or individual mRNAs will continue linearly until the rate of synthesis equals the rate of degradation. The time required to reach this equilibrium is the half-life for a given mRNA (Greenberg, 1972). Frequently, a low concentration of actinomycin D (act D) is used to inhibit incorporation into rRNA while allowing for continued uptake into mRNA.

Pulse-chase protocols with $[^{3}H]$-uridine have also been used to measure eukaryotic mRNA half-lives. Higher concentrations of act D, which inhibit RNA polymerase II transcription, have been used following a pulse of $[^{3}H]$-
uridine to effect a chase more efficiently. The addition of glucosamine and an excess of cold uridine has been shown to effect a very efficient chase in *Drosophila* cells (Levis and Penman, 1977). Glucosamine acts by trapping uridine in the form of UDP-N-acetylhexosamines.

The use of drugs which inhibit transcription permits analysis of the decay of pre-existing mRNAs. Drugs used in such studies include act D (Singer and Penman, 1972; Sheiness and Darnell, 1973; Chung et al., 1981; Dani et al., 1984; Baumbach et al., 1984; Eick et al., 1985; Bird et al., 1986; Fort et al., 1987; Rahmsdorf et al., 1987; Jinno et al., 1988; Jack and Wabl, 1988; Wilson and Triesman, 1988; Swartwout and Kinniburgh, 1989), and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Zandomeni et al., 1982; Efrat and Kaempfer, 1984). Act D intercalates DNA and the latter agent interacts specifically with RNA polymerase II.

**B. COMPLEXITY**

mRNA half-life measurements in many different eukaryotic cells have shown that the mRNA population decays with complex kinetics. Poly(A)+ mRNA in HeLa cells was shown to have complex decay kinetics separable into at least 2 populations whose half-lives were 7 and 24 hours (Singer and Penman, 1973). Similar complex decay kinetics were also seen for the mRNA from rabbit blastocysts (Schults, 1974), human lymphocytes (Berger and Cooper, 1975), mouse kidney (Ouellette and Malt, 1976), and a *Drosophila* cell line (Lengyel and Penman, 1977). A 32P-pulse-chase protocol was used to measure individual mRNA half-lives in the slime mold, *Dictyostelium discoideum* (Shapiro et al., 1988). Individual half-life values ranged from 0.9 to 9.6 hours. These values agreed well with the two major components of 50
minutes and 10 hours for the poly(A)$^+$ population detected in a $[^3H]$-uridine pulse-chase (Casey et al., 1983).

C. NON-SPECIFIC DETERMINANTS OF mRNA DECAY

Individual mRNAs within the same cell can have large differences in their respective decay rates, but the molecular basis for such differences are poorly understood. This section summarizes previous studies on the relationships between mRNA decay rates and the 5' -cap, 3' -poly(A) tail, mRNA size, efficiency of ribosome loading, and mRNA translational status.

1. 5'-Cap Structures

Most eucaryotic mRNAs have a cap (m$^7$G(5')ppp(5')N) at their 5'-termini. Cap structures of mRNAs may differ in several ways: they may contain different bases at their penultimate and adjacent nucleoside, and these bases may or may not be methylated (Banerjee, 1980). The presence of a cap is necessary for efficient translation (Banerjee, 1980; Drummond et al., 1985), and splicing (Konarska et al., 1984). Cap structures are also necessary for maintaining the stability of microinjected RNAs in Xenopus oocytes (Furuichi et al., 1977; Green et al., 1983; Drummond et al., 1985), possibly by protecting against 5' to 3' exonucleolytic activity (Furuichi et al., 1977). No difference was observed in the stability of chicken lysozyme mRNA with either a mono- or a dimethyl cap (Drummond et al., 1985) so it is unlikely that these variations in structure exert different degrees of protection to individual mRNAs.
2. 3'-Poly(A) Tracts

The identification of a function for the 3'-poly(A) tail of mRNA has proven to be difficult. The observation that viruses which exist exclusively in the cytoplasm of infected cells have polyadenylated mRNAs implies a cytoplasmic function and suggests three possibilities: 1) the poly(A) tail is involved with some aspect of protein synthesis, 2) poly(A) is involved in mRNA decay, or 3) the poly(A) tail may be responsible for the determination of mRNA subcellular localization. Experimental support for all three possibilities has been obtained. Only the possible role of the poly(A) tail in mRNA decay will be discussed here.

Two different mechanisms for poly(A) loss have been observed, the first involves a gradual reduction in poly(A) tail length, the second a more abrupt and rapid loss to a poly(A)- or poly(A)-deficient state. In 1973, Sheiness and Darnell demonstrated that the poly(A) tract of HeLa cell cytoplasmic mRNA is progressively shortened following an actinomycin chase. The poly(A) tracts of vegetative mRNAs in *Dictyostelium discoideum* also shorten with time from an initially synthesized length of 110-115 nt down to the steady-state length of 60-65 nt (Palatnik et al., 1979). The poly(A) tail of mouse liver or kidney metallothionein-1 mRNA rapidly shortens from an initial length of 150-200 residues to a steady-state length of 60 nt during the first 5 hr of induction with copper chloride or dexamethasone (Mercer and Wake, 1985). The rate of A loss then slowed from 20 nt per hr to 3 nt per hr. Metallothionein mRNAs with A-tail lengths of less than 30-40 residues were not seen. Mercer and Wake (1985) hypothesize that a processive 3'-&gt;5' exonuclease is involved and that a poly(A) tail length of &gt;30 nts is necessary to maintain stability. Poly(A) shortening appears to require ongoing translation since both
shortening and mRNA decay are inhibited following exposure to emetine in HeLa cells (Sheiness et al., 1975) or following cycloheximide treatment of rabbit reticulocyte or wheat germ translation extracts (Rubin and Halim, 1987). It has been hypothesized that sequences in the 3'-UTR of an mRNA can influence the rate of loss of poly(A) (Littauer and Soreq, 1982; Brewer and Ross, 1988). Specifically, deletion of the 3' AU-rich sequences in the 3'-UTR of c-fos mRNA dramatically slows both its poly(A) shortening and decay rate (Wilson and Triesman, 1988).

In several developmental systems including Xenopus (Colot and Rosbash, 1982), Dictyostelium (Palatnik et al., 1984), and Spisula (Rosenthal et al., 1983), rapid changes in the poly(A) tail lengths of preexisting mRNAs were observed. Following fertilization of Xenopus or Spisula oocytes, specific mRNAs become polyadenylated in the cytoplasm and are recruited onto polysomes. Other mRNAs in the fertilized oocytes, and in developing Dictyostelium cells, undergo a rapid deadenylation which is paralleled by a reduction in their translatability. Although abrupt changes of poly(A) tail length in these developing systems coincide with translational alterations, their effect on mRNA decay is unknown.

The question of whether the loss of the poly(A) tail influences the rate of mRNA decay remains an unresolved one. For some mRNAs such as c-myc (Brewer and Ross, 1988; Swartwout and Kinniburgh, 1989) and c-fos (Wilson and Triesman, 1988), the loss of the poly(A) tail appears to be the initial event of their decay. This rapid loss of the poly(A) tail from c-fos mRNA occurs in a translation-dependent manner (Wilson and Treisman, 1988). Other mRNAs such as those for myosin heavy chain (Medford et al., 1980), and actin (Krowczynska et al., 1985) are stable and located on polysomes
following the loss of their poly(A) tract. My data with the stable PGK1 and CYH2 yeast mRNAs also indicates that while these mRNAs rapidly lose (or become deficient in) their poly(A) tail, their degradation is not closely linked to this loss (Chapter II, this thesis). Furthermore, fractionation of whole cell RNA using thermal elution from poly(U)-Sepharose shows no correlation between the relative length of poly(A) and the decay rate of specific mRNAs in both Dictyostelium (Palatnik et al., 1980; Shapiro et al., 1988), and yeast (Santiago et al., 1987).

Microinjection experiments using either HeLa cells or Xenopus oocytes have also resulted in conflicting data. Enzymatically deadenylated globin mRNAs are both functionally (Huez et al., 1981), and chemically (Marbaix et al., 1975) less stable than their native counterparts. No difference in stability was seen between A+ and A- versions of human interferon mRNA when injected into Xenopus oocytes (Sehgal et al., 1978). Reovirus mRNAs (normally poly(A)+) are stable (McCrae and Woodland, 1981), while enzymatically polyadenylated histone mRNAs show an increased functional stability in oocytes (Huez et al., 1978). Analysis of rabbit globin mRNAs with a series of A-tail lengths from 0-122 adenylate residues indicate that a length of >32 residues results in complete stability (Nudel et al., 1976). Using an in vitro extract derived from K562 erythroleukemia cells, Bernstein et al., (1989) showed that a complex between poly(A) and the poly(A)-binding protein is necessary for the stability of A+ mRNAs.

Destabilization of cellular mRNAs following HSV-1 infection will be described in a later section. The mechanism responsible for the specific decay of cellular mRNAs may initially involve the specific loss of their poly(A) tails. Following HSV-1 infection of Friend erythroleukemia cells, the
levels of poly(A)− histone H3 mRNA remain unchanged for the first 4 hours, while the levels of poly(A)+ α-globin, and β-actin mRNAs decrease progressively (Mayman and Nishioka, 1985). Infection of polyoma-transformed BHK cells with HSV-1 results in cellular mRNA sequences becoming 4-fold more abundant in the nonadenylated RNA fractions (Nakai et al., 1982).

In summary, for some mRNAs, the loss of poly(A) may be the initial event in their degradation. However, the majority of the available evidence suggests that no correlation exists between poly(A) tail length and mRNA stability.

3. mRNA Size versus Half-Life

Early studies suggested that a correlation exists between the length and the decay rate of mRNAs (Singer and Penman, 1973; Spradling et al., 1975; Berger and Cooper, 1975; Meyuhas and Perry, 1979). If mRNA decay was due to non-specific mechanisms, then longer mRNAs should be less stable than shorter mRNAs simply because of differences in target size. Several studies have addressed this question using individual mRNAs. The half-lives and sizes of 15 different mRNAs were measured in Dictyostelium and no correlation was observed between the decay rates and lengths of these mRNAs (as measured on northern blots). Graves et al., (1987) made a series of in-frame deletions within the coding region of the histone H3 mRNA. Each of the shorter histone mRNAs was degraded at the same rate as the endogenous histone mRNA. In a recent study, hybrid mRNAs, comprised of yeast STE3, ACT1, and PGK1 sequences, and differing up to almost fourfold in length were shown to have identical decay rates (R. Parker and A. Jacobson, manuscript in
preparation). Conflicting results were seen when the length of 15 yeast mRNAs was compared to their decay rates (Santiago et al., 1986). The mRNAs in this study fell into two populations with an inverse relationship between mRNA size and decay rate. Half-lives of these mRNAs were measured following the inhibition of transcription by high concentrations of the drug, 1,10-phenanthroline (Santiago et al., 1986). This drug affects a number of cellular processes (Chang et al., 1978; Johnston and Singer, 1978; Krishnamurti et al., 1980), and may exert its effects by chelating cellular zinc. If the nuclease(s) responsible for mRNA degradation are zinc metalloenzymes, they may also be inhibited.

4. Efficiency of Ribosome Loading and mRNA Turnover

Passive protection of mRNAs by ribosomes has long been thought to be a factor in the determination of mRNA half-life (Losson and Lacroute, 1979; Chang and Kan, 1979; Pelsy and Lacroute, 1984; Baumann et al., 1985). In this model, those mRNAs more efficiently loaded onto polysomes would be "protected" from non-specific nucleases. The efficiency of ribosome loading can be measured by fractionating cytoplasmic extracts on sucrose gradients followed by northern blot analysis of the RNA isolated from each gradient fraction. This measures the polysomal distribution and extent of ribosome loading for each mRNA. When probed with specific DNA probes for mRNAs of known decay rates, there was no correlation between the number of ribosomes per unit length of stable versus unstable mRNAs in both yeast (Santiago et al., 1987), and Dictyostelium (Shapiro et al., 1988).
5. Translational Status and mRNA Decay

Although the extent of ribosome loading does not appear to influence mRNA decay rates, the following observations have suggested an intimate association between the translational status of an mRNA and its decay rate:

i. Effect of Protein Synthesis Inhibition

Inhibitors of protein synthesis with different mechanisms of action result in a "superinduction" or stabilization of nearly every mRNA studied. Drugs used in these studies include the elongation inhibitors, cycloheximide and emetine, the initiation inhibitor, pactamycin, and the polysome dissociator, puromycin. Although the elongation inhibitors result in maximal loading of mRNAs on ribosomes, while pactamycin and puromycin have the opposite effect (ribosome runoff with release of the mRNA as a free mRNP), the specific destabilization of histone mRNAs following the inhibition of DNA synthesis is prevented by all of these agents (Stimac et al., 1984; Sive et al., 1984; Baumbach et al., 1984; Bird et al., 1986).

Cycloheximide treatment also stabilizes members of a class of mRNAs whose rapid decay rates appear to be determined, in part, by AU-rich sequences in their 3'-untranslated regions (UTRs). These include the c-myc (Kelly et al., 1983; Dani et al., 1984), c-fos (Fort et al., 1987; Rahmsdorf et al., 1987), interleukin 2 (Efrat and Kaempfer, 1984; Efrat et al., 1984), and β-interferon (Raj and Pitha, 1981) mRNAs. Cycloheximide treatment also reduces the turnover rate of several unrelated mRNAs in the yeast, Saccharomyces cerevisiae (Chapter II, this thesis).
ii. Effect of Nonsense Mutations

In 1979, Losson and Lacroute showed that the introduction of nonsense mutations within the yeast URA3 mRNA reduced its half-life. This effect was position-dependent, in that URA3 mRNAs with 5'-proximal nonsense codons were the most unstable (Losson and Lacroute, 1979). The yeast URA1 mRNA exhibits a comparable polar effect (Pelsy and Lacroute, 1984). Similar observations have been made in mammalian cells. Nonsense codons within the human β-globin (Maquat et al., 1981) and triosephosphate isomerase (Daar and Maquat, 1988) mRNAs reduce their stability 2- to 5-fold and are responsible for β° thalassemia and anemia, respectively. The position effect is also observed in higher eukaryotes, since mouse IgM mRNAs with nonsense mutations in their 5'-region are 10- to 30-fold less stable than mRNAs with nonsense mutations in their 3'-region (Baumann et al., 1985).

Taken together, the experiments with inhibitors and nonsense mutants imply that translation and mRNA turnover are tightly linked and suggest that the relative position of the ribosome on a given mRNA can influence the susceptibility of that mRNA to nuclease attack.

iii. Involvement of Rare (Minor) Codons

When the yeast 3-phosphoglycerate kinase (PGK1) gene is expressed from a high-copy number plasmid, 30-40% of cellular protein is PGK (Chen et al., 1984). However, expression of mammalian genes using the PGK1 promoter and terminator produce 20-fold lower steady-state levels of both mRNA and protein. One possible explanation of these observations is that coding sequences influence levels of gene expression.

Bennetzen and Hall (1982) observed that highly expressed genes in yeast
are biased toward the use of only 25 of the 61 coding triplets. Such restricted codon usage correlates with the levels of the corresponding isoaccepting tRNAs in both yeast (Ikemura, 1982) and *E. coli* (Ikemura, 1981), although the extent of codon bias is much higher in yeast.

To test whether a relationship exists between the presence of rare codons in a mRNA and the rate at which it decays, Hoekema et al., (1987) replaced up to 164 frequent codons of the yeast PGK1 gene (39% of the total PGK1 codons) with rare codons. These changes resulted in a 10-fold reduction in PGK protein synthesis and a 3-fold reduction in steady-state levels of PGK1 mRNA. These reduced levels of PGK1 mRNA may reflect a similar increase in the PGK1 mRNA decay rate, assuming that all constructs had identical transcription rates. Addition of 22 minor codons in the 5'-end of the PGK1 gene had no significant effect, an indication that a smaller number of minor codons can be tolerated. In *E. coli*, rare codons in mRNAs slow ribosome elongation rates (Pedersen, 1984). In one example, insertion of four consecutive rare codons in the highly expressed chloramphenicol acetyl transferase (CAT) gene reduced CAT protein levels to 1/3 of control levels (Robinson et al., 1984). These results suggest that an mRNA with a paused ribosome may have an increased probability of being degraded.

**D. SPECIFIC STRUCTURAL DETERMINANTS OF mRNA STABILITY**

The discussion above suggests that, for most RNAs, non-specific determinants do not make a significant contribution to individual mRNA decay rates, i.e., mRNA decay is not a random process. Consistent with this conclusion, recent evidence, summarized below, demonstrates that specific sequences or regions within individual mRNAs are involved with their
degradation.

1. 3'-Untranslated Regions

The 3'-untranslated regions (UTRs) of many different mRNAs have been identified as a primary determinant of their rapid decay rates (Ross and Pizarro, 1983; Simcox et al., 1985; Meijlink et al., 1985; Lycan et al., 1987; Caput et al., 1986; Rahmsdorf et al., 1987; Reeves et al., 1987; Shaw and Kamen, 1986; Luscher et al., 1985; Graves et al., 1987; Ross and Kobs, 1986; Ross et al., 1986; Jones and Cole, 1987; Shapiro et al., 1988). These mRNAs include those for transiently expressed proto-oncogene, lymphokine, and cytokine mRNAs such as c-myc, c-fos, GM-CSF, c-myb, and β-interferon. These mRNAs have a very rapid decay rate \( t_{1/2} < 30 \text{ minutes} \) when compared to more stable mRNAs such as β-globin or GADPH which have half-lives ranging from 2 hr to 29 hr (Ross and Pizarro, 1983; Dani et al., 1984; Ross and Sullivan, 1985). The 3'-UTRs of these RNAs and those for murine and human tumor necrosis factor (TNF), human lymphotoxin, human colony stimulating factor, human and mouse interleukin 1, human and rat fibronectin, and most of the human and mouse interferons all contain AU-rich sequences (Caput et al., 1986). The importance of these sequences was originally suggested by experiments which showed that deletion of a 67 nt AU-stretch within the 808 nt 3'-UTR of c-fos mRNA resulted in a dramatically increased transformation potential (Meijlink et al., 1985).

The regions identified as having a major influence on the decay rates of the human GM-CSF, c-fos, and c-myc mRNAs are 51 nt, 75 nt, and 140 nt AU-rich segments, respectively. This was demonstrated by using two experimental approaches: the first involves the deletion of these sequences which results in the stabilization of these mRNAs, and the second involves
the destabilization of an mRNA (such as β-globin) following either the insertion of an AU-containing segment into, or replacement of the 3'-UTR of the stable mRNA (Shaw and Kamen, 1986; Fort et al., 1987; Rahmsdorf et al., 1987; Jones and Cole, 1987; Kabnick and Housman, 1988; Shyu et al., 1989). Such experiments suggest that the regions which confer instability are transferable and dominant.

Other well-documented determinants in the 3'-UTR include those found in histone and human transferrin receptor (hTR) mRNAs. Stem-loop structures at the 3'-end of mouse histone mRNAs (Pandey and Marzluff, 1987), and in the 3-UTR of the hTR mRNA (Casey et al., 1988; Mullner and Kuhn, 1988) are necessary and sufficient for mediating the respective cell-cycle and iron-dependent regulation of their stabilities.

2. 5'-Untranslated Regions

Other mRNA regions also appear to have a role in mRNA decay. Chromosomal translocations of c-myc in both murine plasmacytomas or human Burkitt’s lymphoma result in truncated c-myc mRNAs lacking the non-coding exon 1. These truncated c-myc mRNAs have a 3- to 10-fold longer half-life than the full length c-myc RNA (Eick et al., 1985; Rabbitts et al., 1985; Piechaczyk et al., 1985). This observation also holds true for a truncated c-myc mRNA in an in vitro decay system from mouse plasmacytoma P3X cells (Pei and Calame, 1988). However, fusions between c-myc exon 1 and IgCα heavy chain or glyceraldehyde-3-phosphate dehydrogenase mRNAs demonstrate that c-myc exon 1 sequences alone are not sufficient to confer a high degree of instability to these hybrid mRNAs in the above in vitro system. Similar results were obtained in vivo using stably transfected murine fibroblasts;
deletion of the conserved non-coding exon 1 exerts only a small effect on the decay of \textit{c-myc} RNA, but may be required for intramolecular interactions necessary for maximum instability (Jones and Cole, 1987). The inconsistencies between this data and the stability of the truncated \textit{c-myc} mRNA following a chromosomal translocation has been attributed to the differences among the various tumor cell lines used (Jones and Cole, 1987).

3. Involvement of Coding Region Determinants

The cell-cycle regulated decay of histone mRNAs requires that translation proceed to within 300 nts of the 3'-end of these mRNAs (Graves et al., 1987). Thus, introduction of nonsense codons into the 5'-region of cell-cycle regulated histone mRNAs results in mRNA stabilization. A stem-loop structure in the 3'-UTR has been identified as the initial target for histone mRNA degradation (Pandey and Marzluff, 1987). These data suggest that a ribosome-bound nuclease must be delivered to its target or that the translocating ribosome may function to unfold specific mRNA regions, thereby exposing normally concealed cleavage signals.

Insertion of premature nonsense codons into the first 42 amino-terminal codons of \(\beta\)-tubulin mRNA prevents its autoregulated destabilization induced by high levels of unpolymerized tubulin subunits (Cleveland, 1988). Proper regulation occurs only if the four NH\(_2\)-terminal amino acids can emerge from the ribosome, presumably to be recognized via a protein-protein interaction involving free tubulin subunits. A similar mechanism for histone mRNA decay may require the production of a nascent histone peptide of sufficient length which serves as an autoregulatory recognition signal. An autoregulatory mechanism involving the interaction of histone mRNA and free core proteins
has been suggested by experiments which demonstrate that the decay rate for histone mRNAs is accelerated four- to six-fold following incubation in vitro with free histone proteins and a S130 fraction (Peltz and Ross, 1987).

