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Mitochondrial mRNA stability and polyadenylation during anoxia-induced quiescence in the brine shrimp *Artemia franciscana*

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Summary

Polyadenylation of messenger RNA is known to be an important mechanism for regulating mRNA stability in a variety of systems, including bacteria, chloroplasts and plant mitochondria. By comparison, little is known about the role played by polyadenylation in animal mitochondrial gene expression. We have used embryos of the brine shrimp *Artemia franciscana* to test hypotheses regarding message stability and polyadenylation under conditions simulating anoxia-induced quiescence. In response to anoxia, these embryos undergo a profound and acute metabolic downregulation, characterized by a steep drop in intracellular pH (pH_i) and ATP levels. Using dot blots of total mitochondrial RNA, we show that during *in organello* incubations both O_2 deprivation and acidic pH (pH 6.4) elicit increases in half-lives of selected mitochondrial transcripts on the order of five- to tenfold or more, relative to normoxic controls at pH 7.8. Polyadenylation of these transcripts was measured under the same incubation conditions using a reverse transcriptase-polymerase chain reaction (RT-PCR)-based assay. The results demonstrate that low pH and anoxia promote significant deadenylation of the stabilized transcripts in several cases, measured either as change over time in the amount of polyadenylation within a given size class of poly(A)$^+$ tail, or as the total amount of polyadenylation at the endpoint of the incubation. This study is the first direct demonstration that for a metazoan mitochondrion, polyadenylation is associated with destabilized mRNA. This pattern has also been demonstrated in bacteria, chloroplasts and plant mitochondria and may indicate a conserved mechanism for regulating message half-life that differs from the paradigm for eukaryotic cytoplasm, where increased mRNA stability is associated with polyadenylation.

Key words: brine shrimp, *Artemia franciscana*, polyadenylation, mitchondria, mRNA stability, anoxia, quiescence, intracellular pH.

Introduction

In response to physiological cues, a common strategy for regulating gene expression is the modulation of RNA stability. Polyadenylation at the 3′-end of mRNAs is a major structural feature that appears to play an important role in message stability in many cases (Tharun and Parker, 1997). During development, regulated changes in length of the poly(A)$^+$ tail (PAT) of specific messages are correlated with their stability and translation (Richter, 1999). Deadenylation and 5′ decapping are rate-limiting steps in yeast mRNA decay (Muhlrad et al., 1995), while in mammals the 3′ untranslated region (UTR) and RNA binding proteins also have major effects on degradation rates (Ross, 1995). Mechanisms of RNA decay have been elucidated in bacteria (Xu and Cohen, 1995; Sarkar, 1997; Blum et al., 1999), chloroplasts (Hayes et al., 1999; Schuster et al., 1999), plant mitochondria (Gagliardi and Leaver, 1999; Kuhn et al., 2001; Lupold et al., 1999) and the eukaryotic cytoplasm (Mitchell and Tollervey, 2000). Longer PATs are generally associated with longer-lived mRNA species, and in some cases play a direct role in translational regulation as well (Rajagopalan and Malter, 1997; Sachs, 2000). In contrast to the eukaryotic cytoplasm, the degradation rate of poly(A)$^+$ mRNA is increased compared to non-adenylated mRNA in chloroplasts, plant mitochondria, and bacteria. Polyadenylated mRNAs from trypanosomes were also recently shown to degrade more rapidly in the presence of elevated UTP concentrations (Militello and Read, 2000). The unusual mitochondrial genomes and transcriptional mechanisms of trypanosomes, however, make generalizations problematic. Any potential significance to the different roles the PAT may play regarding mRNA degradation in the cytoplasm *vs.* organelles is unclear and, to date, animal mitochondria have not been studied in depth regarding RNA degradation. Although mRNAs in animal mitochondria are known to be polyadenylated (Beutow and Wood, 1978), to our knowledge there are no reports directly assessing the effect of polyadenylation on mitochondrial mRNA stability. We

undertook this study with two aims in mind: first, to quantify the stability of mRNAs under conditions simulating anoxic quiescence in *A. franciscana* mitochondria, and second, to examine the polyadenylation of these mRNAs and possible correlations with stability.

The brine shrimp *Artemia franciscana* is a useful model species for evaluating mitochondrial RNA stability. In response to unfavorable environmental conditions such as anoxia, gastrula-stage embryos of *A. franciscana* are able to enter a quiescent state in which metabolism and development are reversibly downregulated to a profound degree, a state that can last for up to several years (e.g. Clegg, 1997, 2001; Warner and Clegg, 2001). As oxygen is removed from the embryos, the pH_i (intracellular) falls from approximately 7.8 to 6.8 after 60–90 min (Busa et al., 1982; Kwast et al., 1995), and to about 6.3 overnight (Busa et al., 1982). Heat production is reduced by >99.5% with time under anoxia (Hontoria et al., 1993; Hand, 1995), and ATP concentrations are also depressed to low levels (Stocco et al., 1972; Rees et al., 1989). A wide variety of biochemical and gene-expression changes have been observed to accompany the quiescent state. For example, nuclear run-on data show an 80% decrease of ^{32}P-UTP incorporation into RNA after 4 h of anoxic exposure *in vivo*, indicating a substantial downregulation of transcription (van Breukelen et al., 2000); low pH_i is a contributing factor (van Breukelen et al., 2000; Willsie and Clegg, 2001). These and other observations (e.g. Clegg et al., 1999; Hand et al., 2001) indicate that depressed metabolism and increased macromolecular stability are important during quiescence.

In response to anoxia, mitochondrial protein synthesis is decreased by 77% after 30 min, with an 80% decrease at low pH compared to control (aerobic, high pH) mitochondria (Kwast and Hand, 1996a,b). Similarly, exposure of mitochondria *in vitro* to either acidic pH (6.4) or anoxia results in large decreases in transcription rates, and transcriptional initiation is decreased by 50% upon exposure to low pH (Eads and Hand, 2003). A direct effect of O_2 on gene expression in the mitochondrion has thus been demonstrated. Translation in the cytoplasm during entrance to anoxic exposure is also decreased (Clegg and Jackson, 1989; Hofmann and Hand, 1990, 1994), while mRNA pools do not drop (Hofmann and Hand, 1992). These observations suggest that message levels are not limiting to translation. Additionally, dot blots of total RNA from embryos exposed to anoxia for up to 6 h *in vivo* demonstrate that the ontogenetic increase in COXI mRNA is blocked by oxygen deprivation (Hardewig et al., 1996). However, direct evidence that mRNA stability is increased during anoxia has been lacking. We undertook the present study to determine if mRNA can be stabilized by anoxia *in vitro* and if polyadenylation plays a role in message stability.

To investigate the stability of mitochondrial mRNA we used dot blots of total RNA extracted from isolated mitochondria exposed to conditions designed to reflect those prevalent during anoxia-induced quiescence. We also characterized the poly(A)$^+$ tails of these mRNAs using a reverse transcriptase-polymerase chain reaction (RT-PCR)-based assay. We report here that mRNA stability in *A. franciscana* mitochondria is responsive to both lowered pH and anoxia *per se*, and that mRNA species that are undergoing rapid degradation are more polyadenylated, while those being stabilized by anoxia and/or low pH are deadenylated.

