The Journal of Experimental Biology 206, 577-589

doi:10.1242/jeb.00110

Transcriptional initiation under conditions of anoxia-induced quiescence in mitochondria from *Artemia franciscana* embryos

Brian D. Eads[1,2,*] and Steven C. Hand[1,†]

[1]*Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA* and [2]*Department of Environmental, Population and Organismic Biology, University of Colorado, Boulder, CO 80303-0334, USA*

*Present address: Department of Zoology, University of Wisconsin, Madison, WI 53706, USA

†Author for correspondence (e-mail: shand@lsu.edu)

Accepted 28 October 2002

Summary

In response to anoxia, embryos of the brine shrimp *Artemia franciscana* are able coordinately to downregulate metabolism to levels low enough to permit survival for several years at room temperature. In addition to dramatic decreases in free ATP levels and heat production, intracellular pH drops from 7.8 to 6.3 overnight. Use of isolated mitochondria to study transcriptional responses to anoxia offers several advantages: (1) the localized nature of transcript initiation, processing and degradation, all of which may be followed *in organello*; (2) the relatively simple cis- and trans-machinery involved and (3) the ability to provide relevant physiological treatments *in vitro*. In response to anoxic incubation of embryos *in vivo* for 4 h followed by anoxic mitochondrial isolation and anoxic transcription assay at pH 6.4, a significant decrease in overall UTP incorporation (77%) was seen after 30 min relative to normoxic, pH 7.9 controls. A less severe inhibition of transcription under anoxia (52%) was observed compared with controls when pH was raised to 7.9. Similarly, under normoxia, the incubation at low pH (6.4) reduced transcription by 59%. Ribonuclease protection assays showed that the contribution of *in vitro* initiation during the assay fell from 78% at pH 7.9 to approximately 32% at pH 6.4 under either normoxic or anoxic conditions. DNA footprinting of putative transcriptional promoters revealed proteins at regular intervals upstream of the 12S rRNA in the control region, which previously had been indirectly inferred to contain promoters for H-strand transcription. The area between 12 030 and 12 065 contains a sequence in the $tRNA_{leu}$ gene believed to bind the transcription termination factor mTERF or TERM, and we provide the first evidence that this sequence is protein-bound in *A. franciscana.* However, our hypothesis that initiation is reduced at low pH because of a change in DNA binding by mitochondrial transcription factors was not confirmed. We propose that regulation of initiation may be mediated by covalent modification or by protein–protein interactions not detected by footprinting.

Key words: anoxia, transcriptional initiation, mitochondria, *Artemia franciscana*, brine shrimp, pH, hypometabolism, ribonuclease protection assay, DNA footprinting, gene expression.

Introduction

The survival of animals exposed to a chronic lack of oxygen (anoxia) appears to depend upon a coordinated depression of energy consumption and production. Although the physiological mechanisms employed to achieve this are varied (Hochachka et al., 1993; Grieshaber et al., 1994; Hardewig et al., 1996; Somero, 1998; Hand et al., 2001), a correlation exists between the degree of the metabolic depression achieved and the duration of tolerance (Hand, 1998). In response to anoxia, embryos of the brine shrimp *Artemia franciscana* are able coordinately to downregulate metabolism (Warner and Clegg, 2001; Hand et al., 2001) to a degree that allows them to survive for more than 4 years at room temperature (Clegg, 1997). This long-term survival implies that even seemingly minor sources of ATP turnover, such as transcription, which is thought to account for only 10% or less of total energy flux in mammalian cells (Buttgereit and Brand, 1996; Rolfe and Brown, 1997), must be reduced in order to ensure long-term survival. The striking degree of metabolic arrest in *A. franciscana* embryos during anoxia-induced quiescence has been demonstrated for a number of different cellular processes, including translation (Clegg and Jackson, 1989; Hofmann and Hand, 1994), mitochondrial translation (Kwast and Hand, 1996a,b) and nuclear transcription (van Breukelen et al., 2000). However, no evidence has been available regarding mechanisms of mitochondrial transcription. Using isolated mitochondria to study transcriptional responses to anoxia offers several advantages: the localized nature of transcript initiation, processing, and degradation, all of which may be followed *in organello*; the relatively simple cis- and trans-machinery

involved; and the ability to provide relevant physiological treatments *in vitro*. The present study used isolated mitochondria of *A. franciscana* to monitor transcription events *in organello* under anoxia and provide a greater understanding of the mechanisms involved in cellular quiescence.