Two groups have recently used a transient expression assay in which the induction of the \textit{c-fos} promoter (by addition of calf serum) is exploited for a transient burst of \textit{c-fos} synthesis. This technique was used to monitor the decay of various mRNAs encoded by chimeric genes encoding portions of human \textit{c-fos} and either human (Kabnick and Housman, 1988) or rabbit (Shyu et al., 1989) \textit{β}-globin. Kabnick and Housman demonstrated that both the \textit{c-fos} 5' UTR and coding region were able to destabilize the human \textit{β}-globin mRNA, although the major contribution is made by sequences in the 3' UTR. They concluded that the turnover rates for these mRNAs are due to either multiple determinants or to interactions among multiple sequences within the same mRNA.

Interestingly, the results from Shyu et al., (1989) have raised the possibility that degradation of human \textit{c-fos} mRNA occurs via two distinct cellular degradation pathways. This hypothesis is based on experiments in which the \textit{c-fos} mRNA remained unstable after the 75 nt AU-rich element (ARE) was deleted from its 3' UTR. The insertion of this ARE into the rabbit \textit{β}-globin mRNA reduced its half-life from >24 hours to 37+/−6 minutes. This decay was dependent on continued transcription since ActD or DRB treatment reduced the rate of degradation. A second pathway is likely to involve the \textit{c-fos} coding region since substitution of the \textit{β}-globin coding region with that of \textit{c-fos} results in a \textit{t}_{1/2} of 17+/−2 minutes. This effect is independent of transcription, i.e., it occurs in the presence of act D.

Other examples of the involvement of coding sequences in mRNA decay
are the HIS3 and STE2 (Herrick, this thesis), STE3 and MATα1 (R. Parker and A. Jacobson, manuscripts in preparation), and the SPO13 (Surosky and Esposito, 1989) mRNAs of Saccharomyces cerevisiae, and the TFIIIA gene of Xenopus laevis (Harland and Misher, 1988). Data to support the role of coding sequences in each of these cases is derived from experiments in which coding sequences have been deleted or transferred to other stable mRNAs.

E. OTHER FACTORS WHICH INFLUENCE mRNA DECAY

Various internal and external stimuli, including exposure to hormones, viruses, heat shock, changes in growth rate and state of differentiation, position within the cell cycle, and subcellular location of an mRNA can affect cellular mRNA decay rates. These effects are summarized below:

1. Hormones and Half-Lives

Exposure to specific hormones can exert a dramatic effect on mRNA stability. For example: 1) using mammary gland organ cultures and a [3H]-uridine pulse-chase protocol with glucosamine, Guyette et al., (1979) showed that, following exposure to prolactin, the half-life of casein mRNA increased 17-25 fold (5.4 hr vs 92 hr); 2) In rat mammary explants, hydrocortisone treatment increases the t½ of casein mRNA from 1 hr to 20 hr (Chomiczynski et al., 1986); 3) A 30-fold increase in the half-life (16 hr to 480 hr) of vitellogenin mRNA from Xenopus laevis liver was seen following estrogen treatment (Brock and Shapiro, 1983); 4) Mouse fibroblasts which have the human growth hormone (hGH) gene stably integrated show a 2-5 fold induction of hGH expression upon exposure to the synthetic glucocorticoid
hormone, dexamethasone. Using approach to steady-state measurements with [3H]-uridine, it was shown that the hGH mRNA was stabilized from a $t_{1/2}$ of 9 hr to > 50 hr (Paek and Axel, 1987); and 5) Using human KB epidermoid carcinoma cells treated with act D, Jinno et al., (1988) showed that the addition of epidermal growth factor (EGF) prolongs the half-lives for the EGF receptor (from 0.9-2.2 hr to > 6 hr), $\beta$-tubulin, and $\alpha$-actin mRNAs. These effects are specific since there was little change in the decay rate of total cellular mRNA.

Not every exposure to hormones results in stabilization of a specific mRNA. For example: 1) Treatment of cultured rat dermal fibroblasts with dexamethasone destabilized type I procollagen mRNA from a 12 hr to a 6-8 hr half-life (Raghow et al., 1986) (although the same RNA in cultured human foreskin fibroblasts was stabilized following exposure to transforming growth factor $\beta$ (TGF$\beta$) [Raghow et al., 1987]) and 2) apolipoprotein II (apoII) and vitellogenin (VTGII) mRNAs from chick liver are selectively destabilized ($t_{1/2}$ reduced to 1.5 hr from 13 hr) during hormone withdrawal following extended estrogen exposure (Gordon et al., 1988).

Selective destabilization and stabilization of mRNAs are not restricted to hormones since the addition of cAMP to FTO-2B rat hepatoma cells results in a 5- to 6-fold stabilization of phosphoenolpyruvate carboxykinase mRNA (Hod and Hanson, 1988), while exposure of disaggregated Dictyostelium cells to cAMP prevents the destabilization of a large class of developmentally regulated mRNAs (Mangiarotti et al., 1983; Manrow and Jacobson, 1988).

2. Viral Effects on mRNA Turnover

During viral infection of eukaryotic cells, many host cell functions are
inhibited at the expense of viral gene expression. This reduction in cellular gene expression can be a result of viral-induced destabilization of cellular mRNAs. Friend erythroleukemia cells induced to differentiate with 2% dimethyl sulfoxide synthesize large quantities of stable globin mRNA. Infection of these cells with herpes simplex virus I (HSV-I) results in the degradation of preexisting cellular globin mRNA to 15% of control levels (Nishioka and Silverstein, 1977). A similar mechanism appears to operate in vaccinia-infected mouse L cells (Rice and Roberts, 1983). By 3 hours postinfection, the concentrations of β-actin and α-tubulin are reduced to 50% of their normal levels. The exact mechanism involved in either experimental system is unknown. Recent work using Vero monkey cells and mutant HSV-I strains have identified "vhs" mutants which are deficient in the virion shutoff of host protein synthesis. Further characterization demonstrated that vhs mutants are defective in their ability to degrade host mRNA (Kwong et al., 1988).

3. mRNA Half-Lives Change with Position in the Cell-Cycle, with Growth Rate, and State of Differentiation

Changes in post-transcriptional regulation occur following alterations in cellular growth rates. The mRNAs for growth-associated "housekeeping" genes become more abundant (10-50 times higher during S phase) as quiescent cells are stimulated to proliferate by the addition of serum. This phenomenon has been demonstrated for chicken thymidine kinase mRNA (Groudine and Casimir, 1984), mouse dihydrofolate reductase mRNA in Chinese hamster ovary cells (Kaufman and Sharp, 1983), human thymidylate synthase mRNA in human diploid fibroblasts (Ayusawa et al., 1986), and histone mRNAs
(Gallwitz, 1975, Heintz et al., 1983, and DeLisle et al., 1983). With the exception of histone mRNA (5-fold increase), there were no significant increases in the transcription rates of these mRNAs as assayed by in vitro nuclear run-on experiments. The stability of eucaryotic elongation factor Tu (eEF-Tu) mRNA also varies with growth rate (Rao and Slobin, 1988). The half-life of this mRNA in Friend erythroblast cells in stationary phase is 24 hr, but decreases to 9 hr when these cells are actively growing.

Differences in stages of differentiation can also result in the altered post-transcriptional regulation of specific mRNAs. The immunoglobulin heavy-chain (IgH) mRNA has a low steady-state level and a t1/2 of 3 hr in a B-cell lymphoma. Terminal differentiation to a plasmacytoma cell results in a 20 hr half-life and a large increase in IgH mRNA abundance (Mason et al., 1988). Since no increase in the transcription rate was measured between the pre-B and plasma cells, these effects can be explained by differences in the stability of μ mRNA (Jack and Wabl, 1988).

4. Heat Shock and mRNA Decay

Two effects on the stability of specific mRNAs have been observed following heat shock (Petersen and Lindquist, 1988). At the onset of heat shock the hsp70 mRNA is stabilized whereas, following a return to the normal temperature, Drosophila cells destabilize heat-shock specific mRNAs.

5. Subcellular Location and mRNA Turnover

Histone mRNAs are normally translated on free ribosomes associated with the cytoskeleton (Zambetti et al., 1985) and are destabilized following the inhibition of DNA synthesis (Butler and Mueller, 1973). To test if the
subcellular location of the histone mRNA affects this regulation, a chimeric mRNA was constructed in which sequences encoding human histone H3 were fused to sequences encoding the signal peptide from the *E. coli* pBR322 β-lactamase gene. The fusion mRNA was targeted to membrane-bound polysomes and lost the normal destabilization which occurred following DNA synthesis inhibition (Zambetti et al., 1987).

Polysomes are attached to the cytoskeleton in HeLa cells (Lenk et al., 1977). Very little is known about the specifics of the interaction, although it appears that cytoskeletal attachment is obligatory for the translation of some mRNAs (Ornelles et al., 1986). Little is known concerning the relationship of mRNA turnover to cytoskeletal association.

**F. IN VITRO DECAY SYSTEMS**

In *vitro* degradation systems are valuable tools for understanding many aspects of the turnover of specific mRNAs (Ross and Kobs, 1986, Sunitha and Slobin, 1987, and Pei and Calame, 1988). Since 1986, Ross and co-workers have developed and exploited an *in vitro* system derived from K562 human erythroleukemia cells. The system uses purified polysomes and conditions optimal for protein synthesis elongation. Unfortunately, overall levels of protein synthesis are low, and the initiation rate for exogenous substrates is negligible. Nevertheless, decay rates measured *in vitro* are likely to have physiological relevance since the relative decay rates measured *in vivo* for the gamma globin, delta globin, *c-myc*, and H4 histone mRNAs are maintained *in vitro* (Ross and Kobs, 1986). *In vitro*, the unstable H4 histone mRNA is degraded exonucleolytically, initiating at its 3'-terminus and proceeding in a 3'->5' direction. This pathway is identical to that of H4 histone mRNA in
and similar or identical decay intermediates are observed (Ross et al., 1986). The decay pattern seen for the *c-myc* mRNA in the cell-free extract also mimicks or is identical to that observed in cells (Brewer and Ross, 1988, Brewer and Ross, 1989). The degradation of *c-myc* mRNA in *vitro* occurs in the following manner: the mRNA rapidly loses its poly(A) tail, and this loss is followed by the appearance of decay intermediates whose cleavage sites map to the AU-rich sequences in the 3'-UTR. (Rapid deadenylation of the *c-myc* mRNA also appears to be the initial event of its degradation in *vivo* [Swartwout and Kinniburgh, 1989]). Subsequent decay in *vitro* occurs in a 3'->5' direction. Addition of an S-130 fraction from exponentially growing K562 cells accelerates *c-myc* mRNA decay 8- to 10-fold. The destabilizing activity is labile in that it is absent from S130 fractions isolated from cycloheximide-treated cells. This factor is sensitive to heat and micrococcal nuclease, and is resistant to proteinase K, suggesting the involvement of a nucleic acid component.

**G. WHY YEAST AS A SYSTEM?**

The yeast, *Saccharomyces cerevisiae*, was used in this study of mRNA turnover for several reasons. First, a large number of cloned and sequenced genes of known cellular function were readily obtainable from our colleagues. Knowledge of the gene product encoded by an mRNA allows one to circumvent problems with overexpression, e.g., those which occur with the *ACT1* gene (D. Botstein, personal communication). Second, yeast is a simple organism in which to do biochemical manipulations such as DNA transformation, gene replacement, and labeling studies. Finally, the powerful
genetics in yeast should eventually allow for the dissection of mRNA turnover pathways.

**H. SUMMARY OF YEAST mRNA TURNOVER DATA**

The decay rates for both the poly(A)$^+$ population and for individual yeast mRNAs have been measured previously by using many different functional and chemical approaches. Hutchison et al., (1969) used the temperature-sensitive mutant ts136 (which becomes defective in cytoplasmic RNA production following a shift to 36°C) to measure a half-life of 23 min for the decay of polyribosomes to monosomes. ts136 was also used by Koch and Friesen (1979) and Chia and McLaughlin (1979) in a different approach to measure mRNA decay: at various times after a shift to 36°C, cells were pulse-labeled with $[^{35}\text{S}]$-methionine and the proteins were then separated on two-dimensional gels. Koch and Friesen looked at the decay of 30 mRNAs and observed a range of half-life values from 3.5 to greater than 70 min, with the entire population turning over with an average half-life of 16 min. In the study by Chia and McLaughlin, the mRNA population decayed with a half-life of 22 min and individual mRNAs had half-lives ranging from 4.5 to 41 min. In another functional assay, Tonnesen and Friesen (1973) measured $[^{14}\text{C}]$-leucine uptake at 30°C after inhibiting RNA synthesis with either daunomycin or ethidium bromide. They measured a half-life of 21$\pm$4 min for the mRNA population. Hynes and Phillips (1976) used an approach to steady-state labeling procedure with $[^{3}\text{H}]$-adenine to measure the kinetics of incorporation into the oligo(dT)-cellulose selected poly(A)$^+$ population, and measured a half-life of 17 min at 30°C.

A functional half-life of 3.25 min for allophanate hydrolase mRNA was
measured by monitoring the loss of enzyme activity after removal of the inducer, urea (Lawther and Cooper, 1973). The antibiotic, lomofungin, which inhibits yeast RNA polymerases (Cano et al., 1973), was used to inhibit transcription, after which the loss of α-glucosidase and invertase activity was monitored (Kuo et al., 1973). No reduction in activity was seen for either enzyme until 30-40 min after addition of the drug.

Filter hybridization of specific mRNAs to excess cloned DNAs following an adenine pulse-chase protocol was used to measure the half-lives for the following mRNAs: HTB1 (t1/2=15 min; Osley and Hereford, 1981), CYC1 (t1/2=13 min; Zitomer et al., 1979), PPR1 (t1/2=1 min; positive regulator of the URA1 and URA3 genes; Losson et al., 1983), URA3 (t1/2=10.5 min) and 2 abundant unknown mRNAs (t1/2=7 and 14.5 min; Bach et al., 1979). An approach to steady-state labeling analysis, using either [3H]-uracil or adenine labeled RNA followed by hybridization to excess cloned DNAs, was used to measure the half-lives of four coordinately regulated ribosomal protein mRNAs (average t1/2=14+/−2 min) and URA3 mRNA (t1/2=7.3 min; Kim and Warner, 1983). The half-lives for the URA3 (10 min) and FUR4 (2 min) mRNAs (encodes the uracil permease, Chevallier et al., 1982) were also measured in this fashion. Santiago et al., (1986) used the drug 1,10-phenanthroline to inhibit transcription and observed a range of half-lives between 6.6+/−0.67 to over 100 min for the decay of 15 mRNAs. The specifics of the above experiments are summarized in Table I. Sequence or structural determinants involved in the degradation of the mRNAs listed in Table I are unknown.

There are drawbacks to each of the methods described above for measuring mRNA decay rates in yeast. Intercalating drugs (daunomycin and ethidium bromide) and RNA polymerase inhibitors (lomofungin, thiolutin, and
1,10-phenanthroline) affect several metabolic pathways and may, in fact, inhibit mRNA turnover pathways. In general, the measurement of mRNA half-lives using chemical versus functional assays is preferable, although technically more demanding. Pulse-chase and approach to steady-state labeling procedures using tritium have been shown to give consistent data, but cannot be used with mRNAs of low abundance. The ts136 mutant affects the appearance of all cytoplasmic RNAs, a situation which may also alter normal mRNA decay pathways. In Chapter II, I will describe in detail why the use of an RNA polymerase II temperature-sensitive mutant to specifically inhibit transcription and subsequently measure mRNA decay rates is a method preferable to those described above.

Poly(A) Tails in Yeast

A yeast cell contains 3000-4000 different mRNA sequences, most of which are on polysomes, and are polyadenylated. Poly(A)+ mRNA comprises 1-2% of the total cellular mRNA (Hereford and Rosbash, 1977) and yeast cytoplasmic mRNAs have an average poly(A) tail length of 40-60 nt (McLaughlin et al., 1973; Reed and Wintersberger, 1973).

Yeast cells do not have giant heterogeneous nuclear RNA as seen in higher eukaryotes, but instead have nuclear RNA only slightly larger than cytoplasmic RNA (Groner and Phillips, 1975). Following a 15 min pulse of [3H]-adenine in yeast spheroplasts, 10% of total poly(A)+ RNA is nuclear poly(A)-containing RNA. Yeast poly(A) tail lengths appear to fall into 2 classes. One class is heterogeneous in size and shortens with time from an initially synthesized length of 60 residues down to <20 nt in length (Phillips et al., 1979). The second class has a homogeneous length of 20 nt, and may
have a transcriptional origin. In steady-state, 50% of all yeast mRNA molecules isolated on oligo(dT)-cellulose carry poly(A) tails of <32 nt (Groner et al., 1974). (Poly(A) tails <15 nt do not bind oligo(dT)-cellulose at the NaCl concentrations used (0.1 M NaCl; Groner et al., 1974). A substantial amount of pulse-labeled polydisperse RNA fails to bind oligo(dT)-cellulose (McLaughlin et al., 1973), an indication there may be a significant poly(A)− or poly(A)-deficient mRNA population on yeast polysomes. This is consistent with results presented in Chapter II of this thesis.

**Cap Structures in Yeast:**

Poly(A)-containing RNAs in yeast have a 5′-cap which has 2 different structures, mG(5)pppAp (75%) or mG(5)pppGp (25%) (Sripati et al., 1976). This ratio is maintained on mRNAs in both unstressed cells and during heat-shock, indicating that these alternate cap structures are not involved with changes in mRNA stability (Piper et al., 1987). Cap structures in yeast do provide protection from the processive action of a 5′-→3′ exonuclease (Stevens, 1978). An mRNA decapping enzyme has been purified from a high-salt wash of ribosomes which yields digestion products of mGDP and 5′-pRNA (Stevens, 1980). Interestingly, incubation of purified decapping enzyme with a series of synthetic capped RNAs of different sizes demonstrated that the larger RNA substrate was cleaved by as much as 10-fold more efficiently (Stevens, 1988). Unlike higher eukaryotes, there are no internal 6-methyladenosine residues within yeast mRNAs (Sripati et al., 1976; DeKloet and Andrean, 1976). Yeast mRNAs, however, do contain an average of 2 methylated nucleosides/1,200 nts, located in the 7-methyl guanosine residue and the ribose methylated nucleoside to which it is linked (DeKloet and
3' UTRs and mRNA Decay in Yeast

Indirect evidence for the involvement of the 3'-end of histone H2B in its destabilization was provided by Lycan et al. (1987). Osley and Hereford (1981) had observed dosage compensation for TRT1 H2B mRNA following introduction of a second copy of the H2B gene. The mechanism appeared to be post-transcriptional since the half-life was reduced to 7 minutes (from 15 minutes) in the transformant. In recent work, Lycan et al. (1987) used a construct containing a GAL10 promoter-driven histone-lacZ fusion lacking histone 3'-UTR sequences. The histone-lacZ fusion mRNAs did not fluctuate in the cell cycle and were also insensitive to normal H2B dosage compensation as seen upon addition of galactose. In the CYC1 gene, a 38 bp deletion in the 3'-UTR results in aberrant termination leading to polyadenylated transcripts of 1650 and 2400 nts (w.t. mRNA is 630 nts). These mRNAs have only 10% of the steady-state level of the normal RNA, and the defect is thought to alter the stability of the mutant mRNAs (Zaret and Sherman, 1984).

RNases in Yeast

Many different RNase activities have been found in yeast, in large part a result of the work of Audrey Stevens. In 1963, Ohtaka et al., purified an RNase from yeast which hydrolyzes RNA completely, yielding 3' mononucleotides. This enzyme is heat-sensitive, has a pH optimum of 7.5, and is inhibited by zinc ions. A 5'->3' exoribonuclease has been purified 200-fold from a high-salt wash of ribosomes (Stevens, 1978, Stevens, 1979,
This enzyme processively degrades uncapped RNAs in a 5'->3' direction and produces 5'-mononucleotides. It has a pH optimum of 8 to 8.5, requires divalent cations (Mg$^{2+}$ or Mn$^{2+}$), and is stimulated by monovalent cations. It has a mw of 160,000 and also has RNase H activity. Its role in mRNA decay is unclear since preliminary data suggests it may be located predominantly in the nucleus. The decapping enzyme has already been discussed (Stevens, 1980, and Stevens, 1988); its reaction product, 5'-pRNA, is the preferred substrate of the 5'->3' exoribonuclease.

An endoribonuclease activity has been detected from a high-salt wash of ribonucleoprotein particles (Stevens, 1982). This enzyme has pyrimidine cleavage site specificity and results in the limited cleavage of yeast mRNA and rRNA (Stevens, 1985). Poly (A,U) is cleaved rapidly, and this enzyme shows a stringent cleavage of U-A bonds. The decay products terminate with pyrimidine nucleoside 2', 3'-cyclic phosphate. The addition of the small nuclear RNAs U1 and U2 from Novikoff hepatoma cells stimulates the hydrolysis of a [$^3$H]-m$^7$Gppp[$^{14}$C]RNA-poly(A) labeled substrate (labeled with [$^3$H]-methionine and [$^{14}$C]-uridine, Stevens, 1982). This stimulation involves an interaction of the labeled RNA with the small nuclear RNA, and short double-stranded regions may be the site of its hydrolysis.

