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A cell-free extract from yeast cells for studying mRNA turnover

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ABSTRACT

We have isolated a cell-free extract from yeast cells that reproduces the differences observed *in vivo* in the rate of turnover of individual yeast mRNAs. Detailed analysis of the degradation of yeast phosphoglycerate kinase (PGK) mRNA in this system demonstrated that both natural and synthetically prepared PGK transcripts are degraded by the same pathway previously established by us *in vivo*, consisting of endonucleolytic cleavage at a number of 5′-GGUG-3′ sequence motifs within a short target region located close to the 3′-end of the coding sequence followed by 5′→3′ exonucleolytic removal of the resulting fragments. The extract, therefore, is suitable for studying the mechanistic details of mRNA turnover in yeast. As a first application of this system we have performed a limited mutational analysis of two of the GGUG motifs within the endonucleolytic target region of the PGK transcript. The results show that sequence changes in either motif abolish cleavage at the mutated site only, indicating the involvement of the residues in question in selection of the cleavage positions.

INTRODUCTION

Over the past decade it has become abundantly clear that (regulated) differences in mRNA stability are a key factor in the control of gene expression. Different mRNAs within the same cell have half-lives ranging from a few minutes to several hours or even days and the half-life of a number of individual mRNAs changes in response to various stimuli such as hormones, viral infection, cell growth conditions or the cell cycle (see refs. 1–5 for recent reviews).

Differences in decay rate, be it between different mRNAs under the same conditions or for one particular mRNA under different conditions, must be due to interaction between structural features within the transcripts with *trans*-acting factors, either components of the degradation machinery itself or ancillary factors that influence targeting of the transcript for decay. A number of *cis*-acting determinants has recently been identified and found to consist of either a specific nucleotide sequence or a particular local structure. A well-known example of the first type is the AU-rich sequence present in the trailer of a number of mammalian mRNAs encoding lymphokines, interferons and cellular growth factors (6–10), which acts as an autonomous destabilizing element (7). The second type is represented by the stem-loop structure at the 3′-terminus of mammalian histone mRNAs governing their cell-cycle-dependent destabilization (11, 12) and the cluster of hairpin loops in the trailer of the transferrin receptor mRNA involved in regulation of the stability of this mRNA in response to the intracellular iron concentration (13–15).

In contrast to our rapidly expanding knowledge of *cis*-acting stability determinants relatively little is know as yet concerning *trans*-acting factors, including the nucleases involved in mRNA turnover, in particular in eukaryotic cells. In this respect the recent development of mammalian cell-free mRNA decay systems (16–18) constitutes an important step forward since such systems considerably facilitate the identification, purification and characterization of such factors. By means of such a system, for instance, Ross and coworkers have documented the involvement of a 3′→5′ exoribonuclease in the degradation of histone mRNA (19–21) and provided evidence for feed-back control of this degradation by histones (22). Similar systems have led to the identification, among others, of a nucleoprotein factor destabilizing c-*myc* mRNA by binding to its AU-rich sequence (23, 24), a nuclease activity tightly associated with mammalian mRNP particles, that interacts in a highly specific fashion with individual mRNAs (25), an estrogen-induced ribonuclease activity in *Xenopus laevis* liver cells involved in albumin mRNA degradation (26) and an activity induced upon herpes simplex viral infection that destabilizes host mRNA (27).

We have recently begun an investigation of mRNA decay in yeast by introducing defined structural changes in the leader and/or trailer regions of specific yeast mRNAs (28). In the course of these studies we found that insertion of a poly(G) tract into either the leader or trailer of 3-phosphoglycerate kinase (PGK) mRNA causes the accumulation *in vivo* of a 5′- or 3′-terminal degradation intermediate, respectively (29). Further analysis of these intermediates led to the conclusion that PGK mRNA degradation is initiated by endonucleolytic cleavage targeted

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specifically to a short region near the 3'-terminus of the coding sequence and is completed by 5'→3' exonucleolytic removal of the resulting fragments (29).

In order to gain further insight into the nature of the *cis*-acting determinants and *trans*-acting factors involved in degradation of yeast mRNA, we set out to develop a cell-free mRNA decay system from *Saccharomyces cerevisiae* cells using the above data on *in vivo* PGK mRNA turnover as an important criterium for specificity. Here we report the isolation and some of the characteristics of this system, as well as the first results of a mutational analysis of the region constituting the target for endonucleolytic cleavage of the PGK transcript.

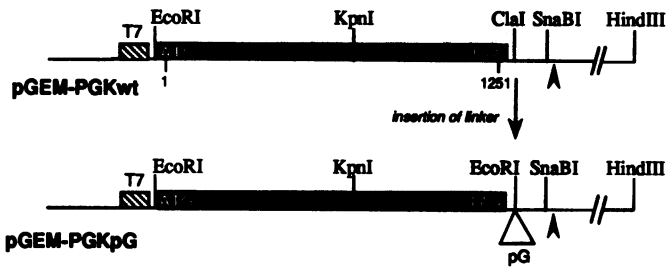

Figure 1. Partial restriction map of the inserts of plasmids pGEM-PGK and pGEM-PGKpG. The arrowhead indicates the *in vivo* transcription termination site. The striped bar corresponds to the T7 promoter region present inb the pGEM3 plasmid. The black bar represents the coding region of the PGK gene.