Materials and methods

Materials

Embryos of *Artemia franciscana* Kellog from the Great Salt Lake, Utah, USA were purchased from Sanders Brine Shrimp Co. (premium grade; Layton, UT, USA). Sucrose for mitochondrial isolation was from Pfanstiehl (Waukegan, IL, USA); other chemicals were the highest quality available from Sigma Co. (St Louis, MO, USA), unless otherwise noted. JM-109 cells, pGEM-T vectors, restriction enzymes, avian myeloblast virus reverse transcriptase (AMV-RT), Taq polymerase, molecular mass markers and dyes were purchased from Promega (Madison, WI, USA), and high-prime kits were from Roche (Indianapolis, IN, USA). Oligonucleotide primers for cloning and PCR were from Ransom Hill Biosciences (Ramona, CA, USA). Radiotracers ([α^{32}P]-dCTP and [α^{32}P]-UTP, 3.7 GBq ml^{-1}, 162.2 GBq mmol^{-1}) and GeneScreen Plus nylon membranes were from NEN/Perkin Elmer (Boston, MA, USA).

Mitochondrial isolation and in organello *treatments*

Mitochondria were isolated from gastrula-stage *Artemia franciscana* embryos and their functional integrity was assessed by respirometry as previously described (Eads and Hand, 1999). *In organello* treatments (low pH, anoxia, low pH and anoxia, and 1.5 mmol l^{-1} exogenous ATP under anoxia) and controls (normoxic, high pH) were performed on mitochondria from the same preparation. Following differential centrifugation, the final mitochondrial pellet was resuspended in 2 ml fortified homogenization buffer (FHB) containing: 500 mmol l^{-1} sucrose, 120 mmol l^{-1} KCl, 10 mmol l^{-1} MES, 10 mmol l^{-1} Hepes, 10 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} $MgCl_2$, 1.0 mmol l^{-1} EGTA, and 0.5% (w/v) fatty-acid-free bovine serum albumin (BSA), pH 7.5 (final mitochondrial protein concentration of 18–20 mg ml^{-1}). For low pH treatment groups, the final mitochondrial pellet was resuspended in 0.6 ml pH 7.0 FHB. Then a 250 μl portion of the mitochondrial suspension was added to 600 μl of FHB (pH 5.9), resulting in a final pH of 6.4. Control mitochondria (250 μl, in pH 7.0 FHB) were added to 680 μl of FHB (pH 8.3) to give a final pH of 7.8. For the anoxia studies, the FHB was made without adding the BSA initially to avoid frothing the mixture and then bubbled vigorously with argon for at least 30 min in a nitrogen-purged glovebag, which was sufficient to drive off all oxygen as measured with a Strathkelvin 1302 Clark-type electrode (Glasgow, UK). BSA powder (0.5% final concentration) was then combined with the anoxic FHB inside the glovebag, and the mixture was titrated with 1 mol l^{-1} KOH to give the appropriate pH in a final 10 ml volume (770 μl for pH 7.9, 320 μl for pH 6.4). FHB was then added to mitochondrial pellets drained of supernatant after the

final centrifugation, and incubated on ice in the purged glovebag for at least 30 min. The pH of the mitochondrial suspensions was assessed with a Radiometer electrode (GK2401C) at the beginning and end of the incubation. All mitochondrial suspensions were preincubated with 100 μg ml^{-1} actinomycin D for 10 min on ice prior to use, and then incubated at room temperature for the experiments. Mitochondrial protein was quantified according to Peterson (1977) using BSA as a standard.

Assessment of RNA degradation in organello

Mitochondria (500 μl), prepared as described above, were held at room temperature for the indicated times prior to RNA extraction. For anoxic studies, mitochondria were transferred to glass vials with screw-tops and teflon sealing discs and submerged in mineral oil at room temperature. Immediately after the incubations, each sample was spiked with 200 ng of a 540 bp fragment of exogenous, synthetic hexokinase (HK) RNA from *Artemia franciscana*, which was produced using a Megascript kit (Ambion; Austin, TX, USA). This step controlled for any differences in RNA isolation among treatments, as well as for any possible RNA degradation during isolation. All manipulations involving RNA were performed under RNase-free conditions. Solutions were treated with 0.1% diethylpyrocarbonate (DEPC) prior to autoclaving (except those containing primary-amine buffers), and glassware was baked at 250°C for 3 h. Total mitochondrial RNA was isolated by guanidinium/phenol extraction as described (Eads and Hand, 1999), DNase treated, quantified by spectrophotometry and subjected to dot-blot analysis essentially as described previously (Hardewig et al., 1996). Northern blots were also performed by the method of Hardewig et al. (1996) to assess quality of RNA preparations and specificity of our probes (see Fig. 1). For dot blots, 5 μg of each mitochondrial RNA sample was combined with 10–20 μg of yeast RNA in a final volume of 98 μl of denaturing solution (80% deionized formamide, 3.7% formaldehyde, 20 mmol l^{-1} phosphate, pH 7.0). Synthetic RNA for each of the mRNA species to be quantified was prepared from linearized plasmids containing the inserts, and known quantities of synthetic RNA from 10 pg to 20 ng were added to 10 μg yeast RNA in denaturing solution. Samples of yeast RNA (25 ng) served as a negative control. These mixtures were denatured at 70°C for 10 min, quick-chilled on ice, and then dilute loading dye was added to each sample prior to application. Gene Screen Plus nylon membranes were blotted and washed according to manufacturer's recommendations on a Minifold-II manifold from Schleicher and Schuell (Keene, NH, USA). After aspiration, the membranes were thoroughly dried and UV-crosslinked, which markedly improved the signal strength. The membranes were prehybridized in 5× SSPE (20× SSPE: 3.6 mol l^{-1} NaCl, 200 mmol l^{-1} sodium phosphate buffer, 20 mmol l^{-1} EDTA, pH 7.2) with 4% SDS at 65°C for at least 2 h prior to addition of gel-purified PCR probes, which were synthesized from mitochondrial genes of *A. franciscana* cloned into pGemT vectors as described previously (Eads and Hand, 1999; Hardewig et al., 1996). Probes (25 ng) were radiolabeled using [α^{32}P]-ATP (3.7 GBq ml^{-1}, 162.2 GBq $mmol^{-1}$; NEN/Perkin Elmer) using a Hi Prime Kit (Roche, Indianapolis, IN, USA) to a specific activity >10^8 d.p.m. μg^{-1}. Following hybridization at 65°C for 36–48 h, membranes were washed twice for 15 min each in 2× SSPE plus 0.1% SDS at 65°C, and twice at room temperature in 0.2× SSPE plus 0.1% SDS for 10 min. The blots were visualized using a Typhoon (Molecular Dynamics**,** Sunnyvale, CA, USA). ImageQuant v. 1.1 by Molecular Dynamics was used for quantitation of radioactivity, and Statview by SAS (Cary, NC, USA) was used for statistical analyses; $P<0.05$ was considered significant.

In order to assess the effectiveness of actinomycin D in inhibiting mitochondrial transcription during incubations to measure RNA degradation, transcriptional run-on assays were performed *in organello* under identical incubation conditions, except that 1.85 MBq ^{32}P-UTP and 5 μmol l^{-1} UTP (specific activity 17.65 GBq mol^{-1}) were added at the beginning of the assays. Incorporation of radiolabel over time was quantified with a filter-based assay, followed by TCA precipitation and scintillation counting as previously described (Eads and Hand, 1999).