Other RNase activities in yeast include a double-stranded RNase (Mead and Oliver, 1983), and two RNase H activities (Wyers et al., 1973, and Wyers et al., 1976). The double-stranded RNase has an optimum temperature of 30°C, is stimulated by KCl and dithiothreitol, and is inhibited by divalent cations. The RNase H activities are not associated with ribosomes, and appear to function by different mechanisms. The abundant RNase H$_1$ (mw of
48,000) liberates 5'-phosphate mononucleotides and may be exonucleolytic. RNase H2 (mw of 21,000) yields a mixture of oligonucleotides with 5'-phosphate termini. The exact cellular function of these enzymes is unknown.

I. SPECIFIC AIMS OF THIS THESIS:

The main objective of this thesis was to begin to elucidate the structural determinants of mRNA stability in the yeast, *Saccharomyces cerevisiae*. The experiments and data have been organized into two chapters in the form of two manuscripts which are going to be submitted for publication. Chapter II describes four procedures that I have developed to measure the decay of both the poly(A) population and individual mRNAs in yeast. This chapter includes data from experiments performed by Dr. Roy Parker, a postdoctoral fellow in Dr. Jacobson's lab. His contributions were invaluable to the study and will be designated clearly as his (see below). Chapter II also compares several properties of stable and unstable mRNAs.

An experimental strategy to identify determinants of mRNA stability is to construct and measure the half-lives of chimeric mRNAs containing regions from stable and unstable mRNAs. Chapter III will summarize the results that I have obtained from the study of various chimeras made between the stable *ACT1* and the unstable *HIS3* and *STE2* mRNAs. The implications of these results, and others obtained by Roy Parker, are discussed in terms of likely mechanisms for mRNA decay in yeast.

Contributions to Chapter II from other individuals include the following: i) the data of Table 3 (ts Pol II half-lives) and Fig. 7D are the results of a collaborative effort between R. Parker and myself, ii) the data of Table 4, Table 5, Fig. 1D and 1E, and Fig. 6 was obtained in experiments done
exclusively by R. Parker, iii) the screening for relative content of rare codons (Fig. 8 and Table 6) was a collaborative effort between A. Jacobson and myself, and iv) the computer analysis for sequence homology or related structures in yeast 3'-untranslated regions was done by A. Jacobson.
<table>
<thead>
<tr>
<th>mRNAs:</th>
<th>t1/2:</th>
<th>Method:</th>
<th>Reference:</th>
</tr>
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<td>Decay of polysomes to monosomes.</td>
<td>23 min</td>
<td>Functional.</td>
<td>Hutchison et al., 1969.</td>
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<td>mRNA population</td>
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<td>Monitored the decay of [35S]-methionine proteins on 2-D gels.</td>
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<td>HTB1 mRNA</td>
<td>15 min</td>
<td>Chemical. [3H]-adenine pulse-chase and hybridization to excess cloned DNAs.</td>
<td>Osley and Hereford, 1981.</td>
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<td>CYC1 mRNA</td>
<td>13 min</td>
<td>Chemical. Same assay as above.</td>
<td>Zitomer et al., 1979.</td>
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<td>Time</td>
<td>Assay Method</td>
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<td><strong>PPR1 mRNA</strong></td>
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<td>Losson et al., 1983.</td>
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<td>10.5 min</td>
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<td>Bach et al., 1979.</td>
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<td>2 abundant mRNAs</td>
<td>7 min + 14.5 min</td>
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<tr>
<td><strong>4 ribosomal protein mRNAs</strong></td>
<td>average of 14+/-.2 min</td>
<td>Chemical. Approach to steady-state labeling with $[^{3}H]$-uracil and hybridization to excess cloned DNA.</td>
<td>Kim and Warner, 1983.</td>
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<td>Chevallier et al., 1982.</td>
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<td><strong>FUR4 mRNA</strong></td>
<td>2 min</td>
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<td><strong>invertase mRNA</strong></td>
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<td><strong>13 random mRNAs (cDNA clones)</strong></td>
<td>Ranged from 6.6+/-.67 to &gt; 100 min.</td>
<td>Inhibited transcription with 1,10-phenanthroline, probed total RNA dot blots with unique probes.</td>
<td>Santiago et al., 1986.</td>
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<tr>
<td><strong>ACT1 mRNA</strong></td>
<td>76.6+/-.15 min</td>
<td></td>
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<tr>
<td><strong>PYK mRNA</strong></td>
<td>59.9+/-.7.8 min</td>
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Chapter II

Determinants of mRNA Stability in *Saccharomyces cerevisiae*:
Identification and Comparison of Stable and Unstable mRNAs

Running title: mRNA decay in *S. cerevisiae*

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ABSTRACT

Large differences exist in the decay rates of individual mRNAs yet the molecular basis for such differences is substantially unknown. We have hypothesized that some insight into the mRNA turnover problem might be gained by comparing the properties of stable and unstable mRNAs. To this end, we have developed a procedure for the measurement of mRNA decay rates in the yeast *Saccharomyces cerevisiae* and applied it to the determination of half-lives for 20 mRNAs encoded by well-characterized genes. The procedure utilizes northern or dot blotting to quantitate the levels of individual mRNAs after thermal inactivation of RNA polymerase II in an rpb1-1 temperature-sensitive mutant. To assess the reliability of half-life measurements obtained in this manner, we have compared the results of this procedure to results obtained by three other procedures (pulse-chase analysis, approach to steady-state labeling, and inhibition of transcription with thiolutin) and also evaluated whether heat-shock alters mRNA decay rates. We find that: i) for most mRNAs, all four procedures yield comparable relative decay rates and ii) there are no significant differences in the mRNA decay rates measured in heat-shocked or non-heat-shocked cells. Of the 20 mRNAs studied, 11, including those encoded by *HIS3*, *STE2*, *STE3*, and *MATα1*, were unstable (t1/2 < 7 min) and 4, including those encoded by *ACT1* and *PGK1*, were stable (t1/2 > 25 min). We have begun to assess the basis and significance of such differences in the decay rates of these two classes of mRNA. Our results indicate that: i) mRNAs encoding "housekeeping" functions tend to be stable, whereas mRNAs encoding tightly regulated functions (e.g.,
mating type) are unstable; ii) there is no correlation between mRNA decay rate and mRNA size; iii) stable and unstable mRNAs do not differ significantly in their poly(A) metabolism; iv) stable mRNAs can maintain their stability in a poly(A)^- or poly(A)-deficient form; v) the degradation of both stable or unstable mRNAs is dependent on concomitant translational elongation; vi) the 3'-untranslated regions of stable and unstable mRNAs do not appear to contain any significant class-specific sequences or structures which could potentially enhance or exclude degradative activities; and vii) the percentage of rare codons present in a given mRNA correlates with the degree of instability of that mRNA.
INTRODUCTION

Differences in the decay rates of individual mRNAs can have profound effects on the overall levels of expression of specific genes (Raghow, 1987; Shapiro et al., 1987). Although the potential importance of mRNA stability as a mechanism for regulating gene expression has been recognized (Brawerman, 1987; Ross, 1989), the structures and mechanisms involved in the determination of individual mRNA decay rates have yet to be elucidated. As an approach to understanding the determinants of mRNA stability, we have begun to compare the properties of mRNAs in Dictyostelium discoideum which differ significantly in their respective decay rates (Shapiro et al., 1988). In this report, we describe our initial efforts to perform a similar analysis of mRNAs in the yeast, Saccharomyces cerevisiae. Our objective was the identification of both stable and unstable yeast mRNAs that were encoded by genes which had already been well characterized. Success in such an endeavor would make it possible to explore the structural determinants of mRNA stability, for example, by analyzing the decay rates of mRNAs transcribed from chimeric genes (Shaw and Kamen, 1986; Fort et al., 1987; Rahmsdorf et al., 1987; Jones and Cole, 1987, Kabnick and Housman, 1988; Shyu et al., 1989).

Decay rates for both the poly(A)$^+$ RNA population and for individual yeast mRNAs have been measured previously using several different functional or chemical assays. Half-lives ranging from 16 to 23 min have been measured for the turnover of the poly(A)$^+$ RNA population, whereas half-lives of individual mRNAs span a broader range from 1 to over 100 min
(Hutchison et al., 1969; Koch and Friesen, 1979; Chia and McLaughlin, 1979; Tonnesen and Friesen, 1973; Santiago et al., 1986; Hynes and Phillips, 1976; Kim and Warner, 1983; Lawther and Cooper, 1973; Chevallier et al., 1982; Osley and Hereford, 1981; Zitomer et al., 1979; Losson et al., 1983 and Bach et al., 1979; Kuo et al., 1973). However, the majority of the assays used in these previous studies were either: i) not potentially useful for the characterization of chimeric mRNAs, ii) not amenable to the simultaneous assessment of the decay rates of large numbers of individual mRNAs, or iii) a source of possible artifacts in the measurements (e.g., by the inhibition of transcription and other cellular metabolic events; (Chang et al., 1978; Johnston and Singer, 1978; Krishnamurti et al., 1980; Casey et al., 1983). Therefore, we sought to establish an alternative procedure for the reliable evaluation of the decay rates of individual yeast mRNAs.

Nonet et al. (1987) have constructed and characterized a conditionally lethal mutant (rpb1-1) with a temperature-sensitive lesion in the largest subunit of RNA polymerase II. In strains harboring the rpb1-1 allele, a shift to 36°C leads to the rapid and selective cessation of mRNA synthesis and to a reduction in the steady-state levels of pre-existing mRNAs (Nonet et al., 1987). The latter reduction is a reflection of ongoing mRNA turnover in the absence of new mRNA synthesis and serves as the basis for the procedure used here: Cells growing at 24°C were abruptly shifted to 36°C by the addition of medium pre-warmed to 48°C and culture aliquots were removed at different times after the temperature shift. RNA was isolated from each aliquot and the relative amounts of individual mRNAs were quantitated by RNA blotting methods. Using this procedure, half-lives were determined for
20 different mRNAs and found to range from 2.5 to 45 min. To establish the validity of decay rates measured in this manner, two types of control experiments were performed: i) those which measured mRNA decay rates by three additional, independent procedures (pulse-chase, approach to steady-state labeling, and inhibition of transcription with thiolutin) and ii) those which showed that mRNA decay rates were not significantly affected by non-specific effects of the temperature shift.

Having identified mRNAs with large differences in their respective decay rates, they were then compared with respect to several physiological or structural parameters which might contribute to the determination of their inherent stability or instability: i) relative decay rates of poly(A)$^+$ and poly(A)$^-$ forms, ii) mRNA size, iii) the dependence of decay on ribosomal translocation, iv) relative content of rare codons, and v) specific sequences or structures within 3'-untranslated (UT) regions. These comparisons show an interesting correlation between mRNA stability and the relative mRNA content of rare codons, but show no significant differences between stable and unstable mRNAs with respect to poly(A) metabolism, size, sensitivity to treatment with cycloheximide, or 3' UT sequences.
MATERIALS AND METHODS

Yeast strains. Four strains of the yeast *Saccharomyces cerevisiae* were used for these experiments. Strain DBY747 (MATa, his3Δ, leu2-3, leu2-112, and ura3-52) was used to analyze mRNA decay in pulse-chase and approach to steady-state labeling experiments as well as in cells treated with thiolutin. Strains Y260 (MATa, ura3-52, and rpb1-1) and Y262 (MATa, ura3-52, his4-539, and rpb1-1; Nonet et al., 1987; kindly provided by Richard Young, M.I.T.) were utilized in temperature-shift experiments in which their thermolabile RNA polymerases were inactivated. Strain N222 (kindly provided by Michael Nonet) has a wild-type RNA polymerase, but is otherwise isogenic with Y262 and was used for several control experiments.

Analysis of mRNA decay in temperature-sensitive RNA polymerase mutants. mRNA decay rates were measured in cultures (100-200 ml) of Y260 or Y262 (5-1X10^7 cells/ml) in which the temperature of the culture was abruptly adjusted from 24°C to 36°C by adding an equal volume of YEPD medium at 48°C and then transferring the culture flask to a shaker bath at 36°C. Aliquots of the culture were removed after 0-100 min at 36°C, cells were harvested by centrifugation, and the cell pellets were frozen on dry ice. Total cellular RNA was extracted from the frozen cells, resuspended in sterile H2O, and stored at -80°C. Integrity of the extracted RNA was monitored by electrophoresis and staining in a denaturing gel. The relative levels of individual mRNAs present at different times after the temperature-shift were determined by northern blotting or RNA dot-blotting. To analyze the decay rate of total poly(A)^+ RNA, cells (strain Y260) were grown in YEPD medium
at 24°C to a density of 5X10^6 cells/ml and labeled for one generation (3.5 h) with 20 μCi/ml of carrier-free 32P-phosphoric acid. The fraction of poly(A)^+ RNA in each sample was monitored by hybridization to poly(U) filters (Palatnik et al., 1980).

**Treatment of cells with thiolutin.** The anti-fungal agent thiolutin, (Jimenez et al., 1973; kindly provided by Dr. Donald Tipper, U. Mass. Medical School) was dissolved in dimethyl sulfoxide at 2 mg/ml. To analyze the effects of thiolutin on total poly(A)^+ RNA, cells (strain DBY747) grown in YEPD medium at 30°C to 5X10^6 cells/ml were labeled with 20 μCi/ml of [3H]-uridine for 1 hr and then exposed to 0, 3 or 6 μg/ml of thiolutin. Culture aliquots (10 ml) were removed and frozen in dry ice after 0, 10, 20, 30, 40 and 60 min of thiolutin treatment and total RNA was extracted. Radioactivity in poly(A)^+ RNA was determined by hybridizing equal volumes of total RNA to poly(U) filters (Palatnik et al., 1980). To measure the effects of the drug on the decay rates of individual mRNAs, thiolutin (3 μg/ml) was added to cells grown at 30°C to 5X10^6 cells/ml in YEPD medium and culture aliquots (40 ml) were removed, centrifuged, and frozen after 0, 10, 20, 40, 60, 80 and 100 min of drug exposure. Total RNA was extracted and 4 μg of RNA from each sample was fractionated in 1 percent agarose-2.2M formaldehyde gels (Lehrach et al., 1977). Individual mRNAs were subsequently quantitated by northern blotting.

**Heat-shock.** To measure mRNA decay rates in the presence or absence of heat-shock, 125 ml cultures of N222 were grown at 36°C or at 24°C in YEPD to mid-log. Decay rates in the absence of heat shock were measured following inhibition of transcription by the addition of 125 mls of 36°C YEPD
containing thiolutin at 6 μg/ml (final concentration=3 μg/ml) to the 36°C culture. Decay rates in the presence of heat shock were measured by the addition of 125 mls of 48°C YEPD containing thiolutin to the 24°C culture. In both cases RNA was prepared at various times after continued incubation at 36°C and the decay of individual mRNAs determined by northern blotting and densitometry.

32PO4 pulse-chase. Cells (strain DBY747) were grown overnight at 30°C in phosphate-depleted YEPD medium (Mortimer and Hawthorne, 1969; Rubin, 1974), diluted to 2-2.5X10^6 cells/ml in the same medium, and grown at 30°C to 5X10^6 cells/ml. The culture was then labeled for 15 min with 0.05-0.1 mCi/ml carrier-free 32P-phosphoric acid (New England Nuclear). To initiate the chase, cells were harvested by centrifugation, washed once in YEPD medium containing 100 mM sodium phosphate, pH 5.8, and then resuspended in the same medium (t=0). At 10, 20, 40, 60, 80 and 100 min after initiating the chase, culture aliquots (75-100 mls) were removed, cells were pelleted by centrifugation, and the cell pellets were frozen on dry ice. RNA was extracted from the frozen cells, resuspended in 400 μl of sterile, DEPC-treated H2O, and stored at -80°C. The specific activity of each RNA sample was determined and the effectiveness of the chase was assessed by comparing the decay in specific activity of the total RNA to the generation time of the cells. Poly(A)+ RNA was purified and an equal mass (0.75-3.0 μg) of 32P-labeled poly(A)+ RNA from each time point of the chase was hybridized to identical filters containing 5-10 sets of dots of excess plasmid DNA complementary to specific mRNAs (see below; Shapiro et al., 1988). After
hybridization, the filters were exposed to X-ray film and the resultant autoradiographs were scanned with a Helena Quick Scan R+D densitometer. Decay curves were generated by deriving the areas under the peaks of the densitometer scans, defining the value of \( t_0 \) as 100 percent, and then calculating the percentage of hybridizable RNA remaining at different times of the chase.

**Approach to steady-state labeling.** Cells (strain DBY747) were grown in phosphate-depleted YEPD medium at 30°C to a density of approximately 4X10^6 cells/ml. Carrier-free \(^{32}\)P-phosphoric acid was added at 100 \( \mu \)Ci/ml and aliquots (47 ml) of the culture were removed after 5, 10, 15, 20, 30, 40, 50, and 70 min of labeling. Total RNA was extracted from frozen cell pellets and poly(A)^+ RNAs were purified. To measure decay rates of individual mRNAs, 1 \( \mu \)g of poly(A)^+ RNA from each sample was hybridized to filters containing dots of specific plasmid DNAs (see below). RNA decay curves were generated by plotting 1-A/A^\infty versus the time of \(^{32}\)PO\(_4\) labeling (where A is the specific activity of an RNA at times t and A^\infty is the specific activity at time \( \infty \); Greenberg, 1972; Kim and Warner, 1983). For total poly(A)^+ RNA the specific activity of the 70 min sample was used as A^\infty and for individual mRNAs the specific activity of the 30 min samples was used as A^\infty.

**RNA extraction.** RNA was extracted by either of two methods. No differences in mRNA decay rates attributable to the method of RNA isolation were observed. Method 1 was a modification of the procedures of Zitomer et al. (1979): Frozen cell pellets of 5-8X10^8 cells were thawed and resuspended in 2.5 ml of sterile, ice cold RNA extraction buffer (0.1 M HEPES, pH 7.4, 0.1 M LiCl, 0.1 mM EDTA) followed immediately by the addition of 50 \( \mu \)l of
diethylpyrocarbonate (DEPC) and 5 grams of 0.45-0.50 mm glass beads (B. Braun Melsungen). Cell suspensions were vortexed vigorously for three 20 sec intervals and then treated, at room temperature, with SDS (final concentration 0.5 percent) and 2.5 ml of phenol:chloroform:isoamyl alcohol (50:48:2). The mixture was agitated on a vortex mixer for twenty sec, centrifuged at 12,000 g for 5 min, and the aqueous layer was removed. The first interface was reextracted with 2.5 ml of additional RNA extraction buffer and the aqueous phase was combined with that obtained in the first extraction. This mixture was subjected to two additional phenol-chloroform extractions followed by a single chloroform extraction. Nucleic acids were precipitated with two volumes of ethanol at -80°C. DNA was removed by two successive washes with ice cold 3.0 M sodium acetate, pH 6.0, during which the insoluble RNA was separated from the soluble DNA by a 15 min centrifugation at 4°C in a microcentrifuge. Following a second ethanol precipitation, the RNA was rinsed with 95 percent ethanol, dried in vacuo, and suspended in sterile water. Alternatively, total yeast RNA was prepared by a modification of the method described by Domdey et al. (1984). Cell pellets were resuspended in 4.5 ml of 50 mM NaOAc, 10 mM EDTA and then mixed with 0.5 ml of 10% SDS and 5 mls of phenol (equilibrated with 50 mM NaOAc, 10 mM EDTA). This mixture was heated to 65°C for 4 min with vigorous vortexing, centrifuged for 5 min at 6,000 rpm in the Sorvall SS34 rotor, and the phenol layer was removed. Phenol (5 ml) was added to the pellet and aqueous layer followed by incubation and mixing at 65°C for 4 min. The mixture was centrifuged as before and the aqueous layer removed and extracted with phenol/chloroform. RNA in the final aqueous layer was
precipitated from ethanol twice and resuspended in sterile water. RNA concentration was determined by absorbance at 260 nm after incubation at 65°C for 5 min. The integrity of each RNA preparation was monitored by electrophoresis in a 1 percent agarose-2.2M formaldehyde denaturing gel and subsequent staining with ethidium bromide.

Purification of poly(A)⁺ RNA. Poly(A)⁺ RNA was purified by an oligo(dT)-cellulose batch procedure. To remove fine particles, oligo(dT)-cellulose (Collaborative Research, Type III) was suspended and allowed to settle in elution buffer (10 mM HEPES pH 7.5, 1 mM EDTA and 0.05 percent SDS) four times. Aliquots of the resin (20 mg) were transferred to 1.5 ml microcentrifuge tubes and equilibrated with binding buffer (20 mM HEPES, pH 7.6, 0.5 M NaCl, 1 mM EDTA and 0.1 percent SDS) by two cycles of gentle shaking and subsequent centrifugation at top speed in a clinical centrifuge. Total cellular RNA (0.1-0.5 mg) in sterile H₂O was heated to 65°C for 5 min, added to an equal volume of 2X binding buffer, mixed with the oligo(dT)-cellulose on a rotating mixer for 15 min at room temperature, and centrifuged as before. The oligo(dT)-cellulose was washed twice with binding buffer (800 μl) followed by two washes (900 μl) with binding buffer containing 0.1 M NaCl. Poly(A)⁺ RNAs were eluted by the addition of 450 μl of sterile H₂O at 37°C. Following centrifugation for 2 min, 400 μl of each eluate was removed and added to 45 μl of 3 M sodium acetate (pH 5.2) and 890 μl of 95 percent EtOH. The elution procedure was repeated (yielding approximately 10-20 percent more poly(A)⁺ RNA) and the two eluates were combined. RNA was either precipitated overnight at -20°C or for 1 hr at
RNA blotting. (i) Northern blots: RNA was electrophoresed in 1 percent agarose, 2.2 M formaldehyde gels and transferred to Zeta-Probe membranes (Bio-Rad) as described previously (Thomas, 1980). Northern blots (and RNA dots) were prehybridized in 50 percent formamide, 4X SSPE, 1 percent SDS, 0.5 percent Blotto, (Johnson et al., 1984) and 0.5 mg/ml of single stranded salmon sperm DNA at 42°C overnight. Hybridization solution was 47 percent formamide, 3X SSPE, 1 percent SDS, 10 percent dextran sulphate, 0.5 percent Blotto and 0.5 mg/ml salmon sperm DNA. Hybridizations were at 42°C for 24-48 hr, followed by washing with: 2X SSC, 0.1 percent SDS (15 min at room temperature); 0.1X SSC, 0.1 percent SDS (15 min at room temperature); and 0.1X SSC, 0.1 percent SDS (15 min, 65°C, twice). Blots were quantitated by autoradiography and densitometry (Shapiro et al., 1988) or by direct quantitation using a Betagen Betascope 603 Blot Analyzer (Betagen Corp., Waltham, MA). Autoradiography was performed at -80°C using a Du Pont Cronex intensifying screen. (ii) RNA dot blots: RNA (3 μg) from each sample was denatured and applied in duplicate dots to Zeta-Probe filters (Shapiro et al., 1988). Filters were hybridized to high specific activity DNA probes (Feinberg and Vogelstein, 1984) as described above for northern blots and quantitated by autoradiography and densitometry.