METHODS

Enzymes and oligonucleotides

Restriction enzymes were obtained from either Bethesda Research Laboratories (Gaithersburg, MD) or New England Biolabs (Beverly, MA) and used according to the supplier's instructions. T4 DNA ligase, polynucleotide kinase and the Klenow fragment of DNA polymerase I were obtained from Bethesda Research Laboratories. Radiolabeled nucleotides were obtained from Amersham (UK). Synthetic deoxyoligonucleotides were prepared in our laboratory using an Applied Biosystems 381A DNA synthesizer.

Plasmid construction

The EcoRI-HindIII fragment containing the yeast PGK gene was isolated from plasmid YEpR5 (30) and cloned in the pGEM3 vector (Promega) under control of the phage T7 promoter (Fig. 1). The resulting clone was designated pGEM-PGKwt. The KpnI-HindIII fragment from pGEM-PGKwt, containing the 3'-terminal part of the PGK coding sequence plus the 3'-flanking region was replaced by the corresponding fragment isolated from YEpR5-pG which carries an 18 nt long poly(G) insertion in the PGK trailer (28), resulting in the clone pGEM-PGKpG.

Plasmid pSEL-PGK was obtained by cloning the KpnI-HindIII fragment from pGEM-PGKwt into the pSelect-1 vector (Promega)

Site-directed mutagenesis

Site-directed mutagenesis of the insert of pSEL-PGK was carried out by means of the pSelect system (Promega) according to the supplier's instructions. The presence of the mutations was verified by sequence analysis (31) with the deoxyoligonucleotide complementary to nt 1337−1353 of the PGK trailer region as the primer.

In vitro transcription

pGEM-PGKwt and pGEM-PGKpG were cleaved at the unique SnaBI site within the trailer region of the PGK insert and *in vitro* transcription was carried out as described previously (32). The integrity of the RNA was checked by agarose gel electrophoresis (32).

Isolation of RNA from yeast cells

Total RNA was prepared from cells of *Saccharomyces cerevisiae* strain 20B12 as described by Zitomer *et al.* (33). Poly(A)$^+$ RNA was isolated by oligo(dT) chromatography (34) and its purity checked by agarose gel electrophoresis. No 26S or 17S rRNA was detected in our preparations.

Preparation of the cell-free degradation extract

Isolation of yeast polysomes was carried out by a modification of the method described by Ross and Kobs (17, 18). *Saccharomyces cerevisiae* strain 20B12 was grown in a 6 liter fermenter (New Brunswick Scientific) on YPD medium [2% (w/v) glucose, 2% (w/v) Yeast extract, 2% (w/v) Bactopeptone (DIFCO)] with continuous stirring (400 rev/min) and aeration (7 L/min) at 30°C. The culture was harvested in early-log growth phase ($10^7-2.10^7$ cells/ml) by centrifugation in a JA10 rotor (Beckman) at 5,000 rev/min for 10 min at 4°C. The cells were washed once with 400 ml distilled water at 24°C. Ten grams of cells (wet weight) were resuspended in a total volume of 400 ml solution A [2.0 mM EDTA (pH 7.0), 10 mM DTT], incubated at 24°C for 45 minutes and pelleted by centrifugation at 5,000 rpm for 10 min at 4°C. The pellet was resuspended in 400 ml buffer B [20 mM Hepes/KOH (pH 7.0), 1.0 M sorbitol] at 24°C. Lyticase (40 mg, Sigma) and phenyl-methyl-sulfonylfluoride (PMSF, 17 mg) were added and the mixture was shaken gently at 24°C until spheroplasts were formed. Spheroplast formation was monitored by comparing the OD_{660nm} in 1.0 M sorbitol to that in H_2O (35). When the ratio increased to three, spheroplasts were pelleted at 3,000 rev/min for 10 min at 4°C, washed with 400 ml ice-cold 1.2 M sorbitol and centrifuged again at 3,000 rev/min. The pellet was resuspended in 2.5 ml RS buffer [10 mM Tris·HCl (pH 7.6), 1.0 mM KAc, 1.5 mM $MgAc_2$, 2.0 mM DTT] at 4°C and 1 mg of PMSF was added. The spheroplasts were broken by 15 strokes with a tight-fitting Teflon pestle homogenizer and the lysate was centrifuged twice in a 50Ti rotor (Beckman) at 20,000 rev/min for 15 min at 4°C. The final supernatant was gently layered on a 30% (w/v) sucrose cushion (10 ml) in RS buffer and centrifuged in an SW41 rotor (Beckman) for 2.5 hr at 40,500 rev/min. The polysomal pellet was dissolved in 1.0 ml RS buffer, divided into aliquots, frozen in liquid nitrogen and stored at −70°C. Each aliquot was thawed only once for use. The preparations typically contained from 90 to 125 A_{260} units per ml and remained active for at least one year.