Measurement of 3′ polyadenylation in mitochondrial RNA

The size of the poly(A)$^+$-tail (PAT) of the same mRNAs measured by dot blots (ATP synthase subunit 6, cytochrome *c* oxidase subunit I, cytochrome *b*, NADH dehydrogenase subunit 1) were assessed essentially as described by (Salles et al., 1999). Briefly, 500 ng of RNA isolated from the degradation experiments described above were used for cDNA synthesis with AMV-RT and an oligo-d(T)-anchor primer (5′-GTTCCACCTCTTTTGGTT$(T)_{15}$-3′). The RNA and primer (~200 ng) were combined in 6 μl of water, denatured at 65°C for 5 min and transferred to 42°C. The mixture was incubated with a final concentration of 1× AMV-RT buffer, 0.5 mmol l^{-1} dNTPs, and 20 units of AMV-RT in a final volume of 20 μl and incubated at 42°C. After 1 h the reverse transcriptase was heat inactivated at 65°C for 20 min, and the undiluted reaction used as template for PCR. The anchor primer [minus the d(T) moiety] and a message-specific primer (0.1 μmol l^{-1}) were used in 50 μl reactions containing 1 μl template, 1× PCR buffer (10 mmol l^{-1} Tris, 50 mmol l^{-1} KCl, 0.1% Triton X-100), 1.5 mmol l^{-1} $MgCl_2$, 0.2 μmol l^{-1} dNTPs, and 2.5 units Taq polymerase (Promega). The message-specific primers used were: ATP6, 5′-TTTATAGTAATATCTTTCTGA; COXI, 5′-GTATTTGAGAGGCCATGATC; CYB, 5′-GCCAACAT-TTCTATTTGATGA; ND1, 5′-AAGATTTTGGGTTACAT-TCAG. Cycling conditions for PCR were: 94°C, 45 s; primer annealing temperature, 1.5 min; 72°C, 2 min; repeated 30 times. Control reactions were run in parallel, including: RNA minus reverse transcriptase; PCR reactions with no cDNA template or no primer; and restriction digestions with no restriction enzyme.

PCR products were then restriction digested with appropriate enzymes to verify specificity and improve resolution of PAT length. Restriction enzymes were chosen

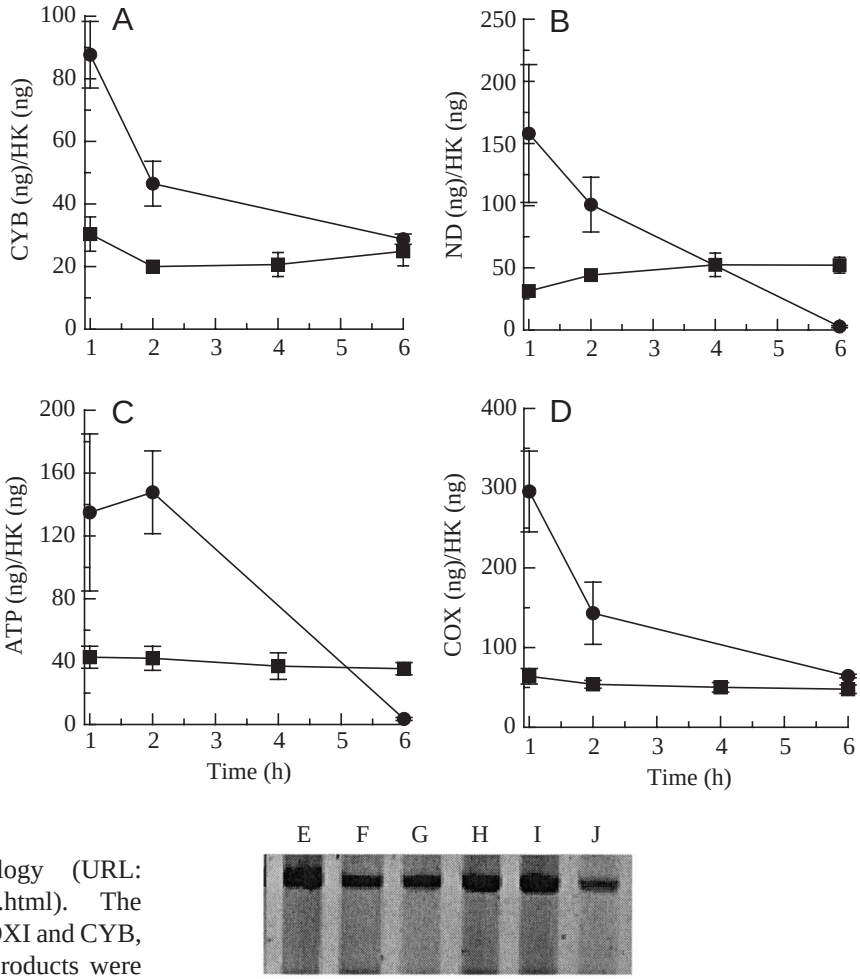

Fig. 1. (A–D) Low pH (pH 6.4) and anoxia *in organello* increase the stability of mRNA pools during 6 h incubations relative to controls (normoxic, pH 7.8). Isolated mitochondria given both treatments were incubated for the indicated times and RNA was extracted and hybridized on dot blots. Filled circles, controls; filled squares, experimental groups. Data were normalized on a nanogram basis to exogenously added HK standards, and homoskedasticity of standards and samples was assessed using Levene's test. Values are means ± S.E.M. (N=3). HK, hexokinase; CYB, cytochrome *b*; ND1, NADH dehydrogenase; ATP6, ATP synthase; COX1, cytochrome oxidase. (E–J) Example of a northern blot of total mitochondrial RNA (2 μg) at time 0 (E–G) and 6 h (H–J) for controls (E,H) *vs.* low pH (F,I) and anoxia (G,J), showing the specificity of the COXI probe against mitochondrial RNA under our experimental conditions.

using Webgene at SUNY Geneseo Biology (URL: www.Darwin.bio.geneseo.edu/~yin/webgene/RE.html). The enzymes were: for ATP6 products, *Eco*RI; for COXI and CYB, *Vsp*I; for NDI, *Hin*DIII. Following digestion, products were phenol-chloroform extracted, ethanol precipitated, washed in 70% ethanol and resuspended in 6 μl 100% formamide. Samples were electrophoresed in non-denaturing 6% polyacrylamide at 100 V, stained with 0.5 μg ml^{-1} ethidium bromide, and visualized on a Molecular Dynamics Typhoon (Amersham Biosciences, Piscataway, NJ, USA) using a 532 nm excitation laser and a 610BP30 emission filter. To quantify polyadenylation, each polyadenylated band in a lane was normalized to the upstream (non-polyadenylated) band in that lane created by digestion. Fluorescence intensity in the lower band was divided by the fluorescence in the upper, non-polyadenylated band using ImageQuant software. This approach accounted for loading differences between lanes and made direct lane-to-lane comparisons possible.

Results

Low pH and anoxia stabilize mitochondrial RNA in organello

The combined effect of anoxia and low pH (6.4) on four mitochondrial genes is shown in Fig. 1A–D, which depicts a stabilization of message levels relative to controls (normoxic, pH 7.8). There were no significant changes in the mRNA levels for cytochrome *b* (CYB), ATP synthase (ATP6), or cytochrome oxidase (COXI) during experimental treatment (P>0.2 for all genes). There was a very slight increase (P<0.05) in NADH-dehydrogenase (ND1) mRNA (Fig. 1B). It is important to note that by spiking the mitochondrial samples with exogenous hexokinase (HK) RNA immediately prior to mtRNA extraction, we were able to establish that our average recovery of total RNA did not change from 0–6 h across all treatments (P>0.05; data not shown). Northern analysis using the COXI primer (690 bp) and 2 μg total RNA extracted from mitochondria exposed to control, low pH or anoxic incubation conditions showed that our probe was specific (Fig. 1E–J).