DNA dot blots. Plasmid DNA was denatured by incubation in 0.4 N NaOH for 15 min at 37°C, followed by quick cooling. DNA dilutions were made in ice cold 0.4 N NaOH and samples of 5 and 10 μg of DNA, each in 450 μl of 0.4 N NaOH, were applied in duplicate to a Zeta-Probe membrane using a Schleicher and Schuell dot blot manifold. After application of DNA, the
wells were rinsed with 500 µl of ice cold 0.4 N NaOH, air dried, and subsequently dried in vacuo for 2 hr at 80°C. Prior to use, filters were washed in a solution of 0.1X SSC and 0.5 percent SDS for 1 hr at 65°C and immediately prehybridized at 45°C for a minimum of 10 min in PHB (50 percent formamide [deionized with Bio-Rad AG 501-X8 resin], 4X SSPE and 1 percent SDS). 32P-labeled poly(A)+ RNA was heated to 65°C for 5 min and an equal mass (1-3 µg) was added to each hybridization reaction. Hybridizations were done in PHB for 24-48 hr at 45°C. After hybridization, the filters were washed with: 2X SSC, 0.1 percent SDS (15 min at room temperature); 0.1X SSC, 0.1 percent SDS (15 min at room temperature, twice); and 0.1X SSC and 0.1 percent SDS (15 min at 55°C). The filters were exposed to Kodak X-Omat AR film at -80°C. For relatively weak hybridization signals, requiring long exposures, a Du Pont Cronex Hi-Plus intensifying screen was used. Care was taken to ensure that the use of intensifying screens did not exaggerate the signal from highly radioactive dots beyond the linear range of the film relative to the weaker signals.

Manipulation of DNA. Enzymes were obtained from Boehringer Mannheim, New England Biolabs, or BRL and were used according to the manufacturers' instructions. Restriction analysis, nick-translation, DNA ligations, and electrophoresis of DNA were performed by standard methods (Maniatis et al, 1982), except that the DNA ligase buffer used was 20 mM Tris pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP and 15 mM NaCl. Transformation of E. coli strain JM83 was done essentially as described by Poteete (1982). The alkaline SDS lysis technique (Birnboim and Doly, 1979) was used for all plasmid DNA preparations. DNA fragments were purified from agarose gels
using the Geneclean Bio 101 kit which uses NaI to solubilize agarose and a silica matrix to bind DNA (Vogelstein and Gillespie, 1979). Labeling of DNA in low melt agarose, using random oligonucleotides as primers, was carried out as described by Feinberg and Vogelstein (1984).

**Rare codon screening.** Codon usage is measured as the frequency that each coding triplet appears per thousand codons. The table of codon frequencies that our data is based on was compiled from the DNA sequence data of 153 yeast genes (Aota et al., 1988). We have arbitrarily defined a rare codon as one which appears 15 or less times per thousand. Using this upper limit, the percentage of rare codons was calculated for 15 mRNAs whose half-lives were measured using the ts Pol II mutant.
RESULTS

Our initial objective was to establish a routine method for the reliable measurement of yeast mRNA half-lives. mRNA decay was analyzed after thermal inactivation of RNA polymerase II in cells harboring the rpb1-1 ts allele (Nonet et al., 1987) and compared to results obtained when transcription was inhibited by treatment of cells with the anti-fungal agent thiolutin (Jimenez et al., 1973; Tipper, 1973) or to procedures in which mRNA decay was measured as a function of changes in its kinetics of labeling with $^{32}$PO$_4$ (i.e., pulse-chase analysis and approach to steady-state labeling). In all four procedures decay rates were measured for both the total poly(A)$^+$ RNA population as well as for selected individual mRNAs. Individual mRNA half-lives were measured either by northern blot or dot blot assays using RNAs isolated at different times after inhibition of transcription or by hybridizing $^{32}$PO$_4$ labeled mRNA (isolated at different time points) to dots of excess cloned DNA.

Table 1 is a list of the cloned DNAs and synthetic oligonucleotides which have been employed in this study. With the exception of pUC8-Sc2676, each plasmid or oligonucleotide used as a hybridization probe encodes a single species of mRNA. pUC8-Sc2676 encodes three mRNAs (HIS3, DED1, and PET56), all of which are readily separated from each other by northern blot analysis (Struhl, 1985).

mRNA decay following thermal inactivation of RNA polymerase II. The decay of the poly(A)$^+$ RNA population following thermal inactivation of RNA
polymerase II is depicted in Fig. 1A. In this experiment, cells (Y260) were labeled with $^{32}$PO$_4$ (20μC/ml) for 3.5 h at 24°C and the relative amount of poly(A)$^+$ RNA in each RNA sample isolated after the shift to 36°C was determined by hybridization to immobilized poly(U). The decay kinetics for the poly(A)$^+$ RNA population are complex, with the fastest and slowest decaying components having half-lives of 5-6 min and 42-43 min, respectively (Table 2).

The relative amounts of individual mRNAs remaining at different times after a temperature shift were determined by RNA blotting assays. Figs. 1B and 1D are examples of dot-blotting and northern blotting experiments, respectively, in which samples of total cellular RNA extracted from cells at 36°C for 0-100 min were hybridized with $^{32}$P-labeled DNA probes complementary to specific mRNAs. Data from such experiments was quantitated either directly (using a Betagen Blot Analyzer) or by autoradiography and scanning densitometry. Decay rates were determined from semilog plots of the percentage of hybridizing material remaining at different times after the inhibition of transcription. Figs. 1C and 1E illustrate the quantitation of the blots in Fig. 1B and 1D and indicate that the mRNAs encoded by ACT1, STE2, cDNA #74, RP29, TCM1, and STE3 have half-lives of 26.9, 4.2, 16.0, 15, 9.8, and 2.5 min, respectively. Direct counting of blots (using the Betagen Blot Analyzer), tends to give decay rates which are 1-2-fold slower than those obtained by autoradiography and densitometry. We attribute this difference to the limited linear response range of X-ray film to β-emissions.

These procedures have been applied to a total of 20 different mRNAs
whose half-lives range from 2.5 to 45 min (Table 3) and for which we have arbitrarily defined three stability phenotypes: unstable (mRNAs with a t\(_{1/2}\)<7 min; e.g., STE2, STE3, HIS3, MFa1, and FUS1), moderately stable (mRNAs with a t\(_{1/2}\)=10-20 min; e.g., TCM1, PAB, and RP29), and stable (mRNAs with a t\(_{1/2}\)>25 min; e.g., ACT1, PGK1, and CYH2). Northern blotting, which has the advantages of indicating the quality of the RNA sample and of resolving more than one mRNA per lane, has been used to determine the majority of the decay rates indicated in Table 3; however, it should be noted that, for a given mRNA, the same decay rate is obtained by either blotting procedure.

Decay of poly(A)+ and poly(A)- mRNAs in rpb1-1 cells. The mRNA half-lives depicted in Table 3 and Figs. 1B, C, D, and E were measured using unfractionated RNA and, thus, did not distinguish between the poly(A)+ and poly(A)- forms of individual mRNAs. Since experiments in mammalian cells have suggested that, for some unstable mRNAs, mRNA degradation may be initiated by deadenylation (Brewer and Ross, 1988; Wilson and Treisman, 1988), we examined the decay rates of poly(A)+ and poly(A)- CYH2, PGK1, STE2, and HIS3 mRNAs. Aliquots (100 µg) of total RNA isolated from Y260 cells after a shift to 36°C were fractionated on oligo(dT)-cellulose and the bound (A+) and unbound (A-) fractions were analyzed by northern blotting. The degree of stringency for the oligo-(dT) selection procedure used in this experiment resulted in no visibly detectable rRNA species (following ethidium bromide staining) on a 1% agarose-2.2 M formaldehyde gel containing poly(A)+ RNA fractionated from the equivalent of 10 µg of total RNA. Although the unbound fraction includes RNAs with short poly(A) tails (<15 adenylate
residues; Groner et al., 1974), for the purposes of discussion this fraction is referred to as poly(A)+ mRNA. Figs. 2A and B show that, after a 10 min lag, poly(A)+ CYH2 mRNA decays with a half-life of 10 min. Poly(A)- CYH2 mRNA increases in relative abundance for the first 15-30 min (reaching a maximum value of 143% compared to t0) after a shift to 36°C and then decays with a half-life slightly longer than that of unfractionated CYH2 mRNA (t1/2=60 min; Figs. 2A, B). Similar changes in the relative abundance of the poly(A)+ and poly(A)- fractions were also observed for the stable PGK1 mRNA (A+ t1/2=7 min; A- t1/2=60 min; Figs. 2C, D). Assuming that the level of overall decay for the poly(A)+ species is negligible and that the poly(A)+ RNA quantitatively chases into the poly(A)- fraction, these results indicate that: i) steady-state levels of poly(A)+ and poly(A)- RNA species at t0 are comparable for these stable mRNAs, and ii) the poly(A) tails of these mRNAs are rapidly shortened to a poly(A)- or poly(A)-deficient state at a rate 3-4-fold faster than the decay rates measured when analyzing total RNA. Also, when total RNA is analyzed, the decay curves for the CYH2 and PGK1 mRNAs are not biphasic, an indication that the poly(A)+ and A- RNAs have identical decay rates (data not shown). Based on these observations, we conclude that the loss of the poly(A)+ tail from these stable mRNAs is not the rate-limiting step in their decay. However, this experiment does not preclude the possibility that the loss of the poly(A) tail is a prerequisite for subsequent steps in the decay of these mRNAs. These results are consistent with a generalized shortening of mRNA poly(A) tracts of steady-state length (30-50 adenylate residues; Phillips et al., 1979) to below the minimal size required for oligo(dT) binding (Groner et al., 1974). The half-life of poly(A)
shortening must be approximately 7-10 min since stable mRNAs (CYH2 and PGK1) ultimately disappear from the poly(A)+ fraction at that rate. The more appreciable lag in the disappearance of CYH2 mRNA suggests that all stable mRNAs may not lose their poly(A) tails at the same rate.

The unstable STE2 mRNA disappeared from both the poly(A)+ and poly(A)− fractions rapidly (Fig. 2E). After a brief lag, poly(A)+ STE2 mRNA decayed with a half-life of 3-5 min (Fig. 2E, F). Similar rapid decay kinetics were observed for the poly(A)+ form of the unstable mRNA encoded by HIS3 (data not shown). Poly(A)− STE2 mRNA showed an initial lag in its decay (approximately 10 min) and then also disappeared with a half-life of 3-5 min (Fig. 2F). It is not clear from these results that the loss of the poly(A) tract from unstable mRNAs is the rate-limiting step in their decay. The percentages of the STE2 poly(A)+ (55%) and poly(A)− (45%) RNA at t₀ are consistent with those expected if the poly(A) tail of STE2 was lost at the same 7-10 min half-life measured for poly(A) tail loss from the stable CYH2 and PGK1 mRNAs. Due to both technical limitations and the fact that the poly(A)+ and poly(A)− STE2 mRNAs decay at an identical rate makes it difficult to conclude whether the deadenylation event is a prerequisite for STE2 degradation. Experiments which demonstrate that the unstable STE2 and STE3 yeast mRNAs contain multiple determinants necessary for their rapid decay (Chapter III, this thesis; R. Parker and A. Jacobson, manuscript in preparation) suggest that the turnover pathway(s) for these mRNAs may be quite complex.
Validity of mRNA decay rates measured in rpb1-1 cells. To evaluate the validity of mRNA half-lives determined in temperature-shifted rpb1-1 cells, two types of control experiments were performed: (i-iii) those which measured mRNA decay rates by three additional, independent procedures and (iv) those which assessed whether mRNA decay rates were influenced significantly by the consequences of heat-shock.

(i) mRNA decay after inhibition of transcription with thiolutin. The antifungal agent thiolutin was previously shown to inhibit all three yeast RNA polymerases both in vivo and in vitro (Jimenez et al., 1973; Tipper, 1973). When used at 3 μg/ml, transcription is effectively inhibited, protein synthetic rates are minimally changed, polysomes decay with a t1/2 of 20 min, and cellular growth (measured as OD$_{650}$) abruptly ceases (Jimenez et al., 1973; D. Herrick, unpublished observations). Decay curves for total poly(A)$^+$ RNA in cells exposed to 0, 3 or 6 μg/ml of thiolutin at 30°C are shown in Fig. 3A. In this experiment cells were labeled with $^3$H-uridine (20 μC/ml) for 1 hr prior to thiolutin treatment and the relative amounts of labeled poly(A)$^+$ RNA were determined by hybridization of total cellular RNA to poly(U) filters. The decay curves for poly(A)$^+$ RNA in cells treated with either 3 or 6 μg/ml of thiolutin were essentially identical and, as in experiments with temperature-shifted rpb1-1 cells, the decay kinetics were complex. In thiolutin-treated cells the most rapidly decaying component of poly(A)$^+$ RNA had a half-life 4-5 min and the slowest decaying component had a half-life of 60-61 min (Fig. 3A; Table 2). The latter decay rate was somewhat slower than the comparable decay component in rpb1-1 cells (Fig. 1A) and was reflected in the decay rates of individual mRNAs (see below).
The relative amounts of individual mRNAs remaining at different times after treating cells with thiolutin were determined by northern blotting as described above (Figs. 3B and 3C). Tables 3 and 4 list the decay rates of mRNAs analyzed in cells treated with thiolutin at 30°C and 36°C, respectively. These data indicate that: a) the relative decay rates of the majority of the mRNAs studied in thiolutin-treated cells and in temperature-shifted rpbl-1 cells were consistent (e.g., in thiolutin-treated cells, the mRNA encoded by STE2 decayed rapidly, the mRNA encoded by ACT1 decayed very slowly, and the mRNA encoded by TCM1 had an intermediate decay rate); and b) mRNAs in thiolutin-treated cells decay faster at 36°C than at 30°C and these faster rates more closely approximate the values obtained by other methods.

(ii) 32PO4 pulse-chase analysis. Fig. 4A shows a decay curve for total cellular RNA in a pulse-chase experiment. In an ideal chase, the specific activity of stable RNA species should be inversely proportional to the increase in cell number during the chase. Since 90-95% of the 32P label at t0 is in rRNA and tRNA (data not shown), the effectiveness of the chase can be approximated from a comparison of the t1/2 for total RNA to the generation time of the cells. In the experiment shown in Fig. 4A the specific activity of total RNA decreased linearly with a half-life of 150 min and the cells had a doubling time of 165 min, suggesting that the chase was effective. As in other approaches, the poly(A)+ RNA population in a pulse-chase turned over with complex kinetics, but the fastest and slowest decay components differed from those seen in experiments in which transcription was inhibited (see above). In Fig. 4A the more rapidly decaying component had a t1/2 of 12 min and the more slowly decaying component had a t1/2 of 29 min.
Decay rates for 11 individual mRNAs were measured by hybridizing poly(A)$^+$ RNA isolated at different times of the chase to dots of cloned DNA (Fig. 4B). (The use of poly(A)$^+$ RNA was necessary because unfractionated RNA yielded excessively high non-specific hybridization to the DNA dots). Autoradiographic data from such experiments was quantitated by scanning densitometry and half-lives measured in this manner ranged from 11 to 26 min (Fig. 4C; Table 3). The mRNA with the fastest decay rate in pulse-chase experiments was that encoded by STE2 ($t_{1/2}=11.3$ min) and the mRNA with the longest decay rate was that encoded by CYH2 ($t_{1/2}=26.1$ min). For the most part, the relative decay rates observed in a pulse-chase were consistent with those observed in temperature-shifted rpb1-1 cells (Table 3). A notable exception, however, was the mRNA encoded by PGK1 which was as stable as the CYH2 mRNA in the temperature-shift experiments, but which decayed twice as fast as that mRNA in a pulse-chase. Since poly(A)$^+$ mRNA was analyzed in this experiment, we interpret the rapid decay of PGK1 mRNA (and other stable mRNAs) in a pulse-chase in terms of the more rapid shortening of its poly(A) tracts observed in Fig. 2. The slower decay rate of the CYH2 mRNA would be consistent with our conclusion (above) that not all stable mRNAs lose their poly(A) tails at the same rate. Because of possible complications from the use of $^{32}$PO$_4$ labeling and from the selection of poly(A)$^+$ RNA, $[^3]$H]-adenine pulse-chase experiments were undertaken to measure decay rates in unfractionated RNA. In these experiments, the mRNAs encoded by ACT1 and PGK1 were very stable, having half-lives of $>$60 min (data not shown).

(iii) Approach to steady-state labeling with $^{32}$PO$_4$. Cells grown in
phosphate-depleted YEPD medium were labeled with $^{32}\text{P}O_4$ (100 $\mu$Ci/ml) and total and poly(A)$^+$ RNAs were purified from culture aliquots after 5-70 min of labeling. RNA decay curves were generated by plotting $1-A/A^\infty$ versus the time of $^{32}\text{P}O_4$ labeling (where $A$ is the specific activity of an RNA at times $t$ and $A^\infty$ is the specific activity at time $\infty$; Greenberg, 1972; Kim and Warner, 1983). The decay kinetics for the poly(A)$^+$ RNA population were complex; extrapolations of the decay curve indicated that the fastest decaying component had a $t_1/2$ of 5-6 min and the slowest decaying component had a $t_1/2$ of 42 min (Fig. 5A; Table 2).

To measure half-lives of individual mRNAs, 1 $\mu$g of $^{32}$P-labeled poly(A)$^+$ RNA from each timepoint was hybridized to DNA dot blots (Fig. 5B) and the extent of hybridization of labeled RNA was monitored as above, i.e., by autoradiography and densitometry. The half-life values for 14 mRNAs measured by this approach are listed in Table 3 and representative decay curves are shown in Fig. 5C. The most stable mRNAs identified by this procedure were those encoded by cDNA #74 ($t_1/2=36.5$ min), ACT1 ($t_1/2=23.0$ min), and RP51A ($t_1/2=22.5$ min) and the least stable was that encoded by STE2 ($t_1/2=5.5$ min). With three exceptions, the relative decay rates measured by approach to steady-state labeling and those derived in rpb1-1 cells were consistent. These exceptions were the mRNAs encoded by PGK1 and CYH2, which were less stable in approach to steady-state labeling experiments than in the temperature-shift experiments, and the mRNA encoded by cDNA #74, which was more stable when measured by approach to steady-state labeling (Table 3). Rapid disappearance of the PGK1 mRNA from the poly(A)$^+$ fraction has been noted above (Figs. 2 and 4), but the reasons for the other two
discrepancies are unclear (see Discussion).

(iv) mRNA decay in heat-shocked, thiolutin-treated cells. In temperature-shifted rpb1-1 cells four mRNAs (those encoded by cDNA #90, RP29, TCM1, and PAB) decayed biphasically, with a break from rapid to slower decay occurring at 30 min after the temperature shift. Since a heat-shock induced destabilization of ribosomal protein mRNAs has been observed previously (Herruer et al., 1988), these results led us to consider the possibility that the heat-shock which occurs in temperature shift experiments could transiently accelerate normal mRNA decay rates. To address this possibility, half-lives of 6 different mRNAs (MFα1, STE3, RP29, TCM1, ACT1, and HIS3) were measured in cells of strain N222 (an isogenic, temperature-independent derivative of Y262) that were incubated in the presence of the transcriptional inhibitor thiolutin with or without a concomittant heat-shock to 36°C. The data of Fig. 6 and Table 4 demonstrate that, after a short lag, mRNA decay rates in cells heat-shocked in the presence of thiolutin are not significantly different from decay rates observed in either thiolutin-treated cells grown at 36°C or temperature-shifted rpb1-1 cells. The lag observed in the decay of the MFα1 mRNA is also seen with the STE3 mRNA (data not shown) and suggests that thiolutin inhibits mRNA turnover for a short period or thiolutin requires 10 min before it begins to act. We conclude, therefore, that mRNA half-life values determined in temperature-shifted rpb1-1 strains are not biased by the consequences of the heat-shock unrelated to the inactivation of RNA polymerase II.