In vitro RNA decay reactions

25−50 ng of *in vitro* synthesized mRNA, or 2 μg of poly(A)$^+$ RNA was renatured in buffer R [10 mM Hepes/KOH (pH 7.5), 50 mM KAc, 0.5 mM EDTA] in a final volume of 10 μl by heating at 85°C for 3 min, cooling to 65°C and allowing the solution to cool slowly to 30°C. Standard decay reactions were set up on ice in a total volume of 50 μl containing 10 mM Hepes/KOH (pH 7.6), 2.0 mM $MgCl_2$, 100 mM KAc, 2.0 mM

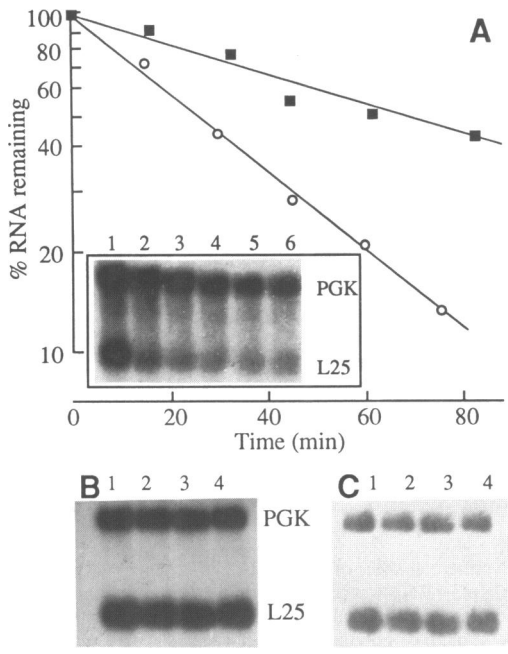

Figure 2. Differential decay of exogenous PGK and L25 mRNA in the yeast polysomal fraction. Panel A: Poly(A)$^+$ RNA (2 μg) was isolated from exponentially growing yeast cells and incubated after denaturation/renaturation treatment under standard reaction conditions with 1 A_{260} unit of polysomal extract in the presence of 40 units of RNasin. Samples were withdrawn at various times thereafter, RNA was isolated and subjected to Northern analysis using probes specific for PGK and L25 mRNA (inset: lane 1, 0 min; lane 2, 10 min; lane 3, 30 min; lane 4, 45 min; lane 5, 60 min; lane 6, 75 min after the start of the incubation). The intensity of the PGK and L25 bands in the Northern blots was determined by laser scanning densitometry: ■: PGK mRNA; ○: L25 mRNA. Panels B and C: Northern analysis using the PGK-specific probe of poly(A)$^+$ RNA incubated under the same conditions as in panel A but without prior heat treatment (panel B) or in the absence of cell extract (panel C). Lanes 1, 0 min; lanes 2, 20 min; lanes 3, 40 min; lanes 4, 60 min after the start of the incubation.

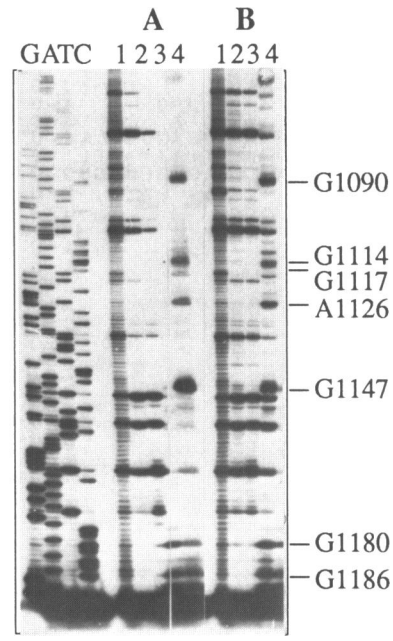

Figure 3. Primer extension analysis of the products of synthetic PGKpG (Panel A) and wild-type PGK (panel B) mRNA after incubation in the yeast cell-free decay system. Synthetic mRNAs were prepared by run-off transcription of plasmids pGEM-PGKpG and pGEM-PGKwt after cleavage with SnaBI. The renatured transcripts (25 ng) were incubated under standard conditions with and without polysomal extract. The RNA was reisolated and analyzed by primer extension analysis with the deoxyoligonucleotide complementary to nt 1219−1240 of the PGK coding region as the primer. The sequencing reaction was carried out on unincubated synthetic wild-type PGK mRNA with the same primer. Lanes 1: unincubated transcript; lanes 2 and 3: control incubations in the absence of polysomal extract for 1 min and 30 min respectively; lanes 4: 1 min incubation in the presence of 0.2 A_{260} units of polysomal extract. The specific 5′-ends detected for the RNA incubated with the extract are indicated. The number indicates the position of the residue with respect to the start of the coding region.