These patterns contrast with control values that showed significant increases in message levels during the first hour of incubation (data not shown), followed by a steep decline from 1–6 h. Calculations of degradation rate constant (k) and transcript half-life ($t_{1/2}$) (Table 1) indicated that marked stabilization relative to controls occurred for ATP6, where $t_{1/2}$ increased by more than 23-fold, from 48.2 to 1113 min. CYB and COXI displayed an increased half-life of 7.5-fold and 7.0-fold, respectively, relative to controls. ND1 displayed the greatest stabilization among all four mRNAs under conditions of anoxia plus low pH. From 1 h to 6 h during the incubation, ND1 levels did not change; the half-life and decay constants were therefore not calculated. To measure the contribution of RNA synthesis to the measured RNA levels under these

Table 1. *Effect of* in organello *treatments on decay rates (*k*) and half-lives ($t_{1/2}$)* of various mitochondrial genes using data from dot-blot analyses*

	Control	Anoxia+low pH	Anoxia	Anoxia+1.5 mmol l^{-1} ATP	Low pH
ATP6					
$k^{\dagger}$	1.44×10^{-2}	6.22×10^{-4}	5.90×10^{-3}	9.67×10^{-4}	5.49×10^{-3}
$t_{1/2}{}^{\dagger}$	48.2	1113	117	716	187
COX I					
k	6.75×10^{-3}	9.58×10^{-4}	1.90×10^{-3}	3.65×10^{-3}	5.49×10^{-3}
$t_{1/2}$	102	723	364	194	126
CYB					
k	1.03×10^{-2}	1.37×10^{-3}	NA	2.75×10^{-3}	2.62×10^{-3}
$t_{1/2}$	67.5	506	NA	252	264
ND1					
k	1.48×10^{-2}	NA	4.16×10^{-3}	8.45×10^{-4}	1.99×10^{-3}
$t_{1/2}$	47.0	NA	167	820	346

ATP6, ATP synthase; COX1, cytochrome oxidase; CYB, cytochrome *b*; ND1, NADH dehydrogenase.

*Decay rates were calculated according to the formula for second-order decay kinetics: $k=2.3/(\text{time}_2-\text{time}_1)\times\log(\text{RNA}_1/\text{RNA}_2)$. Half-life was calculated according to the equation $t_{1/2}=0.693/k$ (Segel, 1976).

$^{\dagger}k$, min^{-1}; $t_{1/2}$, min.

NA, increases in RNA amounts (although not statistically significant) make these assessments inapplicable.

experimental conditions, transcriptional run-on assays were performed (Fig. 2). As anticipated, the actinomycin D-inhibited incorporation was very low compared to the rates measured under optimized conditions (e.g. all nucleotides added and inhibitor-free conditions; Eads and Hand, 1999) (not shown). From 1 h to 6 h incubation, no RNA synthesis was measurable under any of the experimental conditions (Fig. 2), and it is important to note that this period was used for calculating message decay rates and half-lives.

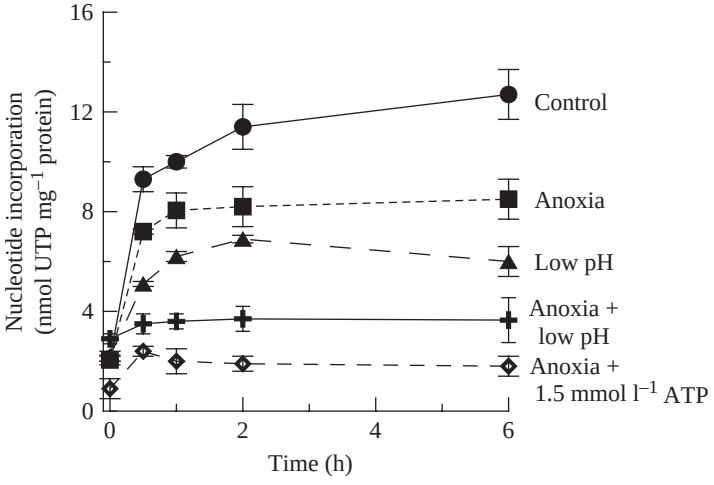

Fig. 2. Conditions of anoxia (filled squares), low pH (6.4; filled triangles), anoxia plus low pH (+ symbols) or anoxia plus 1.5 mmol l^{-1} ATP (open diamonds) *in organello* cause decreased transcription in *A. franciscana* embryos compared to controls (filled circles; normoxic, pH 6.4). Incorporation of ^{32}P-UTP was followed by scintillation counting of TCA-precipitated RNA. Mitochondria were incubated at room temperature with 100 μg ml^{-1} actinomycin D to measure RNA synthesis under conditions identical to those used to assess mRNA half-life. All values are means ± S.E.M. (*N*=3).

Anoxia and low pH can independently stabilize mitochondrial mRNA

To evaluate the contributions of low pH (pH 6.4) and anoxia independently, dot blots and run-on assays were performed on mitochondria incubated under either anoxia or low pH. As shown in Fig. 3 and Table 1, both anoxia and pH 6.4 had stabilizing effects on RNA levels. For example, during treatment with anoxia, no significant change was seen in mRNA levels of any of the four measured mRNA species (Fig. 3; open squares). Increases in transcript half-life reflected the lack of change (Table 1). The most dramatic extension occurred in CYB, from 67.5 min to 1085 min (a 16-fold increase), while COXI and ND1 increased by 3.5-fold and ATP6 by 2.5-fold. Taken together, these data suggest a downregulation of degradation of these mRNAs under anoxia alone.

The effect of low pH, which is a hallmark of anoxic quiescence in *A. franciscana* embryos, was similar to the anoxic effect. As depicted in Fig. 3 (filled squares), levels of all four mRNA species at low pH were unchanged ($P>0.05$) from the first hour of incubation to the 6 h time point. The increase in half-life ranged from 1.2 times for COXI to 7.9 times for ND1, with CYB and ATP6 each increased by 3.9 times (Table 1). These data demonstrate that RNA degradation is depressed at low pH.

Exogenous ATP does not increase the degradation of mitochondrial mRNA

In response to anoxia, *A. franciscana* embryos undergo a net hydrolysis of ATP (Stocco et al., 1972). Although

matrix ATP levels *in vivo* are unknown, anoxia promotes a decline in ATP in isolated mitochondria (Kwast and Hand, 1996a). RNA decay pathways appear to require ATP, either as a cofactor, such as in the bacterial degradosome (cf. Grunberg-Manago, 1999), or for hydrolysis, as in a mitochondrially localized 3′–5′ exonuclease characterized in yeast, which increases activity in response to elevated NTP levels (Min and Zassenhaus, 1993). For these reasons, we hypothesized that the lowered ATP levels under anoxia might be responsible in part for the increased half-lives of mRNA observed under this condition, which proved to be incorrect. As shown in Fig. 4 and Table 1, the addition of 1.5 mmol l^{-1} ATP under anoxia had no effect, or in some cases slightly decreased degradation rates relative to anoxia alone. These trends are reflected by increases in half-lives, from 1.9- and 3.7 fold-increases for COXI and CYB, respectively, to 14.9- and 17 fold-increases for ATP6 and ND1 (Table 1).