Comparisons of stable and unstable mRNAs. Having identified mRNAs
which differ significantly in their respective decay rates, we compared these mRNAs with respect to properties which might, in part, play a role in determining stability or instability. Unless otherwise specified, mRNA decay rates considered in the experiments which follow are those determined in temperature-shifted rpb1-1 cells:

(i) **mRNA decay rate vs. mRNA size.** Previous experiments, in which yeast mRNA decay rates were measured in cells treated with the chelating agent 1,10-phenanthroline, suggested that an inverse correlation existed between the size of an mRNA and its stability (Santiago et al., 1986). Experiments with other organisms, however, failed to demonstrate such a correlation (Graves et al., 1987; Shapiro et al., 1988) and led to the suggestion (Shapiro et al., 1988) that the previously observed correlation may be a consequence of the measurement of mRNA decay rates in phenanthroline-treated cells. To address this discrepancy we have compared mRNA sizes to mRNA decay rates determined by each of the four procedures used here. The data of Fig. 7A-D indicate that no statistically significant correlation between mRNA size and stability was observed with any of the four procedures.

(ii) **mRNA decay rates in the presence of cycloheximide.** A large number of experiments in several different systems have demonstrated a reduction in mRNA decay rates in the presence of cycloheximide or other inhibitors of translational elongation (Stimac et al., 1984; Kelly et al., 1983; Fort et al., 1987; Efrat and Kaempfer, 1984; Raj and Pitha, 1981). The significance of such observations is still unclear. It is unlikely that arrested ribosomes simply "protect" mRNA from nucleases since, in normally growing cells, the ribosome packing density does not differ for stable and unstable mRNAs
Santiago et al., 1987; Shapiro et al., 1988). The possibility exists that essential nucleases are metabolically unstable or that mRNA degradation is dependent on ribosomal translocation through or up to specific mRNA regions (Brewer and Ross, 1989; Graves et al., 1987). Since we had identified mRNAs with large differences in their respective decay rates, we sought to evaluate whether inhibition of translational elongation had comparable effects on degradation pathways which were potentially different. The experiments summarized in Table 5 indicate that the decay rates of stable, moderately stable, and unstable mRNAs are all reduced significantly in the presence of cycloheximide.

(iii) A correlation between the percentage of rare codons and mRNA decay rates. Bennetzen and Hall (1982) observed that highly expressed genes in yeast are biased toward the use of only 25 of the 61 coding triplets. Similar observations have been made by Sharp et al. (1986). Such restricted codon usage correlates with the levels of the corresponding isoaccepting tRNAs in both yeast and E. coli, although the extent of codon bias is much higher in yeast (Ikemura, 1981; Ikemura, 1982). Hoekema et al. (1987) replaced 164 high frequency codons of the yeast PGK1 gene with rare codons and observed a concomitant tenfold reduction in PGK protein synthesis and a threefold reduction in PGK mRNA levels. Assuming comparable transcription rates of the two types of PGK mRNA, this result would suggest a possible relationship between the percentage of rare codons present in a given mRNA and its rate of decay. We have determined codon usage for 15 of the mRNAs studied here (Table 6) and compared the relative content of rare codons (frequency <15/1000) to mRNA decay rates (Fig. 8). Interestingly,
there is a statistically significant correlation between the extent of mRNA instability and the percentage of rare codons.

Are determinants of mRNA instability located within 3'-untranslated regions? Evidence has begun to accumulate which indicates that significant determinants of mRNA instability are located within the 3'-untranslated regions of certain mammalian mRNAs. Of particular note are the AU-rich regions of certain oncogene and lymphokine mRNAs (Shaw and Kamen, 1986; Fort et al., 1987; Jones and Cole, 1987; Rahmsdorf et al., 1987; Kabnick and Housman, 1988; Shyu et al., 1989) and the stem-loop structure of cell-cycle regulated histone mRNAs (Pandey and Marzluff, 1987). Since we have identified different sets of yeast mRNAs which are either stable or unstable, and since all of the mRNAs which we have studied are completely sequenced, we sought to determine whether a particular stability class of yeast mRNAs might also contain class-specific sequences or structures within the respective 3'-untranslated regions. Therefore, the Intelligenetics IFIND:ALIGN algorithms (which perform Wilbur and Lipman similarity searches) were used to compare the sequences of the 3'-untranslated regions of each of the unstable mRNAs to each other. Homologies which appeared in such searches were then compared to the sequences of the 3'-untranslated regions of the stable mRNAs. Likewise, the sequences of all of the respective mRNAs were analyzed for dyad symmetries. Untranslated regions of the respective mRNAs were as defined previously by published experiments or, in the absence of such data, arbitrarily set at 200 nucleotides 3' to the translational terminator. These searches failed to identify any sequence or structural elements which
were unique to the unstable mRNAs or to a subset of the unstable mRNAs. It should be noted, however, that these searches did confirm the existence of 3'-UTR homologies amongst most of the mRNAs which were analogous to those previously identified by Zaret and Sherman (1982) and which have been postulated to play a role in transcriptional termination.
A simple and reliable method for measuring mRNA decay rates in yeast. mRNA decay rates have been measured in yeast previously by several different functional or chemical assays (Hutchison et al., 1969; Koch and Friesen, 1979; Chia and McLaughlin, 1979; Tonnesen and Friesen, 1973; Santiago et al., 1986; Hynes and Phillips, 1976; Kim and Warner, 1983; Lawther and Cooper, 1973; Chevallier et al., 1982; Osley and Hereford, 1981; Zitomer et al., 1979; Losson et al., 1983 and Bach et al., 1979; Kuo et al., 1973). Previous approaches have measured mRNA decay rates by assaying changes in mRNA labeling kinetics (Hynes and Phillips, 1976; Osley and Hereford, 1981; Zitomer et al., 1979; Losson et al., 1983; Kim and Warner, 1983; Bach et al., 1979) or by quantitating the disappearance of mRNA after the inhibition of transcription (Tonnesen and Friesen, 1973; Kuo et al., 1973; Santiago et al., 1986) or RNA processing (Hutchison et al., 1969; Koch and Friesen, 1979; Chia and McLaughlin, 1979). Those experiments which assayed changes in labeling kinetics (pulse-chase and approach to steady-state labeling) employed labeling with $[^3H]$-nucleosides to monitor specific mRNAs and, therefore, precluded consideration of mRNAs with relatively low transcription rates. Moreover, the general experimental protocol in these studies involved the hybridization of labeled RNAs to cloned DNAs immobilized on filters; the latter were then quantitated by scintillation counting and were not readily recycled for future experiments. Most of the experiments which monitored the disappearance of specific mRNAs did so by inhibiting RNA accumulation in a non-specific manner: inhibition of transcription with daunomycin or ethidium bromide...
(Tonnesen and Friesen, 1979), lomofungin (Cano et al., 1973), or 1,10-phenanthroline (Santiago et al., 1986) is accompanied by inhibition of many other biosynthetic pathways (Chang and Yarbro, 1978; D'Aurora et al., 1978). Inhibition of RNA processing in ts 136 inhibits the accumulation of all classes of RNA, not just mRNA (Hutchison et al., 1969). In contrast to these shortcomings of previous methods, the temperature shift procedure used here (with rpb1-1 cells) affords several advantages: i) RNA polymerase II is selectively and rapidly inactivated; ii) there is no requirement for in vivo labeling or consideration of changes in pool sizes; iii) mRNAs of any abundance class can be readily detected and their decay rates measured; and iv) the use of northern blots allows the monitoring of mRNA integrity in parallel with the quantitation of mRNA decay rates, the detection of several mRNAs simultaneously, the reusability of blots for future experiments with different probes, and the quantitation of chimeric and parental mRNAs in the same experiment (Chapter III, this thesis; R. Parker and A. Jacobson, manuscript in preparation).

The reliability of decay rate measurements in temperature-shifted rpb1-1 cells has been addressed in several ways. We have shown that mRNA decay rates are not altered by the consequences of heat-shock (Fig. 6 and Table 4) and that the relative stabilities of most of the mRNAs examined here are consistent regardless of the method used to determine half-lives (Table 3). In spite of the shortcomings of previous procedures alluded to above, mRNA decay rates measured here are generally in good agreement with rates measured previously by others (see Table 3). The reliability of the decay rates measured by the temperature-shift procedure is also addressed by a
recent study in which we compared the decay rates and steady-state levels of 12 chimeric mRNAs to those of their parental mRNAs transcribed from the same promoter (R. Parker and A. Jacobson, manuscript in preparation). For 11 out of 12 chimeric mRNAs, there was good agreement between relative decay rates and relative steady-state mRNA levels. In the only case where the steady-state mRNA level is inconsistent with measured decay rates, the discrepancy is likely to be due an alteration in nuclear turnover or processing of the chimeric mRNA (R. Parker and A. Jacobson, manuscript in preparation).

Two other observations suggest that mRNA decay is proceeding normally in temperature-shifted rpb1-1 cells: i) cycloheximide drastically reduces mRNA decay rates (Table 5) and ii) 5'-proximal nonsense mutations (in the HIS4 mRNA) accelerate mRNA decay rates (R. Parker, unpublished observations). Both types of phenomena have been observed previously in yeast and other organisms (Stimac et al., 1984; Kelly et al., 1983; Fort et al., 1987; Efrat and Kaempfer, 1984; Raj and Pitha, 1981; Losson and Lacroute, 1979; Pelsy and Lacroute, 1984; Maquat et al., 1981; Daar and Maquat, 1988, Baumann et al., 1985).

With regard to the few inconsistencies that were observed between different procedures for determining mRNA decay rates, it is important to note the following: i) cells with thermally inactivated RNA polymerase II and cells treated with thiolutin have ceased to grow whereas cells used for pulse-chase and approach to steady-state labeling experiments are growing exponentially; ii) at t=0, the relative representation and poly(A) status of individual mRNAs in a pulse-labeled RNA sample (analyzed in the pulse-chase and approach to steady-state labeling procedures) will differ from that in the
steady-state mRNA population (analyzed in thiolutin-treated cells and in temperature-shifted rpb1-1 cells); and iii) the failure to detect a very rapid decay component in the mRNA population analyzed by a pulse-chase (Fig. 4), suggests that a completely effective chase was not obtained and that the decay kinetics of total cellular RNA (Fig. 4A) may not be a definitive indicator of chase efficiency. We have previously noted the difficulties of obtaining a completely effective chase with $^{32}\text{PO}_4$ in Dictyostelium (Shapiro et al., 1988; Manrow and Jacobson, 1988).

Identification of stable and unstable mRNAs. The half-lives of individual mRNAs examined in temperature-shifted rpb1-1 cells ranged from 2.5 to 45 min (Table 3). These decay rates are in good agreement with the complex decay kinetics observed for the total mRNA population (Figs. 1A, 3A, 4A, 5A) and indicate that the complexity of the decay curves for the mRNA population reflects the sum of the half-lives of individual mRNAs and that the collection of mRNAs analyzed here is a representative sample of the continuum of mRNA decay phenotypes in the cell. For simplicity, we have arbitrarily divided this continuum into three mRNA stability classes: unstable mRNAs ($t_{1/2}<7$ min), moderately stable mRNAs ($t_{1/2}=10-20$ min), and stable mRNAs ($t_{1/2}>25$ min). mRNAs which are more stable tend to be those involved in "housekeeping" functions (e.g., PGK1, ACT1, and CYH2) whereas mRNAs involved in a tightly regulated function (mating type control) tend to be unstable (e.g., STE2, STE3, FUS1, MATα1, MFα1, and MFα2). Clearly, the availability of a set of well-characterized genes encoding mRNAs representative of the different yeast mRNA decay phenotypes should facilitate
an analysis of the molecular basis of such differences.

What is the basis of the 10-20-fold difference in the decay rates of stable and unstable mRNAs? Possible determinants of mRNA stability can be subdivided into those which are specific and those which are non-specific. Specific determinants are mRNA sequences or structures which either interact directly with components of the cellular turnover machinery or target that machinery to other specific sites. Non-specific determinants are general mRNA features (such as the cap, poly(A) tail, or overall size) which could contribute to mRNA decay rates by promoting or hindering random interactions with non-specific nucleases.

The possibility that the polyadenylation status of an mRNA influences its decay rate has been supported (Marbaix et al., 1975; Nudel et al., 1976; Mercer and Wake, 1985; Brewer and Ross, 1988; Wilson and Triesman, 1988) and refuted (Sehgal et al., 1978; Medford et al., 1980; Krowczynska et al., 1985) by numerous studies in several organisms. An earlier study in yeast (Santiago et al., 1987) found no correlation between mRNA decay rates and poly(A) lengths, an observation supported by the work described here. We find that: i) poly(A)-deficient CYH2 and PGK1 mRNAs are extremely stable (Fig. 2), ii) rapid turnover of the STE2 mRNA is not clearly linked to its deadenylation (Fig. 2), and iii) the decay curves of mRNAs with half-lives longer than the 10-12 min half-life for poly(A) shortening are linear, not biphasic (e.g., Fig. 1C and 1E). From this, and previous data (Palatnik et al., 1980; Shapiro et al., 1988), we conclude that poly(A) lengths do not determine mRNA decay rates and that, at least for the mRNAs examined here,
deadenylation is not the rate-limiting event in mRNA degradation.

Santiago et al. (1986) have suggested that an inverse relationship exists between the size and stability of yeast mRNAs. Half-lives in their study were measured following the inhibition of transcription using high concentrations of the drug, 1,10-phenanthroline. This drug affects a number of cellular pathways (Chang et al., 1978; Johnston and Singer, 1978; Krishnamurti et al., 1980) and may exert its effects by chelating cellular zinc. If the nucleases involved in mRNA degradation include those which are zinc metalloenzymes, they would presumably be inhibited by this drug. To address the possibility that a size/stability correlation is apparent under certain physiological conditions, but not others, we compared mRNA sizes to decay rates obtained by each of the four procedures used here (Fig. 7). Our results do not support a possible relationship between mRNA size and stability. This conclusion is supported by our previous study with Dictyostelium mRNAs (Shapiro et al., 1988) and by recent experiments with chimeric yeast mRNAs (R. Parker and A. Jacobson, manuscript in preparation). In the latter study, large deletions, which caused 2- to 3-fold differences in mRNA lengths, had no affect on mRNA decay rates.

Bennetzen and Hall (1982) observed that highly expressed genes in yeast are biased toward the use of only 25 of the 61 coding triplets. The general trends of this correlation have withstood the analysis of a much larger number of yeast genes (Aota et al., 1988) and are consistent with the levels of the corresponding isoaccepting tRNAs (Ikemura, 1982). The possibility that codon bias (i.e., the percentage of rare codons) influences mRNA decay rates in yeast is suggested by experiments in which a 3-fold reduction in the
steady-state levels of PGK1 mRNA was elicited by a systematic replacement of frequent codons with rare codons (Hoekema et al., 1987). An analysis of the mRNAs examined in this study reveals a correlation between mRNA instability and content of rare codons (Fig. 8 and Table 6). This correlation may reveal an underlying mechanism for regulating mRNA decay rates or may simply reflect the fact that some highly expressed genes have independently evolved high rates of transcription and translation and slow rates of mRNA decay. A role for rare codons may be related to sequence context. When clustered together, rare codons reduce translational elongation rates (Robinson et al., 1984; Pedersen, 1984; Sorensen et al., 1989) to the point that a "paused" ribosome triggers a nucleolytic event. In this regard, it is interesting to note that structural determinants of mRNA stability in the MATα1 and HIS3 genes overlap with regions containing clusters of rare codons (R. Parker and A. Jacobson, manuscript in preparation; Chapter III, this thesis).

Experiments in mammalian cells have demonstrated that sequences within mRNA 3'-UT regions can dictate rapid decay rates (Shaw and Kamen, 1986; Fort et al., 1987; Jones and Cole, 1987; Rahmsdorf et al., 1987; Kabnick and Housman, 1988; Shyu et al., 1989; Pandey and Marzluff, 1987). To assess whether yeast mRNAs of a common stability phenotype share a common 3' UT sequence element (such as the mammalian ARE; Caput et al., 1986; Shaw and Kamen, 1986), computer algorithms were used to search for relevant homologies. No significant class-specific homologies were detected, a result consistent with recent experiments from this lab which show that replacement of the 3'-UTRs of the stable ACT1 and PGK1 mRNAs with those of the HIS3, STE3, and MATα1 mRNAs is not sufficient to increase the decay rates of the
stable mRNAs (Chapter III, this thesis; R. Parker and A. Jacobson, manuscripts in preparation). Of interest, in the same studies, are experiments which demonstrate that sequences which dictate rapid decay can be localized to the coding regions of all three unstable mRNAs (see Chapter III).
Table 1
Cloned DNAs Used to Evaluate mRNA Decay Rates

<table>
<thead>
<tr>
<th>Plasmid/Phage</th>
<th>Gene</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDH6</td>
<td>ACT1 (actin)</td>
<td>3.8 kb EcoRI fragment in pUC9</td>
<td>Ng and Abelson, 1980; Gallwitz and Sures, 1980</td>
</tr>
<tr>
<td>pDH8</td>
<td>PGK1 (phosphoglycerate kinase)</td>
<td>2.95 kb HindIII fragment in pUC9</td>
<td>Mellor et al., 1983</td>
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<tr>
<td>pAB518</td>
<td>STE2 (α-factor receptor)</td>
<td>3.2 kb SalI-BamHI fragment in pRC3</td>
<td>Burkholder and Hartwell, 1985; Nakayama et al., 1985</td>
</tr>
<tr>
<td>pYPA</td>
<td>PAB (poly(A)-binding protein)</td>
<td>3.4 kb EcoRI-HindIII fragment in pUC9</td>
<td>Sachs et al., 1986</td>
</tr>
<tr>
<td>pDH1</td>
<td>HTB1 (histone H2B1)</td>
<td>195 bp PvuII-HindIII fragment in pUC9</td>
<td>Wallis et al., 1980</td>
</tr>
<tr>
<td>pDH2</td>
<td>HTB2 (histone H2B2)</td>
<td>195 bp HindIII-PvuII fragment in pUC9</td>
<td>Wallis et al., 1980</td>
</tr>
<tr>
<td>22, 39, 74, 90</td>
<td>cDNA clones encoding unknown genes</td>
<td>C-tailed inserts of 900 bp average length in G-tailed pBR322</td>
<td>Santiago et al., 1986</td>
</tr>
<tr>
<td>TCM</td>
<td>TCM1 (encodes ribosomal protein L3; trichodermin-resistance)</td>
<td>0.8 kb SalI-HpaI 3' fragment in M13mp9</td>
<td>Schultz and Friesen, 1983</td>
</tr>
<tr>
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<td>CYH2 (encodes ribosomal protein L29; cycloheximide-resistance)</td>
<td>506 bp AccI-EcoRI fragment in M13mp9</td>
<td>Kaufer et al., 1983</td>
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<td>Gene</td>
<td>Fragment</td>
<td>Length</td>
<td>Enzymes</td>
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<td>---------</td>
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<td>HincII 3'-exon</td>
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<td>EcoRI-HinDIII</td>
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</tr>
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<td>STE3 (α-factor receptor)</td>
<td>2.0 kb</td>
<td>HinDIII fragment</td>
</tr>
<tr>
<td>MATα1</td>
<td>MATα1 (matting type regulator)</td>
<td>545 bp</td>
<td>EcoRV-ScaI fragment</td>
</tr>
<tr>
<td>FUS1</td>
<td>FUS1 (required for fusion during mating)</td>
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<td></td>
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<td>fragment</td>
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</table>
Table 2

Fast and Slow Components of Population Decay Curves

<table>
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<th>Experiment</th>
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<tr>
<td>Ts polymerase</td>
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<tr>
<td>Pulse-chase</td>
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<tr>
<td>Approach to steady-state</td>
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<td>42</td>
</tr>
<tr>
<td>Thiolutin</td>
<td>4-5</td>
<td>60-61</td>
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</table>

Half-lives were derived by extrapolation from the decay curves of total poly(A)$^+$ RNAs shown in Figs. 1, 3, 4, and 5. Half-lives of the fast components were corrected for the contribution of the slow components.
<table>
<thead>
<tr>
<th>mRNA</th>
<th>ts</th>
<th>Pol II</th>
<th>Thiolutin</th>
<th>Pulse-Chase</th>
<th>Steady-State</th>
<th>Previous Studies</th>
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<td>CYH2</td>
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<td>#74</td>
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</table>
Individual mRNA half-lives were measured with the four techniques described in the text. References for data from previous studies: 1-Santiago et al., 1986; 2-Kim and Warner, 1983; 3-Osley and Hereford, 1981; and 4-Bach et al., 1979.