DTT, 20 μg of yeast tRNA (Boehringer) and 40 units of RNAsin (Promega). The renatured RNA and an appropriate amount of the polysomal preparation (depending upon its activity) were added, and the mixture was incubated at 30°C. At various times aliquots were taken and degradation was stopped by adding 50 μl stop mix [10 mM EDTA, 300 mM NaAc, 0.1% (w/v) lithium dodecylsulphate]. The samples were extracted twice with 50 μl of 2FC [phenol : chloropform : isoamylalcohol, 25 : 25 : 1 (v/v/v) containing 0.1% (w/v) 2-hydroxyquinoline]. RNA was precipitated from the aqueous phase by addition of 3 vol 96% ethanol. The resulting precipitate was dissolved in sterile distilled water. Ribosomal RNA remained intact during the whole procedure, as judged by ethidium bromide staining of agarose gels.

Northern and primer extension analysis

Northern blotting was carried out by fractionating RNA samples after treatment with glyoxal (34) on 2% agarose gels, followed by transfer to nylon membranes (Amersham). Probes used were: synthetic deoxyoligonucleotides complementary to nt 1337−1353 and nt 1219−1240 of the PGK trailer and coding regions, respectively, labeled at the 5′-end; the EcoRI-KpnI fragment encompassing the 5′-terminal part of the PGK coding region and the TaqI-HpaI fragment from plasmid pBMCY138 corresponding to the 5′-terminal two-thirds of the L25 coding region (36). The latter two probes were labeled by the random priming method as described previously (28) Primer extension analysis was performed as described previously (37). Sequence analysis was carried out by the dideoxy method (31).

RESULTS AND DISCUSSION

Attempts to obtain an *in vitro* mRNA degradation system from yeast cells so far have focused on assaying the decay of endogenous mRNA in either crude extracts or cell-free translation systems (3). No significant degradation was observed, however, in either type of extract. Since polysomal fractions have been successfully used in cell-free degradation systems derived from mammalian cells (17, 18, 20, 22, 23), we decided to test whether a similar fraction from yeast cells could serve as the basis for an *in vitro* system for specific degradation of yeast mRNA.

Preparation of the *in vitro* extract

A detailed protocol for the preparation of the *in vitro* degradation extract is given in the Methods section. Briefly, yeast cells were first converted into spheroplasts by treatment with lyticase and then broken by shearing. A polysomal fraction was isolated by centrifugation through 30% sucrose. The pellet was dissolved

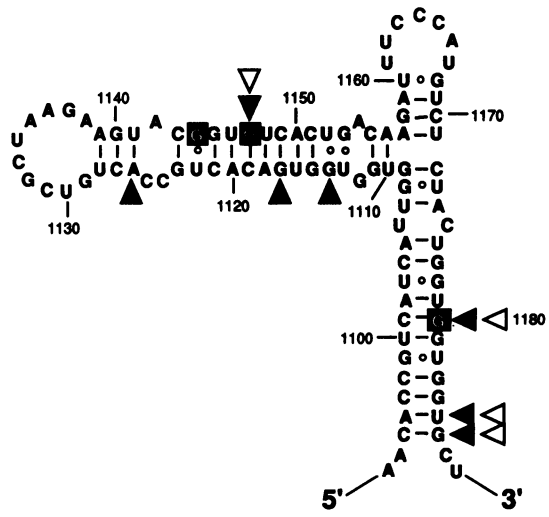

Figure 4. Computer-generated secondary structure model of the region of PGK mRNA acting as the target for endonucleolytic cleavage. The region of PGK mRNA encompassing nucleotides 1093−1188 together with either a 700 nt long upstream, or a 300 nt long downstream sequence were folded using the program FOLD (44). In the latter case both the wild-type and the mutant sequence, including the poly(G) insertion in the 3'-UTR, were analyzed. In all cases the region 1093−1188 was predicted to have the structure depicted. The 5'-ends identified in the primer extension analysis of the *in vitro* decay products of wild-type PGK and PGKpG mRNA are indicated by the black arrowheads. The white arrowheads indicate the 5'-ends of the *in vivo* decay products (29). Residues shown in reversed contrast are those mutated to A in the experiment described in Fig. 8.

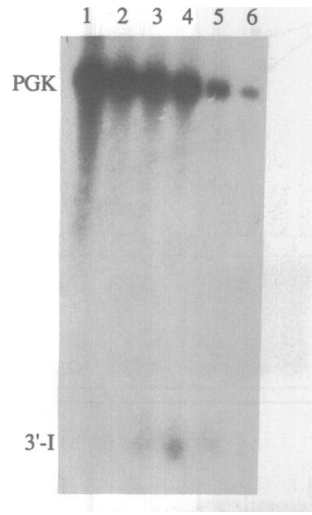

Figure 5. Northern analysis of the products of *in vitro* PGKpG mRNA decay. Synthetic PGKpG mRNA was prepared by run-off transcription of plasmid pGEM-PGKpG after cleavage with SnaBI. The RNA (50 ng) was renatured and incubated with 0.5 A_{260} units of polysomal extract under standard reaction conditions. Samples were withdrawn at various times and subjected to Northern analysis with a deoxyoligonucleotide probe complementary to nucleotides 1337−1353 of the PGK trailer. The intact transcript and 3'-terminal degradation fragment are indicated by PGK and 3'-I respectively. Lane 1: 0 min; lane 2: 2.5 min; lane 3: 5 min; lane 4: 7.5 min; lane 5: 30 min; lane 6: 60 min after the start of the incubation.

in buffer and the resulting extract was stored in small aliquots at −70°C. In order to obtain active extracts two aspects of the procedure proved to be of major importance. First of all, rapidly growing cells (doubling time < 2 h), harvested in early log phase (10^7−2.10^7 cells/ml) should be used. Secondly, spheroplasting should be completed with one hour or less.