Mitochondrial mRNA polyadenylation can be reproducibly quantified

Patterns of gene-specific polyadenylation of mitochondrial mRNAs were quantified using a PCR-based assay referred to as RACE-PAT (Salles et al., 1999) coupled with ethidium bromide fluorescence of electrophoresed products. The reproducibility of the assay was quantified by performing the assays in triplicate and calculating the coefficient of variation for the amount of PAT in each lane (Table 2). Three mitochondrial RNA preparations were used as template for the cDNA reactions, except under conditions of anoxia plus low pH, where two preparations were used. The results of the experiment demonstrated that the quantification of PAT for COXI is quite robust, with the coefficient of variaton (CV) ranging from 3.4 to 10.2 (Table 2). The assay was repeated using ATP6, and the CV ranged from 5.6 to 12.3. Representative gels of RACE-PAT products are shown in Fig. 5. PAT lengths were calculated by subtracting non-PAT portions of the DNA products (i.e. the distance between the restriction site and the 3′ end of the coding region; also subtracting the anchor primer minus the d(T) moiety). Our analyses led us to conclude that evaluating prominent PAT bands for changes was more revealing than choosing size ranges in some cases, hence band sizes being compared vary from gene to gene. PAT length ranged from approximately 300 nucleotides to almost zero, or non-adenylated (e.g. Fig. 5, lanes B5, C6).

Physiological treatments in organello *alter PAT status*

Using the same RNA samples for the PAT assay that we had used for dot blots allowed us directly to compare PAT amounts with observed RNA degradation. Levels of PAT were assessed in several ways: as a function of change in the amount of a given size class (Fig. 6), or as the total amount in all size classes combined at the endpoint of the incubation (Fig. 7). Any number >1 on the ordinate represents an increase in polyadenylation over time for Fig. 6. Change in the mean amount of polyadenylation between 1–6 h was assessed for three separate experiments for each transcript (Fig. 6, with error bars). CYB showed a significant increase in control polyadenylation (P<0.01) over time relative to experimental treatments using Tukey's Honestly Significant Difference (HSD) and Dunnett's test (Fig. 6A). The 2.5-fold increase in total polyadenylation between 1-6 h for CYB under control conditions, for instance, contrasted with a 50% decrease under all experimental treatments (Fig. 6A), which yields an overall change of fivefold in PAT amount. There was also a striking 15-fold increase in amounts of a 300 bp PAT for CYB in controls; this PAT decreased by twofold or more under all experimental treatments (Fig. 6A). At the endpoint of the assay, CYB showed a significant increase in mean PAT amounts relative to all treatments except low pH (Fig. 7).

The same trends were also seen for COXI and ND1, which showed an increase from 1–6 h in polyadenylation of controls for all size-classes except the longest ND1 PAT (Fig. 6B,C). Experimental treatments generally reduced the amounts of PAT accumulated over time for

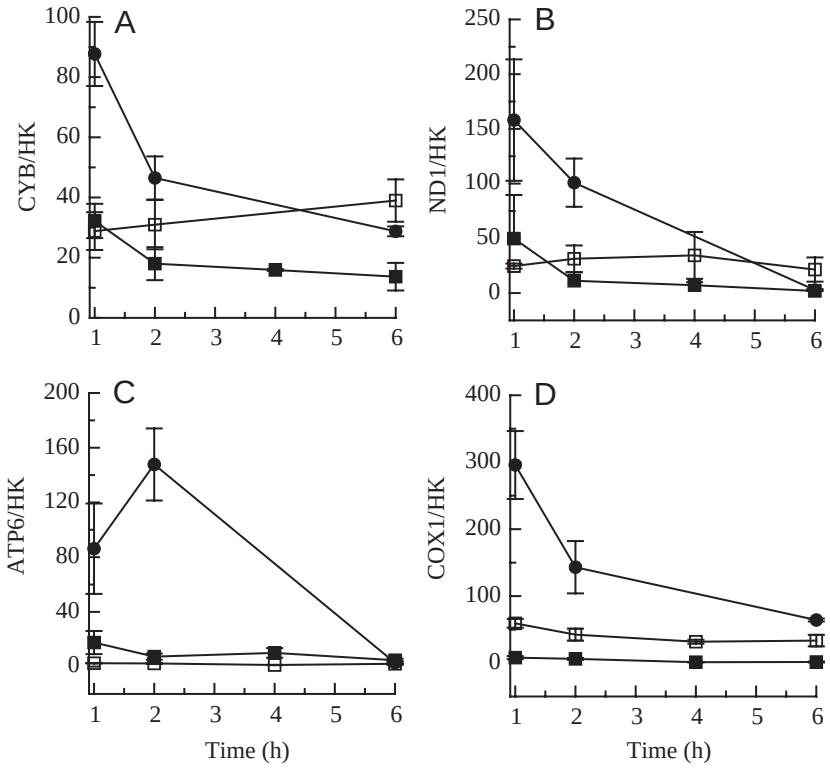

Fig. 3. The effects of either anoxia or low pH (pH 6.4) *in organello* on mitochondrial CYB (A), ND1 (B), ATP6 (C) and COX1 (D) mRNA levels are similar to their combined effects. Filled circles, control incubations (normoxic, pH 7.8); filled squares, incubations at low pH; open squares, anoxic incubations. Data were evaluated and expressed as in Fig. 1.

Table 2. *Reproducibility of assays for quantifying polyadenylation of mitochondrial mRNA*

	Control	Anoxia	Anoxia+1.5 mmol l^{-1} ATP	Anoxia+low pH	Low pH
COXI					
Mean±S.E.M.	8.23±0.24	1.09±0.06	0.84±0.02	1.02±0.03	1.16±0.07
CV	5.08	8.66	3.40	5.88	10.15
ATP6					
Mean±S.E.M.	1.34±0.05	0.82±0.04	1.11±0.07	0.43±0.03	1.39±0.08
CV	5.59	7.21	9.80	12.30	9.54

ATP6, ATP synthase; COX1, cytochrome oxidase; CV, coefficient of variation.

N=3 PAT assays. At least two separate mitochondrial RNA preparations were used for each treatment. The values represent an average ratio of ethidium bromide fluorescence from the bottom (polyadenylated) bands of the PAT products to the top (non-adenylated) band, as measured with Molecular Dynamics Typhoon and ImageQuant software (see Materials and methods for details).

these transcripts, although the results were not significant relative to control amounts (Fig. 6B,C). Decreases in PAT amounts during the assay were evident for all experimental treatments with ND1, by twofold and greater (Fig. 6C). Only the PAT of 130–190 bp under conditions of anoxia plus low pH and a band of 70–120 bp under conditions of low pH showed an increase (1- to 1.5-fold). For COXI, under control conditions the largest increase in PAT amounts was seen in the smallest size class (15–25 bp; 12-fold increase), although longer PATs accumulated as well (Fig. 6B). Also with COXI, when PAT amounts at 6 h were compared between experimental treatments and controls, significant differences were seen for all treatments (Fig. 7). Thus both metrics we used to quantify the amount of PAT in our assays showed some significant increases in polyadenylated fragments under control conditions and a decrease under experimental treatments (Figs 6, 7).

For ATP6, the experimental treatments had less apparent effect on PAT amounts relative to controls, when analyzed as a change in PAT amounts over time (Fig. 6D). Anoxia plus 1.5 mmol l^{-1} ATP had no effect, with identical amounts of PAT present in both bands at 1 and 6 h, and low pH slightly increased PAT amounts above control levels (Fig. 6D). However, when analyzed as the total amount of PAT after the 6 h incubation, control levels were significantly greater than those for experimental treatments, with the exception of anoxia plus ATP, where only a modest difference was noted (Fig. 7). Our measurements of polyadenylation of selected mitochondrial messages indicate in general that amounts of PAT are decreased in the experimental treatments relative to controls. Thus, increased polyadenylation is associated with message instability, and decreased PAT amounts are correlated with stability of message.