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<tr>
<td>PHOS</td>
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<td>9.8</td>
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<td>-</td>
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<td>19.0</td>
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Table 4

Effect of heat-shock on mRNA decay rates

<table>
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<th>+HEAT SHOCK</th>
<th>-HEAT SHOCK</th>
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<td>STE3</td>
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<tr>
<td>TCM1</td>
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<tr>
<td>RP29</td>
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<td>12.5</td>
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</table>

mRNA decay rates were determined for either heat-shocked or non-heated-shocked cultures of N222 following inhibition of transcription with thiolutin (see Materials and Methods for details).
Table 5
Cycloheximide inhibits mRNA decay

<table>
<thead>
<tr>
<th>mRNA</th>
<th>T1/2 (min) -CYH</th>
<th>T1/2 (min) +CYH</th>
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<tbody>
<tr>
<td>ACT1</td>
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<td>&gt;80</td>
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<tr>
<td>PGK1</td>
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<tr>
<td>RP29</td>
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<td>&gt;40</td>
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<tr>
<td>TCM1</td>
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<td>&gt;40</td>
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<tr>
<td>STE3</td>
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<td>MAT@1</td>
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<tr>
<td>HIS3</td>
<td>8</td>
<td>&gt;80</td>
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</table>

The effect of cycloheximide on mRNA decay was determined as follows: a single 250 ml culture of RY262 was grown to mid-log (OD600 = 0.5) in YEPD at 24°C. At t=0, 125 ml aliquots were added to 125 mls of 48°C YEPD, one of which (+CYH) contained cycloheximide at 100 µg/ml. RNA was prepared at various times (0-60 min) and the decay rates of individual mRNAs were quantitated by northern blotting and densitometry.
Table 6

Percentage of rare codons in yeast mRNAs

<table>
<thead>
<tr>
<th>mRNA</th>
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<tr>
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<td>RP29</td>
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<td>HIS3</td>
<td>42.7</td>
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<td>HTB1</td>
<td>9.8</td>
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<tr>
<td>FUS1</td>
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<td>TCM1</td>
<td>14.2</td>
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<td>MATα1</td>
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<td>ACT1</td>
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<td>PGK1</td>
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<td>MFA1</td>
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<td>PAB</td>
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<tr>
<td>CYH2</td>
<td>11.3</td>
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</table>

The percentage of rare codons was calculated as described in Materials and Methods.
FIG. 1. mRNA decay following the inhibition of transcription in a temperature-sensitive RNA polymerase II mutant. (A) To measure decay of the poly(A)+ RNA population, S. cerevisiae strain Y260 was grown in YEPD medium at 24°C to a density of 5X10⁶ cells/ml and labeled for one generation (3.5 h) with 20 μCi/ml of carrier-free ³²P-phosphoric acid. At t=0 an equal volume of YEPD medium preheated to 48°C was added to the culture and shaking was continued at 36°C. Aliquots of the culture (100 ml) were removed 0, 10, 20, 30, 40, 60, and 80 min after the temperature shift and total RNA was extracted. The amount of ³²P incorporated into poly(A)+ RNA was measured by hybridization to poly(U) filters (closed circles). Data are plotted as the percentage of RNA remaining, as compared to t=0, versus minutes at 36°C. Dotted lines indicate the major kinetic components (the rapid decay component (open boxes) has been corrected for the contribution of the slow decay component). (B) To measure the decay of individual mRNAs, the same procedure was followed except that the isotopic label was omitted. RNA (3 μg) from each timepoint was denatured, applied in duplicate dots to Zeta-Probe filters, and probed with 5X10⁶ cpm of ³²P-labeled insert DNA from pDH7 (ACT1), pAB515 (STE2), or cDNA clone #74 (gene unknown). The filters were exposed to X-ray film and appropriate exposures of the resulting autoradiographs were scanned with a densitometer. The figure shows autoradiographs of the dots (different exposures were used for ACT1, STE2, and #74). (C) The figure shows a graph of the decay rates determined from the dot blots shown in Fig. 1B. Symbols are as follows: ACT1 (closed
circles), #74 (open boxes), and STE2 (open circles). (D) Northern blot of mRNA decay in a temperature shift. As in Fig. 1B except that individual mRNAs were assayed by northern blotting. (E) The figure shows a graph of the decay rates determined in the northern blot of Fig. 1D.

**FIG. 2. Decay of poly(A)+ vs. poly(A)- mRNAs.** Poly(A)+ and poly(A)- RNA was purified (using an oligo(dT)-cellulose batch procedure) from 100 µg of total RNA isolated from strain Y260 after 0, 5, 10, 15, 20, 30, 40, and 60 min at 36°C. Ten percent of the total A+ and A- RNA recovered from each timepoint was fractionated on 1% agarose-2.2M formaldehyde gels, transferred to Zeta-Probe membranes, and probed simultaneously with a 2.95 kb HindIII PGK1 fragment and a 0.52 kb EcoRI-HindIII CYH2 random prime labeled fragment. Autoradiographs of the northern blots containing the poly(A)+ and poly(A)- RNAs are shown in Fig. 2A and 2C. Quantitation of the decay rates for CYH2 and PGK1 poly(A)+ (open boxes) and poly(A)- (closed diamonds) mRNAs is shown in Fig. 2B and 2D, respectively. The percentage of CYH2 and PGK1 RNA which is poly(A)+ at t0 is 42 and 43.2 percent, respectively. The decay rate of STE2 poly(A)+ and (A)- RNAs is shown in the northern blot of Fig. 2E, which has 20% of the total A+ and A- RNAs recovered per lane and was probed with a 2.2 kb random prime labeled STE2 SalI fragment. F. Quantitation of the blot in panel E. Symbols are: STE2 poly(A)+ RNA (open boxes), STE2 poly(A)- RNA (closed diamonds), and STE2 poly(A)+ RNA with the 10 min timepoint as t0 (closed boxes, dashed line). STE2 poly(A)+ RNA is 55.3% of the total at t0.
FIG. 3. Decay of poly(A)+ and individual mRNAs following exposure to the anti-fungal agent, thiolutin. A) To measure the decay of the poly(A)+ RNA population, cells (strain DBY747) were grown to 5X10⁶ cells/ml in YEPD medium at 30°C and were labeled with 20 µCi/ml of [³H]-uridine for 1 hr. Thiolutin was then added at either 0, 3, or 6 µg/ml and total RNA was extracted from 10 ml culture aliquots removed after 0, 10, 20, 30, 40, and 60 min. The amount of [³H]-uridine incorporated into poly(A)+ RNA was measured by hybridizing an equal mass of total RNA from each time point to poly(U) filters. The decay curves for the poly(A)+ RNAs shown in Fig. 3A were generated by plotting the percent of radioactivity remaining versus min of exposure to thiolutin. Symbols are as follows: open stars-no thiolutin; closed circles-3 µg/ml; open circles-6 µg/ml. Dotted lines indicate the major kinetic components [the rapid decay component (open boxes) has been corrected for the contribution of the slow decay component]. B) To measure the decay of individual mRNAs, DBY747 cells grown to 5X10⁶ cells/ml at 30°C in YEPD medium were exposed to 3 µg/ml of thiolutin. Aliquots (40 ml) were removed after 0, 10, 20, 40, 60, 80, and 100 min and total RNA was extracted. RNA (4 µg) from each timepoint was fractionated on 1% agarose-2.2M formaldehyde gels. Following transfer to Zeta-Probe paper, each filter was probed with nick-translated plasmid DNA encoding a unique yeast gene. Autoradiographs for the northern blots probed with TCM1, STE2, and ACT1 are shown. C) Autoradiographs were scanned and the area under each peak was calculated. Half-lives were determined from the slope of a plot of the percent of time zero versus minutes of thiolutin exposure. Decay curves for the TCM1, STE2, and ACT1 mRNAs are shown.
FIG. 4. Decay of total, poly(A)$^+$, and individual RNAs during a $^{32}$PO$_4$ pulse-chase. A) Cells (DBY747) were grown in YEPD medium minus phosphate (Rubin, 1974) at 30°C to a density of 4-6X10$^6$ cells/ml. The cells were then pulsed with 100 μCi/ml of $^{32}$PO$_4$ for 15 min, pelleted and washed once in YEPD medium supplemented with 100mM cold NaPO$_4$, and resuspended in this medium to initiate the chase. Aliquots (75-100 ml) of the culture were removed at 10, 20, 40, 60, 80, and 100 min, pelleted, and frozen immediately in dry ice. Total RNA from each timepoint was extracted, poly(A)$^+$ RNA was purified (using an oligo(dT) batch method), and specific activities were calculated. The decay curves for the total (open circles) and poly(A)$^+$ (closed circles) RNA populations were generated by plotting the percent specific activity of the t=0 sample vs. the time of chase. Dotted lines indicate the major kinetic components [the rapid decay component (open boxes) has been corrected for the contribution of the slow decay component].

B) An equal mass of $^{32}$P-poly(A)$^+$ RNA from each timepoint was used to probe identical filters containing dots of excess plasmid DNA which encode single mRNAs. The figure shows autoradiographs of representative dots for the individual PGK1, ACT1, CYH2, and STE2 mRNAs. C) Film exposures in the linear range were scanned and the area under the peaks was calculated. Data are plotted as the percent of RNA remaining at time zero versus time of the chase. Decay curves for the PGK1, ACT1, CYH2, and STE2 mRNAs are shown.
FIG. 5. Decay of individual mRNAs and total poly(A)$^+$ mRNA using a $^{32}$PO$_4$ approach to steady-state labeling procedure. Cells of strain DBY747 were grown in YEPD minus phosphate to $4.2 \times 10^6$ cells/ml. $^{32}$PO$_4$ was added at 100 $\mu$Ci/ml and aliquots of cells were removed after 5, 10, 15, 20, 30, 40, 50, and 70 min of labeling. Total RNA was extracted and the specific activity was calculated. Following a 5 min lag, the increase in specific activity vs. time was linear, indicating that $^{32}$PO$_4$ pools were of constant specific activity. Poly(A)$^+$ RNA was purified using the oligo(dT) batch procedure and specific activities determined for each timepoint. A) A decay curve for total poly(A)$^+$ RNA (closed circles) was generated by plotting $1/A/A_\infty$ versus the length of labeling ($A$ is the specific activity of a given RNA at time $T$; $A_\infty$ was the specific activity after 70 min of labeling). Dotted lines indicate the major kinetic components [the rapid decay component (open boxes) has been corrected for the contribution of the slow decay component]. B) An equal mass of poly(A)$^+$ RNA from each timepoint (1 $\mu$g) was used to probe excess plasmid DNA dots containing unique yeast genes. The figure shows the autoradiographs from individual dots for the PGK1, RP29, TCM1, CYH2, PAB, STE2, and ACT1 genes. C) Autoradiographs were scanned and the areas under the peaks from duplicate DNA dots were calculated. The decay curves shown were generated as described above except that $A_\infty$ was the value from the 30 min timepoint.

FIG. 6. Decay of the MFa1 mRNA with or without heat-shock. The figure shows the decay of the MFa1 mRNA under three conditions: inhibition of transcription in the temperature-sensitive RNA polymerase II mutant (filled
squares), inhibition of transcription with thiolutin in the \((\text{RPB1})\) wild-type strain, N222, at \(36^\circ C\) (filled circles), and inhibition of transcription by thiolutin accompanied by a heat-shock from \(24^\circ C\) to \(36^\circ C\) in N222 (open triangles; see Materials and Methods). The initial lag seen in the presence of thiolutin is reproducible and is seen with both the \(\text{MF}\alpha 1\) and \(\text{STE3}\) mRNAs.

**FIG. 7. mRNA size vs. decay rate.** mRNA sizes, determined by northern blotting, are compared to mRNA decay rates determined by each of four different procedures. Simple regression analysis was used to generate the following statistically insignificant \(R\) values (\(P=0.01\)) for each set of data: pulse-chase-\(R=0.32\); steady-state-\(R=0.10\); thiolutin-\(R=0.44\); and t.s. Pol II-\(R=0.14\). Sizes (in nt) for the mRNAs shown in this figure are: \(\text{URA3}\) (700), \(\text{RP29}\) (620), \(\text{RP51A}\) (580), \(\text{HIS3}\) (830), \(\text{HTB1}\) (610), cDNA \#74 (1050), \(\text{FUS1}\) (1800), cDNA \#90 (1100), \(\text{TCM1}\) (1170), \(\text{MAT}\alpha 1\) (740), \(\text{ACT1}\) (1250), \(\text{MF}\alpha 1\) (800), \(\text{PGK1}\) (1500), \(\text{MFA1}\) (450), \(\text{STE2}\) (1700), \(\text{STE3}\) (1800), \(\text{PAB}\) (2100), \(\text{MFA2}\) (450), \(\text{DED1}\) (2300), \(\text{CYH2}\) (600), cDNA \#39 (380), \(\text{PHO5}\) (620), and \(\text{HTB2}\) (800 and 680).

**FIG. 8. Relative content of rare codons in stable and unstable mRNAs.** Sequences of all mRNAs for which half-lives were measured in temperature-shifted \(\text{rpb1-1}\) cells were scanned for the presence of rare codons, i.e., codons whose frequency of occurrence in a large sample of sequenced yeast genes (Aota et al., 1988) is \(<15/1000\). Percentages of rare codons were calculated for all mRNAs and plotted vs. mRNA decay rate. A simple
regression analysis (P=0.01) generated a significant R value of 0.60.
Figure 1A.

ts Pol II: POLY(A)+ mRNA

[Graph showing the decay of POLY(A)+ mRNA over time at 36°C.]
Figure 1B and 1C.

B

ACT1

STE2

74

0 10 20 30 40 60 80

MINUTES AT 36°C

C

PERCENT OF t₀

MINUTES AT 36°C
Figure 1D and 1E.

D

MINUTES AT 36°C

0 10 20 30 40 50 60 80

STE3

TCM1

RP29

E

% of T0

100

10

1

0 2 4 6

MINUTES AT 36°C
Figure 2A, 2B, 2C, and 2D.

A. POLY(A)+ mRNA

PGK1

CYH2

0 5 10 15 20 30 40 60
MINUTES AT 36°C

B. CYH2 POLY(A)+ and A- mRNA

MINUTES AT 36°C

C. POLY(A)- mRNA

PGK1

CYH2

0 5 10 15 20 30 40 60
MINUTES AT 36°C

D. PGK1 POLY(A)+ and A- mRNA

MINUTES AT 36°C
Figure 2E and 2F.

E

STE2 PROBE

POLY(A)+

POLY(A)-

0 5 10 15 20 30 40 60

MINUTES AT 36° C

F

PERCENT OF TIME 0

MINUTES AT 36° C
Figure 3A.

THIOLUTIN: POLY(A)+ mRNA

PERCENT OF TIME 0

MINUTES OF THIOLUTIN
Figure 3B and 3C.

B

TCM1

STE2

ACT1

MINUTES OF THIOLUTIN EXPOSURE

C

TCM1

STE2

ACT1

MINUTES OF THIOLUTIN EXPOSURE
Figure 4A.

PULSE-CHASE: TOTAL and POLY(A)+ RNA

PERCENT OF TIME 0

MINUTES OF CHASE
Figure 4B and 4C.

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LENGTH OF CHASE (MINUTES)

C

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<tr>
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LENGTH OF CHASE (MINUTES)
Figure 5A.

STEADY-STATE: POLY(A)+ mRNA

MINUTES OF 32P-LABELING

1 - A/A∞
Figure 5B and 5C.

### B

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<thead>
<tr>
<th>Gene</th>
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MINUTES OF $^{32}$P-LABELING

### C

- **RP29**: $1/A_0$ against minutes of $^{32}$P-labeling.
- **TCM1**: $1/A_0$ against minutes of $^{32}$P-labeling.
- **CYH2**: $1/A_0$ against minutes of $^{32}$P-labeling.
- **PAB**: $1/A_0$ against minutes of $^{32}$P-labeling.
- **STE2**: $1/A_0$ against minutes of $^{32}$P-labeling.
- **ACT1**: $1/A_0$ against minutes of $^{32}$P-labeling.

MINUTES OF $^{32}$P-LABELING
Figure 6.

PERCENT OF TIME 0

MINUTES AT 36°C
Figure 7.

mRNA Size vs. Half-Life

A. PULSE-CHASE

B. STEADY-STATE

C. THIOLUTIN

D. t.s. Pol II

mRNA Half-Life (min) vs. mRNA Length (nt)
Figure 8.

% RARE CODONS vs. HALF-LIFE

% RARE CODONS

HALF-LIFE
Chapter III

Structural Determinants of mRNA Stability in Yeast

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SUMMARY

In a previous study, decay rates of individual yeast mRNAs were measured following the inhibition of transcription in a temperature-sensitive RNA polymerase II mutant (Chapter II, this thesis). In this study, the HIS3 mRNA was shown to decay rapidly, with a half-life of 7 min, whereas the ACT1 mRNA was 4-fold more stable, decaying with a half-life of 30 min. To attempt to define the determinants responsible for the observed decay rates, chimeric genes were constructed which encoded various regions of these two mRNAs. The hybrid genes were transformed into yeast on centromere-containing vectors and the half-lives of the chimeric mRNAs transcribed from these genes were measured.

To examine whether the 3'-untranslated region (UTR) of HIS3 can confer instability to the ACT1 mRNA, a chimeric gene was constructed in which DNA encoding the 3'-UTR of ACT1 was replaced with the corresponding region of HIS3. Two similar constructs were made. In the first, ACT1 was fused to HIS3 such that the reading frame was maintained, while the second construct contained a frame-shift resulting in an mRNA with no translational termination codon. The hybrid mRNA containing the HIS3 3'-end fused in-frame to the 5'-end of ACT1 decays at a rate similar to the endogenous ACT1 mRNA in these cells. This decay rate is approximately 2-fold slower than that measured for the endogenous ACT1 mRNA in the untransformed Y260 strain. Interestingly, the nearly identical mRNA lacking a stop codon decays as rapidly as the HIS3 mRNA. mRNA from the reciprocal in-frame construct (HIS3-5'-ACT1-3') was unstable, suggesting that HIS3 instability
determinants are located within the \texttt{HIS3} 5'-UTR or coding sequence. A 411 nucleotide (nt) deletion within the coding region of either \texttt{HIS3} or \texttt{HIS3-ACT1} chimeric genes resulted in a 3-fold stabilization of the respective encoded mRNAs. Insertion of this 411 nt fragment in-frame into the entire \texttt{ACT1} gene had no destabilizing effect on the hybrid mRNA, although the \texttt{HIS3} mRNA was stabilized three-fold in this transformant. We conclude that the instability of \texttt{HIS3} mRNA is not dictated by a single, transferable element (sequence or structure), but may involve interactions between multiple determinants within the mRNA. Similar experimental approaches lead us to draw the same conclusion about the unstable mRNA encoded by the yeast \texttt{STE2} gene.
INTRODUCTION

Individual mRNAs can have large differences in their respective decay rates, yet the sequence or structural determinants which control mRNA turnover are mostly unknown (Brawerman, 1987; Ross, 1989). In order to identify determinants of mRNA stability in the budding yeast, Saccharomyces cerevisiae, we have measured the decay rates of 20 mRNAs following the inhibition of transcription in a temperature-sensitive mutant of RNA polymerase II (Chapter II, this thesis). This screening has allowed us to conclude the following: i) the half-lives of individual yeast mRNAs can differ at least 16-fold; ii) there is no correlation between the length of a mRNA and its decay rate; iii) there is a correlation between the decay rate of an mRNA and its relative content of rare codons; and iv) the poly(A) tails of stable mRNAs decay 3- to 4-fold more rapidly than the remainder of the mRNA. In agreement with our studies of stable and unstable mRNAs in Dictyostelium discoideum (Shapiro et al., 1988), there is also no correlation between the stability of yeast mRNAs and either poly(A) tail lengths or the efficiency of ribosome loading (Santiago et al., 1987).

Given, therefore, that non-specific determinants do not regulate mRNA stability, we have initiated a search for specific cis-acting sequences or structures which may be responsible for the rapid decay of specific unstable mRNAs. A large body of literature from mammalian systems implicates the involvement of the 3'-untranslated region (UTR) in the rapid decay of mRNAs
encoded by a class of transiently expressed proto-oncogene, cytokine, and lymphokine genes (Caput et al., 1986; Meijlink et al., 1985; Shaw and Kamen, 1986). These regions, specifically the AU-rich sequences within them, are transferable and dominant, since these 3'-UTRs can confer instability when fused to normally stable mRNAs (Shaw and Kamen, 1986; Jones and Cole, 1987; Kabnick and Housman, 1988; Shyu et al., 1989). Moreover, stem-loop structures in the 3'-UTR of histone (Pandey and Marzluff, 1987) and transferrin receptor mRNAs (Casey et al., 1988; Mullner and Kuhn, 1988) have also been identified as the initial targets of degradation. These observations led us to investigate whether the 3'-UTR, or other regions, from an unstable yeast mRNA (HIS3) could confer instability to the stable ACT1 mRNA. Our results show that the 3'-UTR of HIS3 mRNA is not sufficient to dictate rapid decay of hybrid mRNAs and that a 411 nt region of the HIS3 coding sequence is necessary, but not sufficient, for HIS3 mRNA instability. Related experiments with the unstable mRNA encoded by the STE2 gene demonstrate that rapid decay of this mRNA is attributable to at least two separable structural determinants, at least one of which is located in coding sequences.
MATERIALS and METHODS

Yeast strains. Strain Y260 (MATa, ura3-52, and rpb1-1; Nonet et al., 1987) was kindly provided by Richard Young (M.I.T.).

Yeast transformation. Transformation of strain Y260 was performed using the lithium acetate procedure (Ito et al., 1983).

Culture media. Medium and plates lacking uracil were used to select transformants carrying derivatives of the plasmid pSE360H. This medium consists of yeast nitrogen base medium (without amino acids; Difco Laboratories) supplemented with ammonium sulfate (1mg/ml) and glucose (2%) as sources of nitrogen and carbon, respectively. Minimal medium was also supplemented with adenine (20 μg/ml) and amino acids (40μg/ml), as needed.

Manipulation of DNA. With the exception of the STET mini-prep procedure (Holmes and Quigly, 1981), all procedures involving DNA have been described previously (Chapter II, this thesis).

Plasmids. The source of the yeast ACT1 gene was a 3.8 kb EcoRI fragment from the plasmid DIA1 (obtained from C. Pikielny). This EcoRI fragment was cloned into the EcoRI site of pUC9, creating plasmid pDH6. The source of the HIS3 gene was the plasmid pUC8-Sc2676 (obtained from K. Struhl) which
contains a 1.75 kb BamHI fragment containing the entire HIS3 gene cloned into pUC8. The source of the STE2 gene was the plasmid pAB518 (obtained from D. Jenness) which contains the entire STE2 gene on a 3.2 kb Sall-BamHI fragment. The yeast centromere-containing vectors used in all constructions were either YCp50 (Parent et al., 1985) or pSE360 (Elledge and Davis, 1988; obtained from Stephen Elledge). The latter is a 5.9 kbp plasmid containing the multiple cloning site (MCS) from pUC18 within the lacZ gene. This plasmid contains the CEN4, ARS1, and URA3 yeast sequences. The plasmid pSE360H was made by R. Parker by filling in the HinDIII site at the URA3 junction, making the HinDIII site within the MCS unique.