Differential mRNA decay *in vitro*

An important criterium for specificity of an *in vitro* mRNA degradation system is whether the differential mRNA decay observed *in vivo* is reproduced in the system (16−18). Fig. 2 shows a comparison of the *in vitro* decay rates of PGK and ribosomal protein L25 mRNA carried out by incubating deproteinized total poly(A)$^+$ yeast RNA in the extract and following the disappearance of the two transcripts by Northern analysis (Fig. 2A). Apparent half-lives were determined by densitometric scanning of the hybridization signals (inset in Fig. 2A). Absolute half-lives cannot be determined in cell-free degradation systems because the rate of mRNA decay depends upon the ratio between substrate and extract (18; P. Vreken, unpublished observations). As is clear from these data, the PGK transcripts, despite their larger size and, thus, presumably greater susceptibility to attack by nonspecific nucleases, disappear at a rate approximately 2.7 (± 0.4; n = 5) times slower than the L25 transcripts. This value is in good agreement with the ratio of about three between the half-lives of the two mRNAs *in vivo* (28, 38). Similar experiments showed the *in vitro* decay rates of actin and L25 mRNA to be in a ratio of 2.3 (± 0.4; n = 3) : 1 (data not shown), in agreement with the slightly shorter *in vivo* half-life of actin transcripts compared to PGK mRNA (39). Thus, the yeast polysomal extract indeed faithfully reproduces the differences in degradation rates observed *in vivo* and should allow analysis of the molecular basis for these differences.

Degradation of exogenous mRNA is absolutely dependent upon heat-treatment prior to incubation with the extract involving denaturation at 85°C and renaturation by cooling slowly from 65° to 30°C. This indicates that the conformation of the mRNA is important for its recognition by the degradative machinery. Furthermore, it should be noted that the RNA is added free of protein which, therefore, may be dispensible for this recognition.

The *in vivo* degradation pathway of PGK mRNA is mimicked *in vitro*

Primer extension analysis of the *in vivo* decay products of wild-type and mutant PGK mRNA has demonstrated that degradation of this mRNA is initiated by endonucleolytic cleavage of the (deadenylated) transcript within a target region of about 100 nt (approximately positions 1100−1200)[1] located closely upstream from the translation stop codon (position 1251). Three products with 5'-ends at positions 1147, 1180 and 1186 were detected. Furthermore, we have obtained evidence that the products of this endonucleolytic cleavage are removed principally by 5'→3' exonucleolytic degradation (29). These data, which constitute the only information so far available on the pathway followed during *in vivo* decay of a particular yeast transcript, provide us with additional, more rigorous criteria in judging the fidelity of the *in vitro* degradation system.

[1]All coordinates are relative to the A of the translation start codon.

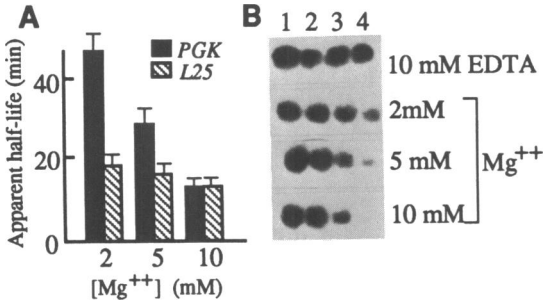

Figure 6. Effect of magnesium ion concentration on the decay rates of PGK and L25 mRNA *in vitro*. Panel A: 2 μg yeast poly(A)$^+$ RNA was incubated with 1 A_{260} unit of polysomal extract at various Mg^{++} ion concentrations. Apparent half-lives for PGK and L25 mRNA were determined as described in the legend to Fig. 2. Panel B: Decay of synthetic wild-type PGK transcripts upon incubation with the polysomal extract at various Mg^{++} ion concentrations as analyzed by Northern hybridization. Lane 1: 0 min; lane 2: 20 min; lane 3: 40 min; lane 4: 60 min after the start of the incubation.