Discussion

In this study, we analyzed the degradation patterns and quantified polyadenylation of selected mitochondrial mRNAs from *A. franciscana* embryos exposed *in organello* to conditions simulating anoxia-induced quiescence. Experimental treatments *in vitro*, which included low pH (pH 6.4), anoxia, low pH and anoxia, and incubation with 1.5 mmol l^{-1} exogenous ATP under anoxia, promoted increased stability of all four transcripts examined, relative to the normoxic controls at pH 7.9. We examined the polyadenylation of the same mRNAs under identical conditions using a RT-PCR

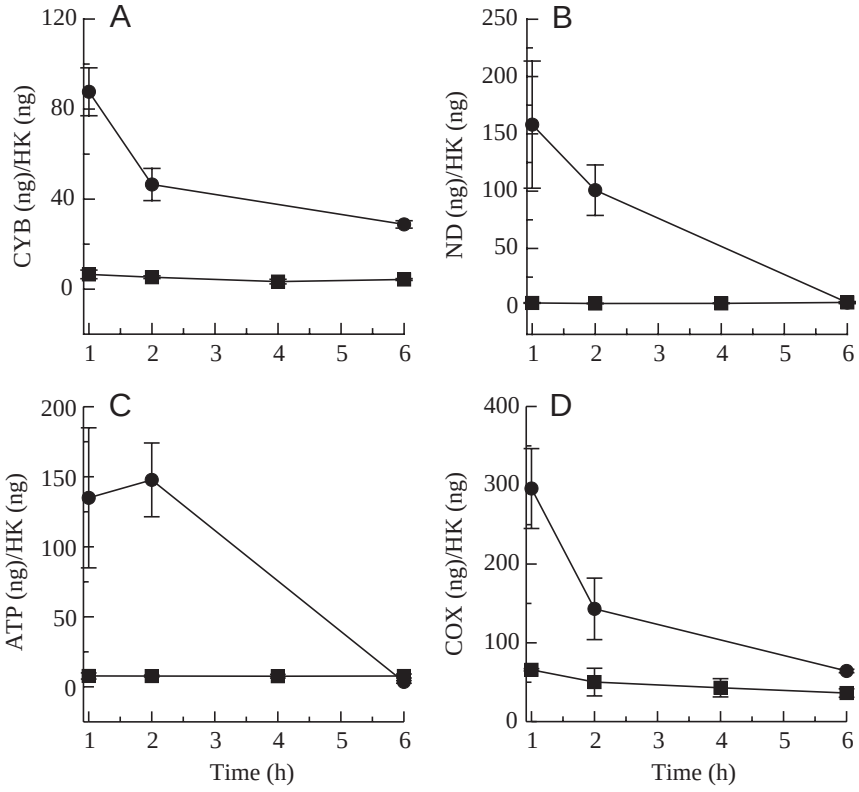

Fig. 4. Anoxia plus 1.5 mmol l^{-1} ATP (filled squares) has a stabilizing effect on mitochondrial mRNA levels. Filled circles, control incubations (normoxic, pH 7.8). Data were analyzed and expressed as in Fig. 1.

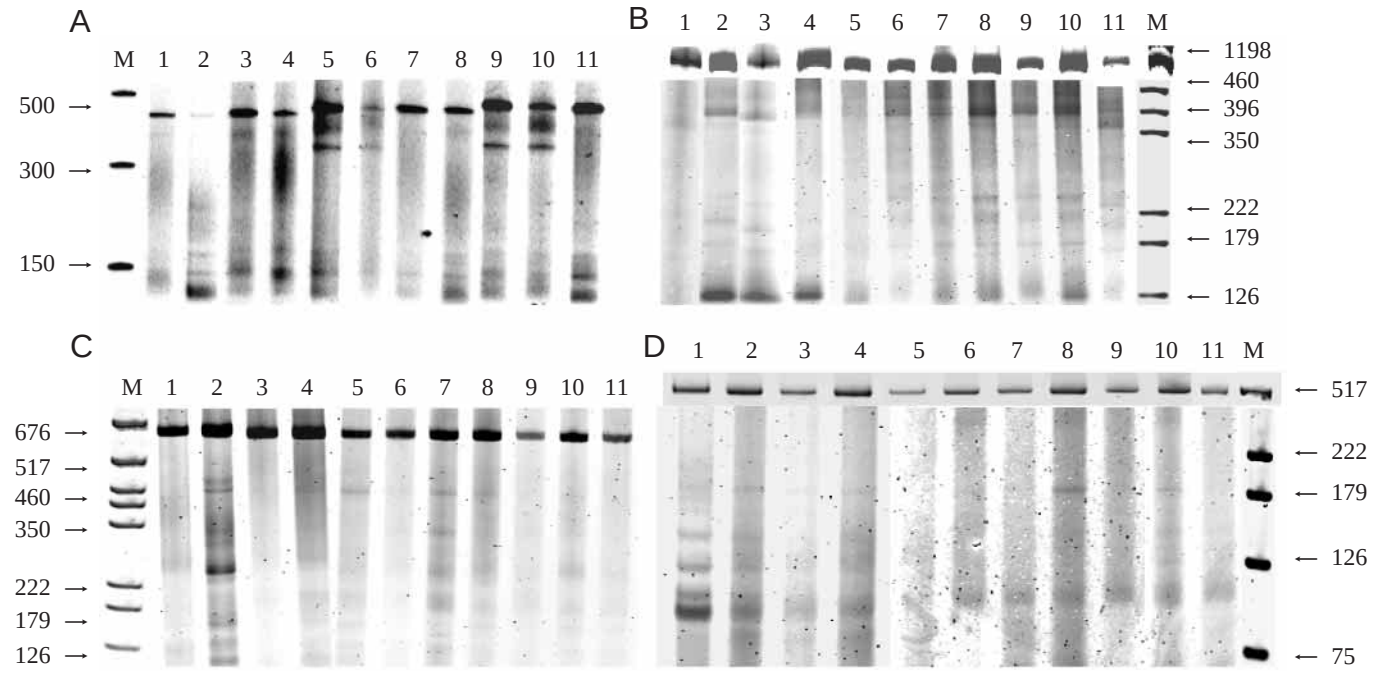

Fig. 5. Polyadenylation of mitochondrial mRNAs of *A. franciscana* embryos under control and various experimental treatments *in organello*. Following RACE-PAT, products were electrophoresed on 6% polyacrylamide at 100 V and stained with ethidium bromide. The fluorescence intensities for polyadenylated bands in each lane were quantified using ImageQuant software and normalized to the intensity of the upstream, non-adenylated band (uppermost band) and used in Figs 6 and 7. COXI (A), CYB (B), ND1 (C) and ATP6 (D); lane 1, control (30 min); lane 2, control (6 h); lane 3, low pH (30 min); lane 4, low pH (6 h); lane 5, low pH plus anoxia (1 h); lane 6, low pH plus anoxia (6 h); lane 7, anoxia (1 h); lane 8, anoxia (6 h); lane 9, ATP plus anoxia (0 h); lane 10, ATP plus anoxia (1 h); lane 11, ATP plus anoxia (6 h). M, markers (nucleotides). For CYB (B) and ND1 (C), lanes from two different gels are combined, and in all cases contrast has been adjusted among lanes to enhance visualization. For presentation purposes in this figure, the high intensity of the reference bands in B and D were uniformly reduced so the polyadenylated bands could be viewed at proper intensity.

based assay, which revealed that the 3′ tails were extensively polyadenylated under control conditions (normoxia, pH 7.9), but that after 6 h incubation most treatment groups showed large and significant decreases in polyadenylation. Although the sizes of PAT did not typically change from treatment to treatment, our method showed that the relative amounts of each size class of PAT did change over time. This observation is important for several reasons. First, the assay was able to detect a range of PAT sizes in all samples, and it provided a semi-quantitative measure of relative abundance for each size. Both PCR amplification and ethidium bromide staining are sensitive to the size of the nucleic acid strand in question, so the results are not strictly quantitative (Salles et al., 1999). However, the use of restriction digests after the PCR amplification confirmed that the reaction for each message was specific (and no amplifications had been initiated at internal sites within the transcripts), as well as providing a way to normalize each reaction to the upper, non-polyadenylated band. Therefore, quantitative differences between time points or treatments reflected the PAT status of the mRNA as originally isolated. The results of these experiments demonstrated that under conditions reflecting anoxia-induced quiescence, mitochondrial transcript degradation is decreased, and the increased half-life is associated with reduced levels of mRNA polyadenylation.