**Construction of chimeric genes.**

1. **ACT1-5’-STE2-3’**

   The ACT1 5’-fragment was obtained by digesting plasmid DIA1 to completion with EcoRI, followed by gel purification of the resulting 3.8 kb fragment. This fragment was then cut to completion with BamHI and ScaI to generate the 1,796 bp ACT1 5’-fragment. This fragment contains the ACT1 promoter and the ACT1 coding region minus the 39 COOH-terminal amino acids. The STE2 3’-fragment was obtained by complete digestion of plasmid pAB518 with Sall and BamHI, followed by gel purification of the 0.98 kb STE2 fragment. This was then cut to completion with PvuII and HinDIII to generate the 246 bp STE2 3’-fragment which contains the 15 COOH-terminal amino acids and 3’-RNA processing signals from STE2. The ACT1 5’- and STE2 3’-fragments were ligated into pUC9 cut with BamHI and HinDIII in a three-piece construction. The hybrid gene insert is 2,042 bp and the ACT1-
STE2 junction is in-frame. The 2,052 bp hybrid gene fragment was obtained by complete digestion with EcoRI (site within pUC9 polylinker) and partial digestion with HindIII (internal HindIII site in ACT1 sequence). This fragment was ligated into the EcoRI and HindIII sites of YCp50. This vector contains the URA3 gene, ARS1, CEN4, and the hybrid gene transcribed in the direction opposite that of the β-lactamase gene.

2. STE2-A

An 82% deletion of STE2 coding sequences was constructed by deleting an internal 1,059 bp PvuII fragment. In a three piece ligation, the STE2 promoter and 61 NH2-terminal amino acids present on the 1,032 bp SalI-PvuII fragment was ligated with the PvuII-HindIII STE2 3'-fragment and the episomal vector YEp352 cut with SalI and HindIII. The YEp352 vector (Hill et al., 1986; kindly provided by John Hill) contains the URA3 gene as a selectable marker and 2μ circle sequences necessary for replication. Similar 2μ based vectors give rise to 20-100 copies per cell (Clark-Walker and Miklos, 1974; Gerbaud et al., 1979; Chevallier et al., 1980).

3. ACT1-5'-HIS3-3' with or without a translational stop codon

A. No stop codon. This hybrid gene was constructed in two steps. The first involved a 3-piece ligation of the 1,796 bp BamHI-ScaI ACT1 5'-fragment, the 234 bp KpnI-XhoI HIS3 3'-fragment, and pUC19 cut with BamHI and SalI. The 3'-overhang from the KpnI digest was removed by exonuclease digestion with T4 DNA polymerase (2 hr incubation at room temperature). The hybrid gene was excised from pUC19 and transferred into YCp50 cut with
EcoRI and HinDIII by digesting partially with HinDIII, purifying the linear plasmid, cutting to completion with EcoRI, and then purifying the hybrid EcoRI-HinDIII fragment.

B. Stop codon included. This hybrid gene was constructed by using a cloning strategy similar to that described above except that one of the junctions was different. To restore the normal HIS3 reading frame, the same ACT1 5'-fragment was fused to a HIS3 Asp718'-XhoI 3'-fragment. The sequence of both ACT1-5'-HIS3-3' junctions was confirmed by DNA sequencing. Sequencing of double-stranded plasmid DNA primed with the M13 reverse primer was done using the Sequenase protocol and reagents (U.S. Biochemical Corp.).

4. HIS3-5'-ACT1-3'

The ACT1 3'-fragment was generated by cutting pDH6 to completion with KpnI and EcoRI. This fragment contains the sequence for the 75 C-terminal amino acids and 3'-UTR of the ACT1 gene. This fragment was ligated into pUC8-Sc2676 cut to completion with KpnI and EcoRI. This removes the 11 C-terminal amino acids and 3'-UTR of the HIS3 gene, and when fused to the ACT1 3'-fragment, results in an open reading frame which uses the normal stop codon of the ACT1 mRNA. The plasmid can be recut with KpnI, confirming that the sequence of the junction is correct. The plasmid was subsequently digested with BamHI and EcoRI and the hybrid gene fragment was cloned into pSE360H at the BamHI and EcoRI sites within the polylinker.
5. **HIS3-Δ**

pUC8-Sc2676 was cut to completion with Asp718 and was filled-in with the Klenow fragment of DNA polymerase I. The DNA was then cut to completion with Ball and the resulting large fragment was gel-purified, ligated, and transformed into JM83 cells. This generates a 411 bp deletion in the **HIS3** coding region whose junction is in-frame. The DNA fragment containing the deletion was cut out with EcoRI and HindIII and was ligated into pSE360H cut at the EcoRI and HindIII sites in the polylinker. Sequence analysis of the Ball-Asp718’ **HIS3-Δ** junction was done using the Sequenase kit (USB Corp.) and CsCl-purified **HIS3-Δ** pUC8 double-stranded DNA. Two oligonucleotides were used as primers: a 38mer complimentary to the T7 promoter and 16 nt of **HIS3** 5’-DNA adjacent to the mRNA cap site, or a 24mer of **HIS3** 3’-DNA complimentary to the region surrounding the major transcriptional termination site. Out of 6 mini-prep DNAs sequenced, three were in-frame and three had a deletion of a C at the junction resulting in a frame-shift and loss of the **HIS3** reading frame. The latter truncated **HIS3** genes encode mRNAs with no translational stop codon.

6. **HIS3-Δ-5’-ACT1-3’**

The **HIS3-5’-ACT1-3’/pSE360** plasmid was cut with Asp718, filled-in with Klenow enzyme, cut with Ball, gel purified, ligated, and transformed to generate the identical 411 bp deletion as described in the **HIS3-Δ** construction.

7. **ACT1-5’-HIS3-ACT1-3’**

The **HIS3-5’-ACT1-3’** plasmid was cut to completion with BamHI and Ball
(unique site), and the resulting large fragment was then gel purified. The ACT1 5'-fragment was excised from pDH6 with KpnI and its 3'-overhangs were made blunt by exonuclease digestion with Klenow enzyme. This DNA was then cut to completion with BamHI. The BamHI-KpnI' ACT1 5'-fragment was gel purified and ligated into the above plasmid. The sequence of the ACT1-5'-HIS3 junction was confirmed using CsCl-purified DNA and Sequenase (USB Corp.) and an ACT1 5' 17mer oligonucleotide (5'-ACTTACAACCTC-CATCAT-3') which primes 50 nt upstream of the junction.

Analysis of mRNA decay in a temperature-sensitive RNA polymerase II mutant. Measurement of mRNA half-lives using this procedure has been described previously (Chapter II, this thesis).

RNA extraction. RNA was extracted as described previously (Chapter II, this thesis).

Riboprobes. The 2,871 bp pGEM-4 plasmid (Promega) was used for all riboprobe constructions. This plasmid contains a multiple cloning site flanked by either the T7 or SP6 bacteriophage promoters. Using either the DNA-dependent T7 or SP6 RNA polymerase allows for in vitro transcription of sense or anti-sense RNAs. A HIS3 3'-riboprobe was constructed by inserting the 476 bp Ball-PstI fragment of the yeast HIS3 gene into pGEM-4 cut with SmaI and PstI. To prepare a probe, this plasmid was linearized with EcoRI and transcribed using the T7 promoter. An ACT1 3'-riboprobe was made by cloning the 409 bp DraI-HinDIII ACT1 fragment into the SmaI and HinDIII
sites of pGEM-4 and transcribing the inserted DNA from the SP6 promoter.

**In Vitro transcription.** Both the T7 and SP6 (Melton et al., 1984) transcription incubation mixtures contained 40 mM Tris-HCl, pH 7.5, 6mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each of rATP, rUTP, and rGTP, 1 unit/μl RNasin (Promega), 50 μCi α³²P-CTP 400 Ci/mmol (Amersham), and either 20-50 units of SP6 RNA polymerase (Boehringer) or T7 polymerase (purified and kindly provided by Reid Gilmore). Transcriptions were for 1 hr at 40°C for SP6 polymerase or 1 hr at 37°C for T7 polymerase. DNA templates were digested for 15 min at 37°C with 1 unit of RQ1 DNase (Promega) for every μg of plasmid template. The RNA transcripts were then purified by G-50 spin chromatography and by single phenol-chloroform-isoamyl alcohol (50:48:2) and chloroform extractions, followed by ethanol precipitation. Riboprobes were heated to 65°C for 5 min, and duplicate aliquots were precipitated with TCA and counted. For each hybridization, 0.5-1.0X10⁶ CPM of riboprobe was added per ml of hybridization solution.

**Northern blots.** Total RNA (10-40 μg/lane) was fractionated on 1% agarose, 2.2 M formaldehyde gels buffered in 40 mM MOPS, pH 7, 10 mM sodium acetate, and 1 mM EDTA (Lehrach et al, 1977). RNA was transferred to Zeta-Probe blotting membranes (Bio-Rad) in 10X SSC as described by Thomas, 1980. Total yeast RNA fractionated on agarose-formaldehyde gels was electroblotted to Zeta-Probe (Bio-Rad) membranes as described by the manufacturer. RNAs were transferred overnight in 1X TAE buffer (10 mM Tris base, 5 mM sodium acetate, and 1 mM EDTA, pH 7.8) at 30 V at 4°C in
a vertical electrophoretic transfer apparatus. Northern blots were stripped by two 20 min washes at room temperature with a boiling solution of 0.1X SSC and 0.1% SDS.

**Hybridization conditions for DNA probes labeled by random priming.** Gel-purified DNA fragments were labeled using the random-priming technique of Feinberg and Vogelstein (1983, 1984). Following transfer, northern blot filters were air-dried, baked in vacuo for 2 hr at 80°C, and then washed for 1 hr at 65°C in 0.1X SSC, 0.5% SDS. Prehybridizations were at 42°C for 4-24 hr in 50% formamide, 10X Denhardt’s solution [1X=0.02% (w/v) each of bovine serum albumin, ficol, and polyvinylpyrolidone], 1% SDS, 5X SSC (1X SSC=0.15 M NaCl, 0.015 M sodium citrate), 50 mM sodium phosphate pH 6.5, and 500 μg/ml boiled, sonicated salmon sperm DNA (Sigma). Hybridization solution consisted of 50% formamide, 2X Denhardt’s solution, 1% SDS, 5X SSC, 25 mM sodium phosphate, pH 6.5, and 250 μg/ml boiled, sonicated salmon sperm DNA. Probes labeled in a randomly primed reaction were boiled for 5 min before the addition of 0.2-1.0X10^6 CPM/ml of hybridization solution. Hybridizations were done at 42°C for 16-48 hr. Filters were washed as follows: Once, for 15 min at room temperature (R.T.) in 2X SSC, 0.1% SDS, once for 15 min at R.T. in 0.1X SSC, 0.1% SDS, and once, for 15 min at 55°C in 0.1X SSC, 0.1% SDS. After washing, filters were exposed to Kodak X-Omat AR film at -80°C using a Cronex Hi-Plus intensifying screen. Exposures within the linear range of the film response were quantitated on a Hoeffer densitometer. Alternatively, the total radioactivity present in each RNA band was quantitated directly using a Betascope 603 Blot Analyzer, (Betagen Corp.,
Waltham, MA.).

**Hybridization conditions for riboprobes.** The protocol used was that described by Stratagene. Prehybridizations (4-24 hr) and hybridizations (16-24 hr) were done at 65°C in 50% formamide, 5X SSC, 1X PE [1X PE = 50 mM Tris, pH 7.5, 1% SDS, 5 mM EDTA, 0.2% polyvinylpyrrolidone (mw 40,000), 0.2% ficoll (mw 400,000), 0.1% w/v sodium pyrophosphate], and 150 μg/ml boiled, sonicated salmon sperm DNA. Washes were done twice for 15 min each in 2X SSC and 0.1% SDS at 65°C. The overall background was checked either with a hand-held monitor or on the Betascope. Where necessary, higher stringency washes were done for 15 min at 65°C in 0.1X SSC, 0.1% SDS. Films or filters were quantitated as described above.
RESULTS

Experimental strategy.

We have employed a ts mutant of RNA polymerase II (RY260; Nonet et al., 1987) to inhibit transcription following a shift from 24°C to 36°C. Using this technique, the half-lives of 20 yeast mRNAs have been measured following quantitation of northern blots containing equal amounts of total RNA extracted from cells at various times after the shift (Chapter II, this thesis). As a result of this screening, we have identified stable and unstable yeast mRNAs which differ from each other by as much as 16-fold. Our general experimental strategy to identify stability determinants exploits these differences by constructing chimeric genes containing different regions of DNA encoding stable and unstable mRNAs. The hybrid genes are transformed into strain RY260 on centromere-containing vectors, the use of northern blots enables us to resolve hybrid mRNAs from their endogenous parents, and allows for the measurement of multiple mRNAs on the same blot.

An analysis of a role for possible non-specific determinants such as size (Chapter II, this thesis), poly(A) tail length and efficiency of ribosome loading, (Santiago et al., 1987) indicates that these factors do not make a major contribution to the regulation of yeast mRNA decay rates. This has led to the hypothesis that the presence or absence of specific destabilizing sequences or structures dictate the stability of individual mRNAs in yeast. This model predicts that hybrid mRNAs made between various regions of stable mRNAs are stable, this is the case as both PGK1-5'-ACT1-3' and ACT1-5'-PGK1-3' mRNAs are stable (R. Parker and A. Jacobson, manuscript in preparation).
To identify possible instability determinants, we have constructed, expressed, and measured the half-lives of hybrid mRNAs containing regions of the stable ACT1 gene (tl/2=25-30 min), and unstable HIS3 (tl/2=6-8 min) and STE2 (tl/2=4.2 min) genes. Maps of the w.t. ACT1, HIS3, and STE2 genes are shown in Fig. 1A, 1B, and 7A, respectively.

Part 1. Structural determinants of HIS3 mRNA instability.

The 3'-end of HIS3 mRNA does not destabilize the ACT1 mRNA. To test whether the 3'-end from the unstable HIS3 mRNA could confer instability to the stable ACT1 mRNA, a hybrid gene was constructed as shown in Figure 1C. In this construction, DNA encoding the 3'-UTR and 39 C-terminal amino acids of ACT1 were replaced in-frame with DNA encoding the 3'-UTR and 11 COOH-terminal amino acids from HIS3. The chimeric gene was transformed into strain RY260 on the centromere-containing vector, YCp50, and the half-lives of the hybrid and parental mRNAs were measured by northern blotting as described in Materials and Methods. Using a probe from the 3'-end of the HIS3 gene, northern blots of RNA isolated from cells expressing the chimeric gene identify the expected bands shown in Fig. 2A: the 1.34 kb ACT1-5'-HIS3-3' hybrid mRNA and the 0.83 kb endogenous HIS3 mRNA. Quantitation of this blot yields the decay curves shown in Figure 2B. In this experiment, the endogenous HIS3 mRNA decayed with a half-life of 9 min, while the hybrid mRNA decayed with a half-life of 79 min. The half-life of the endogenous ACT1 mRNA was measured on an identical northern blot probed with a ACT1 3'-fragment complementary to the 3'-end of actin mRNA and was
shown to have a similarly long decay rate. From these results we conclude that the 3'-end of the HIS3 mRNA did not destabilize the ACT1 mRNA.

**Destabilizing effect of the HIS3 3'-end lacking a termination codon.** The results in Fig. 2C and D depict the decay rate of a very similar chimeric ACT1-5'-HIS3-3' mRNA. This mRNA differs from the previous mRNA by four nucleotides at the junction of the ACT1 and HIS3 sequences such that the 11 COOH-terminal amino acids of HIS3 are now out of frame and no stop codon occurs in the remainder of the mRNA (see Fig. 1C). Fig. 2C shows a northern blot of RNA from cells expressing this mRNA, probed with a HIS3 3'-riboprobe. A long exposure (48 hours) was required to obtain a detectable signal for this mRNA, indicating that it has a very low steady-state level. The decay curves shown in Fig. 2C indicate that the endogenous HIS3 and ACT1-5'-HIS3-3' mRNAs had half-lives of 6 and 9 min, respectively.

**Instability of HIS3 mRNA is due to determinants in its 5'-end.** If HIS3 3'-end sequences in their normal context are unable to confer instability, then destabilizing sequences should exist in the 5'-end of the HIS3 mRNA. This was assayed by constructing a chimeric gene in which DNA encoding the 75 COOH-terminal amino acids and 3'-UTR of ACT1 was fused in-frame to the reciprocal 5'-portion of HIS3. The resulting hybrid gene, shown in Fig 3A, was ligated into the centromere-containing vector, pSE360H, which was then transformed into RY260. Using a HIS3 5'-probe, a northern blot of RNA isolated from a transformant identifies the hybrid mRNA (0.975 kb) and the endogenous HIS3 mRNA (Fig. 3B). In a temperature-shift experiment the
hybrid mRNA had a half-life of 4.5 min and the endogenous HIS3 had a half-life of 6 min (Figs. 3B and 3C). These results suggest that the determinants of decay for the HIS3 mRNA reside either in the 5'-UTR or 5'-coding portion of the HIS3 mRNA.

Deletion of 411 nt of HIS3 coding sequence stabilizes the HIS3-Δ mRNA. To begin to identify the sequences or structures within the 5'-end of the HIS3 mRNA responsible for its rapid decay, a 411 nt deletion was made within the HIS3 coding region. The end points of the deletion were confirmed so as to maintain the HIS3 reading frame (see Fig. 4A and Materials and Methods). Using a HIS3 5'-probe, northern blot analysis of a strain harboring this HIS3-Δ gene identifies the expected 0.41 kb mRNA (Fig. 4B). In a temperature-shift experiment the HIS3-Δ mRNA and the endogenous HIS3 mRNA decayed with half-lives of 22 min and 8.5 min, respectively (Figs. 4B and 4C). These results indicate that a deletion of 411 nts from the HIS3 coding sequence decreases the decay rate for the resulting mRNA. Interestingly, a HIS3 mRNA containing a frame shift as a consequence of the deletion was virtually undetectable on northern blots (data not shown) and must, therefore, be extremely unstable.

A HIS3-5'-ACT1-3' chimeric mRNA lacking 411 nt of HIS3 is stable. To evaluate the effects of a HIS3 coding region deletion on the stability of the HIS3-5'-ACT1-3' chimeric mRNA, the HIS3-Δ-5'-ACT1-3' hybrid gene shown in Fig. 5A was constructed. This gene is identical to the HIS3-5'-ACT1-3' construct used in the experiments of Fig. 3 except that it contains the 411
nt deletion of HIS3 described above. Using an ACT1 3'-probe, northern blots of RNA isolated from cells transformed with this chimeric gene identify the hybrid mRNA (0.56 kb) and show that its half-life (18 min) is 3-4-fold slower than the HIS3-5'-ACT1-3' mRNA (t1/2=4.5 minutes; Figs. 5B and 5C). In this experiment, the endogenous ACT1 mRNA had a half-life of 25.5 min (Figs. 5B and 5C). These results indicate that the same element or sequences involved in the rapid decay of HIS3 mRNA also contribute to the rapid decay of the HIS3-5'-ACT1-3' chimeric mRNA.

Insertion of the 411 nt HIS3 coding sequence fragment is not sufficient to destabilize the ACT1 mRNA. The above experiments suggest that determinants necessary for the rapid decay of the HIS3 mRNA reside in a 411 nt region within the HIS3 coding sequence. To determine whether these 411 nt are sufficient to confer instability to a stable mRNA, this HIS3 fragment was inserted in-frame into the coding region of an otherwise intact ACT1 gene (see Fig. 6A). This hybrid gene was transformed into strain RY260 on the centromere-containing vector, pSE360H, and a temperature shift to inactivate RNA polymerase II was performed. Using an ACT1 3'-ScaI fragment as probe, northern blotting was used to measure decay rates for the endogenous and chimeric mRNAs (Figs. 6B and 6C). The half-life value of both the hybrid ACT1-5'-HIS3-ACT1-3' mRNA and the endogenous ACT1 mRNA was 65.5 min (Fig. 6C).

An independent experiment with a different transformant and using the BamHI-Xhol ACT1 5'-fragment as probe, resulted in a 56.5 min half-life for the ACT1 mRNA and a 61 min half-life for the ACT1-5'-HIS3-ACT1-3' hybrid
mRNA (data not shown).

The blot shown in Fig. 6b was stripped and rehybridized with a probe specific for the HIS3 mRNA (Fig. 7A). This blot identifies the 1.7 kb ACT1-5'-HIS3-5'-ACT1-3' hybrid mRNA and the 0.83 kb HIS3 mRNA. Quantitation of this blot (Fig. 7B) indicates that: i) the hybrid mRNA has a half-life comparable to that measured using the ACT1 probe (t1/2=64 min) and ii) the HIS3 mRNA now decays with an initial half-life of 21.5 min, three fold more stable than any previous measurement. This result was reproduced with mRNA from an independent transformant (data not shown). Following this initial half-life of 21.5 min, decay of the HIS3 mRNA slows dramatically such that, after 60 min at 36°C, 40% of the original mRNA still remains.

Part 2. Structural determinants of STE2 mRNA decay.

Additional evidence for the involvement of coding region sequences in the turnover of yeast mRNAs comes from two experiments with the unstable mRNA (t1/2=4.2 min) encoded by STE2 (the yeast gene for the α-factor mating pheromone receptor [Jenness et al., 1983]).