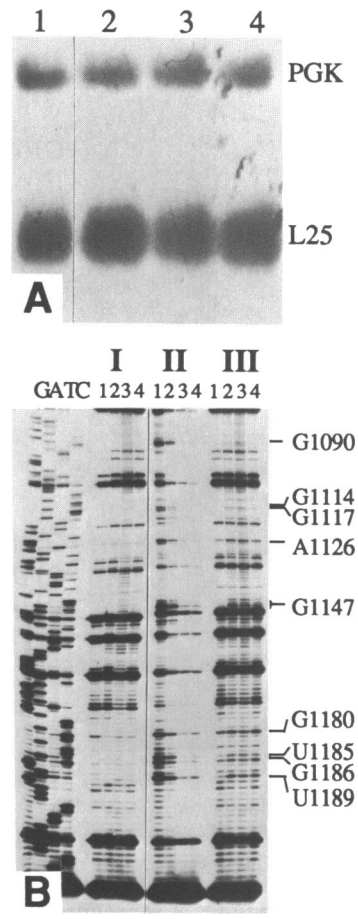

Figure 7. Inactivation of the *in vitro* decay system by heat. Panel A: 2 μg of yeast poly(A)$^+$ RNA was incubated under standard reaction conditions with 1 A_{260} unit of polysomal extract which had been heated for 5 min at 65°C. Decay was followed by Northern hybridization with a mixture of PGK- and L25-specific probes. Lane 1: 0 min; lane 2: 30 min; lane 3: 60 min; lane 4: 90 min after the start of the incubation. Panel B: Synthetic wild-type PGK mRNA was incubated under standard conditions without polysomal extract (I), or with 0.1 A_{260} unit of either untreated polysomal extract (II) or the same extract heated for 5 min at 65° C (III). Samples were taken at various times and the RNA analyzed by primer extension with the deoxyoligonucleotide complementary to nt 1219−1240 of the PGK coding region as the primer. Lanes 1: 1 min; lanes 2: 10 min; lanes 3: 30 min; lanes 4: 60 min after the start of the incubation. Sequencing was carried out with the same primer on unincubated synthetic wild-type PGK mRNA.

In order to determine whether the endonucleolytic cleavage of PGK mRNA observed *in vivo* also occurs in the *in vitro* system, we incubated synthetic PGK mRNA, prepared by run-off transcription of the cloned gene, with the polysomal extract and analyzed the products by primer extension using a deoxyoligonucleotide complementary to nt 1219−1240 as the primer. In a parallel experiment we also analyzed the fate of a synthetic PGKpG transcript, which carries an 18 nt long poly(G) insertion in the trailer (see Fig. 1) that does not influence the *in vivo* degradation pathway. However, the poly(G) tract acts as a barrier for the 5′→3′ exonuclease responsible for removal of the endonucleolytic cleavage products *in vivo* and, thus, causes the accumulation of a degradation intermediate (29).

The results of the primer extension analysis shown in Fig. 3 clearly reveal the same set of specific 5′-ends for both the wild-type and mutant transcript. Two of these ends, located at positions G_{1147} and G_{1180}, are identical to those detected by primer extension analysis of the *in vivo* decay products of both wild-type PGK and PGKpG transcripts. A third one (G_{1186}) is identical to the additional 5′-end observed *in vivo* for the wild-type transcript only (29). As shown previously, all three of these G residues correspond to the 3′-terminal nucleotide of a 5′-GGUG-3′ motif, that is repeated several times in the target region (40; see Fig. 4). The other four 5′-ends, corresponding to G_{1090}, G_{1114}, G_{1117} and A_{1126}, were not detected in our *in vivo* analysis. Strikingly, however, both G_{1114} and G_{1117} also correspond to the 3′-terminus of a 5′-GGUG-3′ sequence motif (Fig. 4), whereas G_{1090} is the 3′-terminal nucleotide of a 5′-GCUG-3′ sequence (40). Only the 5′-end at A_{1126} does not map to a sequence that is in any way related to GGUG.

In Fig. 5 the fate of synthetic PGKpG mRNA in the cell-free extract was analyzed by Northern hybridization with a probe complementary to a portion of the trailer region downstream from the poly(G) insertion. This analysis shows the formation of a small 3′-terminal fragment, the size of which (~150 nt) is in agreement with the distance between the 5′-end of the poly(G) tract and the 3′-terminus of the synthetic run-off transcript, which ends at the SnaBI site about 30 nt upstream from the *in vivo* transcription termination site (see Methods). This fragment, therefore, is analogous to the 180 nt long degradation intermediate previously found to accumulate in yeast cells transformed with the intact PGKpG gene, which extends from the 5′-end of the poly(G) tract to the transcription termination site (29). Furthermore, the kinetics of appearance and disappearance of the 150 nt long product are consistent with a precursor-product relationship between PGKpG mRNA and this fragment. Taken together, these data demonstrate that both the endonucleolytic cleavage of PGK mRNA and the subsequent 5′→3′ exonucleolytic removal of the fragments are reproduced in the *in vitro* extract even when a synthetic, non-polyadenylated and uncapped transcript is used. The *in vitro* produced degradation intermediate appears to be somewhat more heterogeneous in size than its *in vivo* counterpart, possibly due to aspecific nuclease activity.