Previous work in this laboratory and others has shown that a downregulation of gene expression occurs in *A. franciscana* embryos during quiescence (reviewed by Hand, 1998). For example, nuclei isolated from embryos exposed to 4 h of anoxia *in vivo* decrease incorporation of [α-^{32}P]-UTP by >79% relative to controls, with a further decrease to 88% of control values after 24 h of anoxic incubation (van Breukelen et al., 2000). The acidification of pH_i during anoxia accounts for over half of the effect seen *in vitro* (van Breukelen et al., 2000). In addition, isolated mitochondria show a marked decrease in [α-^{32}P]-UTP incorporation rate either under anoxia (89%) or at pH 6.4 (74%; Eads and Hand, 2003). Furthermore, new transcriptional initiation during the assay was shown to decrease dramatically at pH 6.4, from 77% at pH 7.9 to 31% at pH 6.4 (Eads and Hand, 2003). The decrease in transcription seen under anoxia is mirrored by the blockage of the ontogenetic increase in both actin and COXI mRNA by anoxia and aerobic acidosis *in vivo* (Hardewig et al., 1996). These observations point to a sharp decrease in anabolic activity for these reactions, coupled to decreased degradation of RNA pools, to preserve stores during prolonged quiescence.

In the context of the current study, it is appropriate to note that protein synthesis and degradation studies also support the hypothesis that gene expression is arrested under anoxia, and

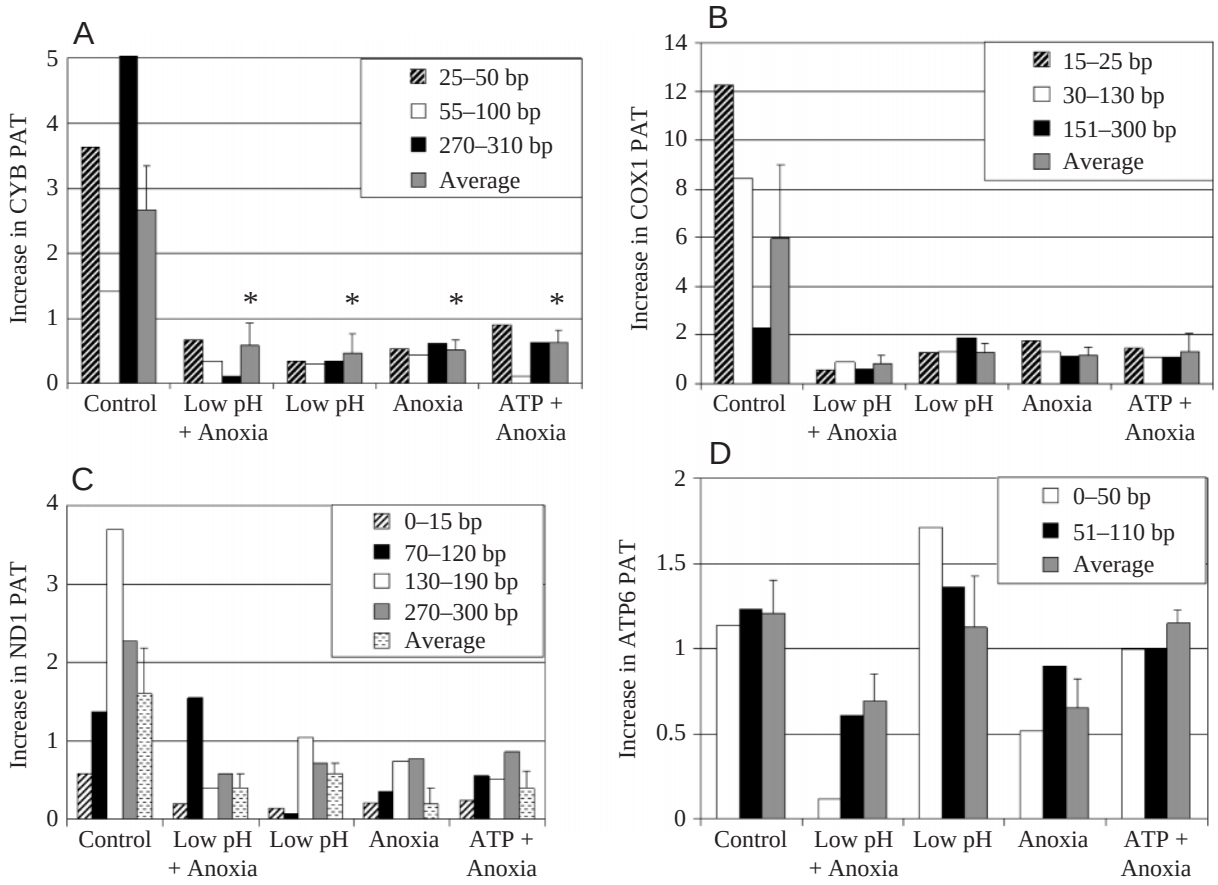

Fig. 6. Size-class-specific changes in amount of poly(A)$^+$ tail (PAT) over time as a function of *in organello* treatment. Mitochondria were incubated under the indicated conditions, RNA extracted and PAT analysis performed. Increases represent the ratio of PAT amounts present after 6 h compared to 1 h in the size classes indicated. (A–D) The four mRNA species studied: CYB (A); COXI (B); ND1 (C); ATP6 (D). Asterisks indicate significant differences from controls. Values without error bars are data taken directly from gels shown in Fig. 5; values with error bars represent mean changes in total polyadenylation from 1–6 h for N=3 separate experiments.

that the pH_i drop plays a significant role (reviewed by Hand, 1997). Mitochondrial protein synthesis is decreased under conditions of low pH and anoxia (Kwast and Hand, 1996a,b), and under anoxia the half-life of cytochrome *c* oxidase protein is increased by 77-fold (Anchordoguy et al., 1993). By analogy, we expected to see an increase in mRNA half-life under anoxia due to a decrease in degradation, which was indeed the case, although not to the same extent as estimated for protein. Because mitochondrial mRNA levels remain virtually unchanged during the period when translation is arrested (the present study; Hardewig et al., 1996), it seems clear in *A. franciscana* that message limitation during anoxia is not responsible for the rapid depression of mitochondrial protein synthesis.