Role of the 3'-end of STE2 mRNA. To determine whether the 3'-UTR from the STE2 mRNA could confer instability to the stable ACT1 mRNA (t1/2=30 min), a 246 bp PvuII-HindIII fragment encoding the 15 COOH-terminal amino acids and 3'-UTR of STE2 was fused in-frame to the ScaI site of the ACT1 gene. This site is identical to that used for the ACT1-5'-HIS3-3' hybrid gene constructions (Fig. 1C). The maps for the STE2 gene and ACT1-5'-STE2-3' hybrid gene are shown in Figs. 8A and 8B. The hybrid gene was transformed
into RY260 on the centromere-containing vector, YCp50, and half-lives of the endogenous ACT1 and STE2 mRNAs, and the ACT1-5'-STE2-3' hybrid mRNA were measured by northern blotting (Figs. 9A and 9B). Decay of the ACT1 mRNA was measured using an ACT1 3'-probe (Fig. 2A) and decay of the STE2 and ACT1-5'-STE2-3' mRNAs was measured with a STE2 3'-probe (Fig. 9A). Quantitation of these blots indicated that the ACT1 and STE2 mRNAs decayed with half-lives of 22 min and 4.4 min, respectively, and that the ACT1-5'-STE2-3' hybrid mRNA decayed with an intermediate half-life of 12 min (Fig. 9B).

Deletion of STE2 coding sequence results in increased stability. To ask whether sequences or structures within the STE2 coding region are involved with the instability of this mRNA, a truncated gene (STE2-Δ) with an 82% deletion of internal coding sequence was constructed (Fig. 8C). The STE2 reading frame was maintained in this deletion and the gene was expressed from the high-copy number episomal plasmid, YEp352. Half-lives of the STE2 and STE2-Δ mRNAs were measured on the same northern blot as shown in Fig. 10A. Quantitation of this blot yields half-lives of 5 and 9.5 min for the STE2 and STE2-Δ mRNA, respectively (Fig. 10B).
DISCUSSION

Structural determinants of mRNA stability in yeast can be studied by analyzing decay rates of chimeric mRNAs. A strategy which has been frequently used to identify structural determinants involved in mRNA turnover has been to analyze the decay of hybrid mRNAs expressed from chimeric genes containing regions of stable and unstable mRNAs (Shaw and Kamen, 1986; Fort et al., 1987; Rahmsdorf et al., 1987; Jones and Cole, 1987; Kabnick and Housman, 1988; Shyu et al., 1989). We have used a similar strategy to identify the instability determinants of the \textit{HIS3} and \textit{STE2} mRNAs in \textit{Saccharomyces cerevisiae}.

The half-lives of chimeric and parental mRNAs are quantitated on northern blots of total RNA isolated after thermal inactivation of Pol II in an RNA polymerase II ts mutant (Chapter II, this thesis). We are confident that measurement of the half-lives of chimeric mRNAs using this protocol is a reliable method for several reasons: i) the use of northern blotting allows for a direct visualization of an mRNAs integrity and does not depend on a functional assay, also, both parental and hybrid mRNAs can be compared on the same blot; ii) there is good agreement between the decay rates and steady-state levels in 11 of 12 hybrid mRNAs (R. Parker and A. Jacobson, manuscript in preparation); and iii) hybrid mRNAs constructed between regions of stable mRNAs are themselves stable (R. Parker and A. Jacobson, manuscript in preparation). This is an important observation as it demonstrates that the stability of individual mRNAs is not dependent on the overall structure of each specific mRNA. Interestingly, the stability determinants of the four unstable yeast mRNAs studied in our laboratory all map to sequences in their
coding region (Chapter III, this thesis; R. Parker and A. Jacobson, manuscripts in preparation). The determinants of decay for the yeast SPO13 mRNA have also been mapped to the 5'-coding region (Surosky and Esposito, 1989). This may suggest a mechanism of mRNA decay quite different from that involving the different 3'-UTR elements which are implicated in higher eukaryotes.

3'-UTRs are not sufficient to confer rapid mRNA decay in yeast. There have been many examples in the mRNA turnover literature describing the role of sequences or structures in the 3'-UTRs of many mRNAs as being the primary determinant responsible for their rapid decay (Ross and Pizarro, 1983; Simcox et al., 1985; Meijlink et al., 1985; Lycan et al., 1987; Caput et al., 1986; Rahmsdorf et al., 1987; Reeves et al., 1987; Shaw and Kamen, 1986; Luscher et al., 1985; Graves et al., 1987; Ross and Kobs, 1986; Ross et al., 1986; Jones and Cole, 1987; Shapiro et al., 1988). Specifically, the region identified as having a major influence on the decay rates of the human GM-CSF, c-fos, and c-myc mRNAs are a 51 nt, 75 nt, and 140 nt AU-rich segment, respectively. This was demonstrated using two experimental approaches: the first involves the deletion of these sequences resulting in the stabilization of these mRNAs, and the second involves the insertion into or replacement of the 3'-UTR of a stable mRNA such as β-globin. Such experiments suggest that these regions that confer instability are transferable and appear to be dominant.

The replacement of the 3'-end of the stable ACT1 mRNA with the 3'-end of the unstable HIS3 mRNA did not increase its decay rate (Fig. 2A and 2B). This is also true when the 3'-UTR of ACT1 or PGK1 is replaced with the 3'-UTR of either the STE3 or MATα1 mRNA (R. Parker and A. Jacobson,
An intermediate half-life of 12 min is seen for a hybrid mRNA with the 5'-sequences of ACT1 fused in-frame to the 3'-end of the STE2 mRNA (Fig. 8A and B). Partial destabilization of an ACT1 reporter mRNA is also seen when fused to 600 nt of 3'-coding and noncoding sequence of the STE3 mRNA (R. Parker and A. Jacobson, manuscript in preparation). Finally, two very different mRNAs with the 3'-end of HIS3 fused out of frame and which lack a translational stop codon are at least 10-fold less stable than similar mRNAs with the normal HIS3 termination codon. The mechanism responsible is completely unknown.

A 411 nt region of HIS3 coding sequence is necessary but not sufficient to confer HIS3 mRNA instability. The 3'-end of the HIS3 mRNA in its normal context lacks any ability to confer the instability of HIS3 mRNA to the stable ACT1 gene. Measurement of the half-life for the mRNA encoded by the reciprocal HIS3-5'-ACT1-3' hybrid gene construct indicates HIS3 instability determinants are located either in its 5'-UTR or coding region. A deletion of a 411 nt region of HIS3 coding sequence results in a 3-4-fold slower half-life than that of the full-length HIS3 mRNA. This is true also when the 3'-end of the HIS3 mRNA is replaced with the 3'-end of the ACT1 mRNA. Taken together, this data suggests that this 411 nt of coding region sequence is necessary for HIS3 instability. The experiments in Fig. 6 and 7 in which this 411 nt of HIS3 coding sequence is inserted into an otherwise intact ACT1 gene demonstrates this sequence is not sufficient on its own to reduce the stability of the hybrid ACT1-5'-HIS3-ACT1-3' mRNA. At present, we do not understand the reason for this apparent inconsistency (see below).
Decay of the **STE2 mRNA** is determined in part by sequences within its **coding region**. A hybrid mRNA with the 3'-end of the unstable **STE2** mRNA fused in-frame to the 5'-end of the stable **ACT1** mRNA has an intermediate decay rate which is approximately half that of **ACT1** mRNA (Fig. 9). The increased stability (nearly two fold) of the **STE2** mRNA with an 82% deletion within its coding region suggests that an instability determinant may reside within this sequence (Fig. 10). Taken together, this data implies that the rapid turnover of **STE2** mRNA may require multiple instability determinants. A similar conclusion was reached in a more detailed study of the determinants of **STE3** mRNA turnover (R. Parker and A. Jacobson, manuscript in preparation). The **STE3** mRNA encodes the analogous receptor for mating pheromone in α cells, and has a very similar rapid decay rate. The sequences responsible for the decay of the **STE3** mRNA are separable and independent and have been mapped to the 5'-coding region (between codons 13 and 179), and to 600 nt of 3'-coding and noncoding sequence. These results are suggestive of a common degradation pathway(s) as being involved in the decay of these related mRNAs.

**Role of coding region sequences in the decay of other eukaryotic mRNAs.** Other detailed studies monitoring the decay of chimeric mRNAs have implicated the involvement of coding region sequences in the decay of the human **c-fos** (Kabnick and Housman, 1988; Shyu et al., 1989) and **Xenopus** TFIIIA mRNA (Harland and Misher, 1988). Kabnick and Housman demonstrated that both the **c-fos** 5'UTR and coding region were able to destabilize the human β-globin mRNA, although the major contribution is made by sequences in the 3'-UTR. They conclude that the turnover rates for these mRNAs are
due to either multiple determinants or to interactions between multiple sequences within the same mRNA.

Interestingly, the results from Shyu et al., 1989, have raised the possibility that degradation of human c-fos mRNA occurs via 2 distinct cellular degradation pathways. This hypothesis is based on constructions in which the 75 nt AU-rich element (ARE) was deleted from the c-fos 3'-UTR, this c-fos mRNA remained unstable. The insertion of this ARE into the rabbit β-globin mRNA reduced the half-live from >24 hours to 37+/-6 minutes, this decay was dependent on continued transcription as actinomycin D or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) treatment prolonged the decay rate. The second pathway involves the c-fos coding region as the substitution of the β-globin coding region with that of c-fos results in a t1/2 of 17+/-2 minutes. This mechanism is independent of transcription. Also, 180 nt of coding sequence from the Xenopus TFIIIA gene (encodes a 5S gene transcription factor) inserted into the 3'-UTR of the CAT mRNA results in a half-life of <30 minutes compared to 2.5 hours for normal CAT mRNA.

The cell-cycle regulated decay of histone mRNAs requires that translation proceed to within 300 nts of the 3'-end of these mRNAs (Graves et al., 1987). Thus, introduction of nonsense codons into the 5'-region of cell-cycle regulated histone mRNAs results in mRNA stabilization. A stem-loop structure in the 3'-UTR has been identified as the initial target for histone mRNA degradation (Pandey and Marzluff, 1987). These data suggest the involvement of a ribosome-bound nuclease which must be delivered to its target. Alternatively, the translocating ribosome may function to unfold specific mRNA regions, thereby exposing normally concealed cleavage signals.
Insertion of premature nonsense codons into the first 42 amino-terminal codons of β-tubulin mRNA prevents its autoregulated destabilization induced by high levels of unpolymerized tubulin subunits (Cleveland, 1988). Proper regulation occurs only if the four NH2-terminal amino acids can emerge from the ribosome to be recognized via a protein-protein interaction, presumably involving free tubulin subunits. A similar mechanism for histone mRNA decay may require the production of a nascent histone peptide of sufficient length, which serves as an autoregulatory recognition signal. An autoregulatory mechanism involving the interaction of histone mRNA and free core proteins has been suggested, as the decay rate for histone mRNAs is accelerated four-to six-fold following incubation in vitro with free histone proteins and a S130 fraction (Peltz and Ross, 1987). A similar mechanism may be responsible for the 2-fold slower decay rate for ACT1 mRNA in strains expressing two stable copies. The overexpression of actin protein may somehow be reducing the rate of decay of its own mRNA in some unknown fashion.

**Role of rare codons in coding sequence dictated instability.** Previously, we observed a correlation between the decay rate of an mRNA and its relative content of rare codons (Chapter II, this thesis). Those mRNAs with a high percentage of rare codons are less abundant and less stable than mRNAs with a low percentage of rare codons. With this in mind, a closer look at the rare codon content of the hybrid mRNAs examined here reveals the following: i) the stable ACT1 mRNA contains 17.3% (65 of 376) infrequent codons, ii) the unstable HIS3 mRNA has 43.6% (96 of 220) rare codons, and iii) the 411 nt of coding sequence necessary for the instability of HIS3 mRNA
has 48% rare codons and includes two stretches of 6, and one stretch of 4 consecutive rare codons.

Bennetzen and Hall (1982) observed that highly expressed genes in yeast are biased toward the use of only 25 of the 61 coding triplets. Rare codons in E. coli mRNAs slow ribosome elongation rates (Pedersen, 1984; Sorensen et al., 1989). In one example, insertion of four consecutive rare codons in the highly expressed chloramphenicol acetyl transferase (CAT) gene reduces CAT protein levels to 1/3 of control levels (Robinson et al., 1984). This may help to explain the effects which rare codons have on mRNA decay rates: if a ribosome-bound nuclease recognizes either a specific sequence or secondary structure for the cleavage event, then a nuclease on a paused ribosome may have an increased probability of interacting with its target.

An alternative explanation for the reduced steady-state levels of mRNAs containing a high percentage of rare codons invokes an adaptation of the ribosome translocation model for nuclear-cytoplasmic transport (Urlaub et al., 1989). In this model, mRNAs are pulled through nuclear pores cap-first via the mechanism of translocation during protein synthesis. A dihydrofolate reductase (dhfr) mRNA with a nonsense codon in its 5'-end has a greatly reduced steady-state level because translocation terminates and the stalled mRNA is degraded in the nucleus. If a significant stretch of rare codons results in a reduction or pause in elongation, those mRNAs could be degraded at a rapid rate inside the nucleus.

A final possible explanation for the reduced decay rates seen in the ACT1-5'-HIS3-ACT1-3' plasmid-containing strain would be due to a reduction in the rate of protein synthesis. Treatment with the elongation inhibitor,
cycloheximide, results in the stabilization of all yeast mRNAs analyzed (R. Parker, this thesis). If the presence of mutant actin protein resulted in the reduction or cessation of protein synthesis, this could cause a reduction in mRNA decay rates.
FIGURE LEGENDS

Fig. 1. Structural maps of the w.t ACT1, w.t. HIS3, and hybrid ACT1-5'-HIS3-3' genes. The important restriction sites and boundaries of the w.t ACT1 (Fig. 1A) and HIS3 (Fig. 1B) genes are shown. The structure of the hybrid ACT1-5'-HIS3-3' gene is shown in Fig. 1C. Different fill designs have been used to identify the regions donated from each parental gene. Details for each of the hybrid plasmid constructions are described in Materials and Methods.

Fig. 2. Decay of HIS3 and ACT1-5'-HIS3-3' mRNAs with (2A and 2B) or without (2C and 2D) a translational stop codon. The northern blots contain 25 µg of total RNA/lane isolated from Y260 (transformed with one of the ACT1-5'-HIS3-3' plasmids) after the designated min at 36°C. The HIS3 and ACT1-5'-HIS3-3' mRNAs were detected with a HIS3 3'-riboprobe. B. Quantitation of the blot in 2A for the decay of HIS3 (open boxes) mRNA, and the ACT1-5'-HIS3-3' (plus stop codon; closed diamonds) hybrid mRNA. D. Quantitation of the blot in 2C for the decay of HIS3 (open boxes) mRNA, and ACT1-5'-HIS3-3' (no stop codon; closed diamonds).

Fig. 3. Decay of HIS3 and HIS3-5'-ACT1-3' mRNAs. A. Structural map of the HIS3-5'-ACT1-3' hybrid gene. B. Northern blot showing the decay of the HIS3 and HIS3-5'-ACT1-3' mRNAs. Each lane contains 10 µg of total RNA isolated from Y260 transformed with the HIS3-5'-ACT1-3' plasmid. The blot was probed with the random prime labeled HIS3 5'-fragment. The first 0 min timepoint contains 10 µg of total RNA from the untransformed Y260 strain.
C. Quantitation of the blot in panel B. The open boxes are the endogenous HIS3 mRNA, closed diamonds the HIS3-5'-ACT1-3' hybrid mRNA.

**Fig. 4. Decay of HIS3 and HIS3-∆ mRNAs.** A. Structural map of the HIS3-∆ gene. B. Northern blot showing the decay of the HIS3 and HIS3-∆ mRNAs. Each lane contains 10 µg of total RNA isolated from Y260 transformed with the HIS3-∆ plasmid. The blot was probed with the HIS3 5'-probe. C. Quantitation of the blot in panel B. The open boxes are the endogenous HIS3 mRNA, closed diamonds the HIS3-∆ mRNA.

**Fig. 5. Decay of ACT1 and HIS3-∆5'-ACT1-3' mRNAs.** A. Structural map of the HIS3-∆5'-ACT1-3' gene. B. Northern blot showing the decay of the ACT1 and HIS3-∆5'-ACT1-3' mRNAs. Each lane contains 10 µg of total RNA isolated from Y260 transformed with the HIS3-∆5'-ACT1-3' plasmid. The blot was probed with the ACT1 3'-riboprobe. C. Quantitation of the blot in panel B. The open boxes are the endogenous ACT1 mRNA, closed diamonds the HIS3-∆5'-ACT1-3' mRNA.

**Fig. 6. Decay of ACT1 and ACT1-5'-HIS3-ACT1-3' mRNAs.** A. Structural map of the ACT1-5'-HIS3-ACT1-3' gene. B. Northern blot showing the decay of the ACT1 and ACT1-5'-HIS3-ACT1-3' mRNAs. Each lane contains 7.5 µg of total RNA isolated from Y260 transformed with the ACT1-5'-HIS3-ACT1-3' plasmid. The blot was probed with a random prime labeled SacI ACT1 3'-fragment. The first 0 min lane has RNA from untransformed RY260, the second has RNA from the HIS3-5'-ACT1-3'-transformed strain, and the third
has RNA from an independent transformant of the \textit{ACT1-5'-HIS3-ACT1-3'} plasmid. C. Quantitation of the blot in panel B. The closed diamonds are the endogenous \textit{ACT1} mRNA, open boxes are the \textit{ACT1-5'-HIS3-ACT1-3'} mRNA.

Fig. 7. Decay of \textit{HIS3} and \textit{ACT1-5'-HIS3-ACT1-3'} mRNAs. A. The northern blot shown in Fig. 6B was stripped and probed with the \textit{HIS3} 5'-probe. The RNAs are the same as described in Fig. 6. B. Quantitation of the blot in panel A. The closed diamonds are the \textit{ACT1-5'-HIS3-ACT1-3'} mRNA, open boxes are the endogenous \textit{HIS3} mRNA.

Fig. 8. Structural maps of the w.t \textit{STE2}, \textit{ACT1-5'-STE2-3'}, and \textit{STE2-A} genes. The important restriction sites and boundaries of the w.t \textit{STE2}, \textit{ACT1-5'-STE2-3'}, and \textit{STE2-A} genes are shown in Fig. 8A, 8B, and 8C, respectively.

Fig. 9. Decay of \textit{ACT1}, \textit{STE2}, and \textit{ACT1-5'-STE2-3'} mRNAs. A. The top and bottom panels show identical northern blots of 7.5 \(\mu\)g of total RNA per lane isolated from strain Y260 transformed with the \textit{ACT1-5'-STE2-3'} plasmid. The northern blot in the top panel was probed with a random prime labeled Scal \textit{ACT1} 3'-fragment, the northern blot in the bottom panel was probed with a random prime labeled PvuII-HindIII \textit{STE2} 3'-fragment. Each set of RNAs contains 7.5 \(\mu\)g of total RNA (0 min) from untransformed Y260. B. Quantitation of the blots in panel A. The open circles are \textit{ACT1} mRNA, open boxes the \textit{STE2} mRNA, and the closed circles are the \textit{ACT1-5'-STE2-3'} hybrid mRNA.
Fig. 10. Decay of the STE2 and STE2-Δ mRNAs. A. Northern blot showing the decay of the STE2 and STE2-Δ mRNAs. Each lane contains 7.5 μg of total RNA isolated from Y260 transformed with the high-copy number STE2-Δ plasmid and includes a 0 min timepoint from untransformed Y260. This blot was probed with a random prime labeled 2.3 kb STE2 fragment. B. Quantitation of the blot in panel A. The open boxes are the STE2 mRNA, the closed diamonds are the STE2-Δ mRNA.
FIGURE 1A, 1B, and 1C.

A. w.t. ACT1 GENE: mRNA-1.38 kb

B. w.t. HIS3 GENE: mRNA 0.83 kb

C. ACT1-5' - HIS3-3' GENE: mRNA-1.34 kb
FIGURE 2A, 2B, 2C, and 2D.
FIGURE 3A, 3B, and 3C.

A

**HIS3-5'-ACT1-3' GENE: mRNA-0.97 kb**

B

**HIS3-5' PROBE**

C

**PERCENT OF TIME 0**

**MINUTES AT 36°C**
FIGURE 4A, 4B, and 4C.

A

HIS3-Δ GENE: mRNA-0.42 kb

B

HIS3-5' PROBE

C

MINUTES AT 36° C
FIGURE 5A, 5B, and 5C.
FIGURE 6A, 6B, and 6C.
FIGURE 7A, and 7B.

A

HIS3-5' PROBE

ACT1-5'-HIS3-ACT1-3'→

HIS3-5'-ACT1-3'→

HIS3 →

MINUTES AT 36°C

B

PERCENT OF TIME 0

100

10

1

MINUTES AT 36°C
A.

w.t. **STE2** GENE: mRNA-1.47 kb

```
Sal I | CAP SITES | ATG | Pvu II | Pvu II | TGA | HinD III | AATAAA
```

B.

**ACT1-5'-STE2-3'** GENE: mRNA-1.37 kb

```
CAP SITES | ATG | INTRON | Sca I/Pvu II | Kpn I | TGA | HinD III | AATAAA
BamHI | 5' UT
```

C.

**STE2-Δ** GENE: mRNA-0.44 kb

```
Sal I | CAP SITES | ATG | Pvu II/Pvu II | TGA | HinD III | AATAAA
```
FIGURE 9A and 9B.
FIGURE 10A and 10B.

A

STE2 PROBE

STE2

STE2-Δ

MINUTES AT 36° C

B

PERCENT OF TIME

MINUTES AT 36° C
References:


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