It remains to be seen whether the extra 5′-ends detected *in vitro* are due to a partial perturbation of the endonucleolytic cleavage process or whether they represent *bona fide* decay products that

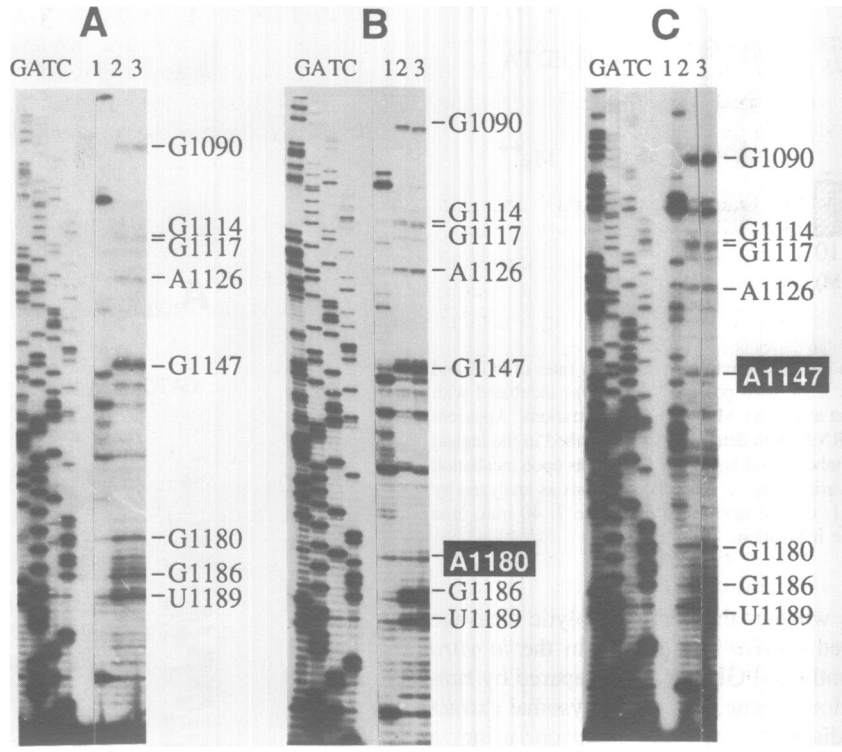

Figure 8. *In vitro* mutational analysis of the endonucleolytic target region of the PGK transcript. G→A transitions were introduced into the KpnI-HindIII fragments of the portion of the wild-type PGK gene present in plasmid pSEL-PGK at positions 1180 as well as at 1144 and 1147 simultaneously (*cf.* Fig. 4). Synthetic transcripts were prepared from both mutant as well as the wild-type plasmid. The renatured transcripts were incubated under standard conditions with 0.1 A_{260} unit of polysomal extract and the products were analyzed by primer extension using the deoxyoligonucleotide complementary to nt 1219–1240 of the PGK coding region as the primer. Panel A: wild-type transcript. Panel B: G_{1180}→A mutant transcript. Panel C: G_{1144}→A plus G_{1147}→A mutant transcript. Lanes 1: 30 min incubation without polysomal extract. Lanes 2 and 3: 10 min incubation in the presence of polysomal extract (duplicate experiments). In each case the sequencing reaction was carried out with the same primer on unincubated transcript.

were undetectable *in vivo, e.g.* because of a low incidence of cutting at these sites or a higher sensitivity of these products to the exonuclease. The fact that virtually all the extra 5′-ends detected *in vitro* map to the ultimate G residue of a 5′-GGUG-3′ motif, however, strongly suggests that they result from cleavage by the same enzyme that produces the 5′-termini at G_{1147}, G_{1180} and G_{1186}.

Properties of the cell-free degradation system

A number of properties of the *in vitro* degradation system were investigated. These are summarized below.

RNase inhibitor – The yeast extract is low in aspecific nuclease activity since differential decay is observed for PGK and L25 transcripts that differ in length by about a factor three, even in the absence of the placental ribonuclease inhibitor RNasin (not shown). However, addition of 40 units of RNasin increased the average ratio between the apparent half-lives of the two transcripts from about 2.4 to 2.7, in closer agreement with the *in vivo* value. In this respect the yeast system falls between that derived from mouse plasmacytoma cells, where no addition of RNasin was required (18) and that isolated from human erythroleukemia cells (17) where addition of this inhibitor, or one derived from liver cells, was found to be absolutely necessary to obtain differential decay. It should be noted that the yeast nucleases responsible for specific mRNA degradation were refractory to the action of RNAsin, even at concentrations up to 10 times higher than used under standard conditions (data not shown).

Mono- and divalent cations – Fig. 6 shows that degradation of synthetic PGK mRNA in the yeast extract is absolutely dependent upon the presence of Mg^{++} ions since addition of 10 mM EDTA blocks degradation completely (panel B). The optimum Mg^{++} concentration is around 2 mM. A further increase results in more rapid degradation of both PGK and L25 mRNA. However, the effect is considerably more pronounced for the former, leading to almost identical half-lives for the two transcripts, and, thus, loss of an important criterium for specificity, at 10 mM Mg^{++} (Fig. 6A). A similar pattern of dependence upon Mg^{++} ions was observed in mammalian *in vitro* mRNA decay systems (17, 18).

Stevens (41, 42) has isolated a Mg^{++}-dependent 5′→3′ exoribonuclease from yeast cells possibly corresponding to the enzyme that removes the products of the endonucleolytic cleavage of PGK mRNA. Since this enzyme (designated exoribonuclease-1) is sensitive to potassium ions, we also tested the effect of increasing concentrations of potassium acetate on the *in vitro* degradation of synthetic PGK mRNA. We found no change in the apparent half-life of the transcript up to a concentration of 300 mM (data nor shown), which inhibits

exoribonuclease-1 completely (42). Thus, it is unlikely that this enzyme plays a role in removal of the PGK mRNA fragments. Recent work has implicated exoribonuclease-1 in the degradation of a fragment of internal transcribed spacer 1 produced in one of the rRNA processing steps in yeast (43).