Taken together, these data show that both pH and anoxia are able to influence mitochondrial RNA metabolism independently of one another, which may indicate that the influence of these two factors are mediated by separate mechanisms. Distinct

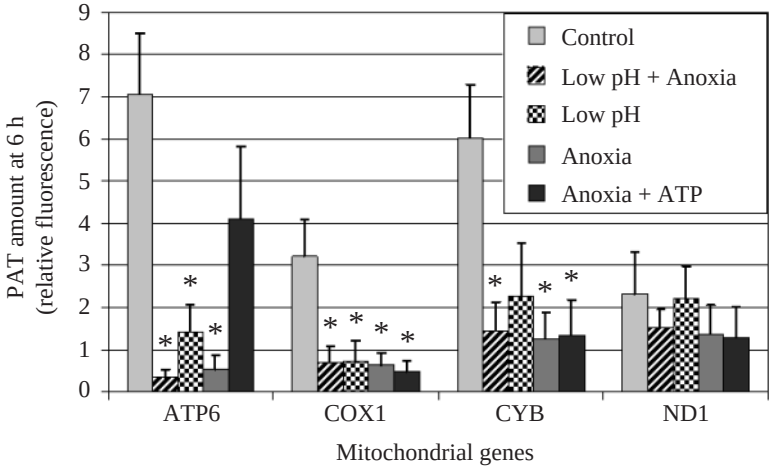

Fig. 7. Levels of total polyadenylation in all size classes combined are lower under all experimental treatments relative to control conditions (normoxia, pH 7.8). The amounts of fluorescence in poly(A)$^+$ tails (PAT) from the gels in Fig. 5 were normalized and 6 h time points compared. Asterisks indicate significant differences from controls for the mean of N=3 separate experiments.

signaling cascades or different target processes could be used to affect the arrest of RNA metabolism. For example, it has been shown that transcription initiation is sensitive to low pH but not anoxia (Eads and Hand, 2003). On the other hand, our data showing that conditions of anoxia and low pH combined have an effect on mRNA half-life similar to that of either treatment alone may indicate a common target. One parsimonious explanation is that the acidic pH_i during anoxia is a general inhibitor, while there are specific O_2-sensitive processes that also influence gene expression. Identifying the *cis*- and *trans*-elements of RNA metabolism in mitochondria is important for understanding the regulatory mechanisms of gene expression.

Although the role of nucleo-cytoplasmic polyadenylation in gene expression has been studied extensively in developing organisms (cf. Bashirulla et al., 2001; Richter, 1999), there are little data available on the role of the PAT in mitochondrial RNA metabolism. In both *Xenopus* sp. and *Drosophila* sp., changes in PAT length of cytoplasmic message are known to signal a variety of important gene expression events, including mRNA localization, translation/silencing and degradation (Richter, 1999), while very little is known about these processes in animal mitochondria (reviewed by Taanman, 1999; Scheffler, 1999). Although poly(A)$^+$ length has been examined in parallel with mRNA half-life in murine mitochondria, only ribosomally bound mRNAs were used in the analysis (Avadhani, 1979). Two classes of mRNA with distinct half-lives were present on the ribosomes and these mRNAs possessed PATs of 55–75 nucleotides (Avadhani, 1979). However, any inference about the relationship between mRNA half-life and PAT length in this case is confounded with translation. Given that only 10% of mitochondrial mRNA may be bound by ribosomes (Scheffler, 1999), it may be misleading to use only ribosomally bound mRNA in examining the relationship between PAT length and message half-life.

A report of mRNA degradation in trypanosome mitochondria documents the presence of a UTP-dependent mechanism that preferentially and rapidly degrades poly(A)$^+$ RNA, as well as a UTP-independent process that does not depend on a PAT (Militello and Read, 2000). Considering the unusual features of trypanosome mitochondrial transcription involving UTP such as editing and 3′ uridylation, it is not clear that the UTP-dependent pathway of degradation is representative of mitochondrial RNA metabolism in general. However, the rapid degradation of polyadenylated mRNAs in mitochondria described here for *A. franciscana* embryos may reflect a more general phenomenon. Similar reports exist for plant mitochondria (Gagliardi and Leaver, 1999; Kuhn et al., 2001), chloroplasts (Hayes et al., 1999; Schuster et al., 1999), and bacteria (Sarkar, 1997; Blum et al., 1999; Carpousis et al., 1999). Thus it appears that polyadenylation has an opposite effect on mRNA stability in mitochondria to that seen in the cytoplasmic compartment of eukaryotic cells.

Mechanisms for altering mRNA stability during oxygen limitation that do not involve polyadenylation have received attention for a few selected mRNA species in mammalian cells (reviewed by Bunn and Poyton, 1996). For instance, the increased stability of mRNAs for erythropoietin and vascular endothelial growth factor during hypoxia has been documented, and appears to be due in part to the activity of 3′-UTR binding proteins that protect the mRNAs from degradation (McGary et al., 1997; Claffey et al., 1998). Several specific RNA-binding activities have also been documented for metabolic enzymes, including glutamate dehydrogenase and catalase (Hentze, 1994), glyceraldehyde-3-phosphate dehydrogenase (Nagy and Rigby, 1994) and lactate dehydrogenase (Pioli et al., 2002). Similarly, ribosomal proteins from chloroplasts are able to stabilize ribosome-free mRNAs (Nakamura et al., 2001). However, *Artemia* mitochondrial genes lack UTR binding sites, which are the apparent basis of the activities described above, and argues against this scenario. An alternative mechanism for stabilizing mRNA, the lack of ATP as a substrate for nuclease activity, is also unlikely to be operating in *A. franciscana* because exogenous addition of 1.5 mmol l^{-1} ATP did not increase message degradation rate (Fig. 4).

It is appropriate to speculate as to why the relationship between polyadenylation and decreased stability of mRNA in prokaryotes/organelles is counter to that observed for the nucleo-cytoplasmic compartment. In the eukaryotic cytoplasm, PATs are associated with mature, fully processed messages and are generally required for successful translation, although the role of the PAT in transcript degradation and translation is complex (Mitchell and Tollervey, 2000). Addition of a PAT is not required in *Artemia* to create a complete stop codon for most genes (Valverde et al., 1994), and is likely not required for translation. In fact, recent unpublished data for *A. franciscana* embryos (S. Hand, B. Eads, D. Jones) indicate that ribosomal RNA in the mitochondrion is polyadenylated while that in the cytoplasmic compartment is not, thus implying that mitochondrial polyadenylation is not being used as a translation signal. Others have noted the possibility that the PAT plays a more general role in RNA metabolism in bacteria (Li et al., 1998). The lower overall levels of polyadenylation in bacteria and many organelles than in the eukaryotic cytoplasm further indicates that PATs may not be required for successful translation. Rather, in these less complicated systems, polyadenylation of mitochondrial message might provide an organizing center for nuclease(s) to begin digesting the 3′ end. Recent work in potato mitochondria (Gagliardi et al., 2001) provides support for the idea that 3′ polyadenylation promotes 3′–5′ degradation. While differences in the mechanism of degradation may exist between plant and animal mitochondria on one hand, and chloroplasts and eubacteria on the other, the relationship between elevated polyadenylation and increased degradation appears to have been conserved between these groups (Gagliardi et al., 2001). Animal mitochondria typically contain very little non-coding DNA and have extremely compacted coding regions, while chloroplasts and plant mitochondria are generally much larger and contain genes not found in animal mtDNA, relatively more noncoding

DNA, and intergenic spacers and UTRs that could serve as *cis*-processing signals (Scheffler, 1999). Perhaps nuclear genes have subsumed roles in animal mitochondria that remain mitochondrially encoded in other groups. The machinery responsible for organellar RNA degradation is generally unknown, although some mitochondrially localized nucleases have been isolated and characterized in yeast (Margossian and Butow, 1997; Min and Zassenhaus, 1993) and in various other organisms (see Morales et al., 1992; Alfonzo et al., 1998; Puranam and Attardi, 2001). Certainly the involvement of polyadenylation in mitochondrial mRNA turnover and translation is only beginning to be understood.

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