Temperature – Fig. 7A shows that exogenous PGK and L25 mRNA, isolated from yeast cells, do not significantly decrease over a period of 90 min when added to an extract that was heated at 65°C for 5 min prior to incubation. Since the initial endonucleolytic cleavage of PGK mRNA produces fragments that are easily detectable by Northern hybridization (29) this result indicates that the endonuclease responsible for this cleavage is heat-sensitive. This conclusion is confirmed by the data in Fig. 7B which shows a primer extension analysis of the products resulting from incubation of wild-type PGK mRNA with the heat-treated extract for different times (panel III). Comparison with a similar analysis using the untreated extract (panel II) shows that the levels of the 5′-termini diagnostic for the endonucleolytic cleavage (*cf.* Fig. 3) are drastically reduced, although small amounts remain detectable. Furthermore, in the heat-treated extract the kinetics of appearance and disappearance of these 5′-ends are considerably slower. The pertinent bands reach maximum intensity after approximately 10 min, compared to 1 min in the untreated extract, after which they remain essentially constant. The latter observation suggests that not only the endonuclease but also the 5′→3′ exonuclease removing the endonucleolytic cleavage products is heat-sensitive. In this experiment a further two specific 5′-ends, at U_{1185} and U_{1189}, are visible, both of which were also detected *in vivo* in some cases (29)

Pei and Calame (18) too reported their *in vitro* decay system prepared from mouse plasmacytoma cells to be heat-sensitive. However, in this case heating at 65°C was insufficient to inactivate the system. Instead the extract had to be boiled for 15 min.

Mutational analysis of the endonucleolytic target site

The data described in the previous sections demonstrate that, despite earlier failures, it is possible to prepare an extract from yeast cells in which major features of *in vivo* mRNA decay are reproduced. To illustrate the utility of this system we have made a first attempt to identify the sequence requirements for endonucleolytic cleavage of PGK mRNA by a limited mutational analysis of two of the GGUG motifs within the 1100/1200 endonucleolytic target region. The two motifs chosen were 1144–1147 and 1177–1180, which give rise to the major 5′-ends detected *in vivo* (G_{1147} and G_{1180}; 29). In the first case the GGUG sequence was changed into AGUA, while the second motif was converted into GGUA (*cf.* Fig. 4). In order to try to facilitate this type of mutational analysis and further limit the region of the PGK transcript required for correct endonucleolytic cleavage, the sequence changes were introduced by site-directed mutagenesis into the pSEL-PGK vector, which carries the KpnI-HindIII fragment of the PGK gene (*cf.* Fig. 1). The wild-type and mutant transcripts, encompassing the 3′-terminal 750 nt of the coding region and about two-thirds of the trailer were then prepared by *in vitro* run-off transcription after cleavage with SnaBI. Fig. 8A shows the results of primer extension analysis carried out after incubation of the transcript having the wild-type sequence. Clearly, the specificity of the endonucleolytic cleavage is not significantly affected by the lack of the 5′-terminal 500 nt of the PGK coding region and leader, since the pattern of bands conforms very well to that observed in Figs. 3 and 5.

Figs. 8B and C depict the results of primer extension analysis after incubation of the two mutant transcripts in the *in vitro* extract. In both cases the band corresponding to the mutated motif is drastically reduced in intensity, whereas the other bands are unaffected.

While these data clearly implicate the 3′-terminal G residue of the two GGUG motifs in the selection of the respective cleavage sites by the endonuclease, they do not prove that the motif as a whole is required. Nevertheless, the GGUG motif is the only sequence conservation of any consequence shared by the various cleavage sites, suggesting that it may well be important. It should be noted, however, that if such motifs are indeed directly involved in cleavage site selection, they are not sufficient in themselves but must be part of a more complex set of features recognized by the endonuclease. This can be concluded from the fact that 5′-ends could not, or at best only very weakly, be detected at two of the seven GGUG motifs (1108–1111 and 1180–1183; see Fig. 4) within the 1100/1200 target region (Figs. 3, 5 and 8). Furthermore, none of the nine additional copies of the GGUG motif present in PGK mRNA upstream from this region (40) is a target for endonucleolytic cleavage *in vivo* (29). Since there is no obvious conservation between the sequences surrounding the various GGUG motifs participating in 5′-end formation (Fig. 4), it is likely that the endonuclease requires the motif to be embedded in a particular higher-order structure. As shown in Fig. 4, the 1100–1200 region can be folded into a distinct secondary structure, which is even conserved to a considerable extent among PGK transcripts from various organisms (data not shown). So far, however, there is no experimental evidence to support this structure. Also this structure does not immediately suggest a plausible explanation for the lack of 5′-end formation at G_{1111} and G_{1183}. Structure mapping in combination with further mutational analysis will, therefore, be required to gain more insight into the nature of the features recognized by the endonuclease that initiates degradation of PGK mRNA.

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