During the first hour of anoxic exposure, intracellular pH (pHi) in *A. franciscana* embryos drops by approximately one full unit, from 7.8 to 6.8, and embryos respond with a rapid and profound arrest of both anabolic and catabolic processes that are triggered in part by this drop (Clegg, 2001; Hand, 1998). For example, run-on assays using isolated nuclei from *A. franciscana* embryos demonstrated a decrease in transcription of over 80% in response to anoxic incubation and by 55% under artificial acidification (van Breukelen et al., 2000). The current study used isolated mitochondria in a similar fashion to measure transcription, with the added experimental use of an anoxic treatment *in vitro* based upon the ability of mitochondria to re-initiate transcription *de novo*. The use of nuclear run-on assays to provide a 'snapshot' of transcription at the time of nuclear isolation is predicated on the observation that new initiation of transcription does not occur *in vitro* (e.g. Stallcup et al., 1978). For isolated mitochondria, however, it has been demonstrated that transcription initiation can occur in isolated organelles *de novo*; i.e. radioisotope incorporation is not strictly a reflection of transcriptional activity *in vivo* but, to a large extent, reflects incubation conditions *in vitro* (Gaines and Attardi, 1984). In fact, over half of the measured transcriptional activity in mitochondria of HeLa cells seemed to result from initiation (Gaines and Attardi, 1984), and so it was of interest to us to measure new initiation in our assay system. We therefore developed a ribonuclease protection assay (RPA) to measure [α-^{32}P]UTP incorporation in upstream and downstream portions of the 12S rRNA, which in *A. franciscana* mitochondria lies immediately downstream of the major heavy strand (H strand) transcriptional promoter (Carrodeguas and Vallejo, 1997). Our results establish not only that new initiation contributes substantially to transcription in isolated mitochondria but also that at low pH (6.4) this activity is largely inhibited.

Regulation of mitochondrial transcription could logically be expected at the level of initiation, but information regarding this process in *A. franciscana* is lacking. In all metazoan mitochondria studied to date, transcriptional initiation occurs in an area typically ≤1 kb known as the control region (Taanman, 1999). Previous studies with isolated mitochondria have demonstrated the utility of DNA footprinting analysis for documenting protein–DNA interactions correlated with changes in transcription, specifically at transcriptional promoters in the control region (Ghivizzani et al., 1993, 1994; Cantatore et al., 1995; Micol et al., 1997; Enríquez et al., 1999). We therefore chose to examine promoter occupancy to test the hypothesis that reductions in *de novo* initiation at low pH were caused by polymerase or transcription factor absence by using methylation interference and primer extension analysis. Our results indicate that promoter occupancy does not change with *in vitro* incubation conditions, so decreased re-initiation at acidic pH is not due to a lack of DNA–protein interactions but to other possible mechanisms such as covalent modification.

Materials and methods

Mitochondrial isolation and in vitro *treatments*

Artemia franciscana Kellog embryos (Great Salt Lake population) were obtained from Sanders Brine Shrimp Co. (Ogden, UT, USA) and stored at –20°C until use. Embryos were hydrated in ice-cold tapwater for 4 h, incubated aerobically in 0.25 mol l^{-1} NaCl for 8 h, dechorionated and washed as described by Eads and Hand (1999). Mitochondria were isolated by differential centrifugation, and respirometry was performed to assess functionality as previously described (Kwast et al., 1995; Eads and Hand, 1999). The final mitochondrial pellet was resuspended in 0.6 ml of fortified homogenization buffer (FHB), which consisted of 0.5 mol l^{-1} sucrose (Pfansteihl, Waukegan, IL, USA), 10 mmol l^{-1} MES [2-(N-morpholino)ethanesulfonic acid], 10 mmol l^{-1} Hepes, 100 mmol l^{-1} KCl, 10 mmol l^{-1} KH_2PO_4, 3.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EGTA and 0.5% (w/v) bovine serum albumin (BSA; fatty acid free, fraction V; Sigma Chemical Co., St Louis, MO, USA), pH 7.0 at room temperature. *In organello* treatments (pH 6.4 and anoxia) and controls (pH 7.9 and normoxia) were performed on mitochondria from the same isolation. From the point of homogenization onwards, all manipulations were performed under RNase-free conditions. Diethylpyrocarbonate (DEPC; 0.1%) was used to treat solutions, all glassware was baked at 250°C for 4 h, and only sterile or autoclaved plastics were used.

For low pH treatments, 250 µl of mitochondria were added to 600 µl of FHB (pH 5.9), resulting in a final pH of 6.35, while another 250 µl of mitochondria were added to 680 µl of FHB at pH 8.3 to give a final pH of 7.88. The pH was measured with a Radiometer electrode (model GK2401C) at the beginning and end of the incubation. For *in vitro* anoxia, the FHB was made without adding the BSA initially to prevent frothing of the buffer during gassing. The mixture was vigorously bubbled with argon in a nitrogen-purged glovebag for more than 30 min, which was sufficient to drive off any oxygen measurable with a Strathkelvin model 1302 polarographic oxygen electrode (defined here as nominally anoxic). The FHB was then added to 0.5% BSA (final concentration) that had equilibrated in a nitrogen-purged glovebag, and 1 mol l^{-1} KOH was added to give the appropriate pH (770 µl for pH 7.88, 320 µl for pH 6.35 in 10 ml total). This FHB was then added to the mitochondrial pellets, which had been drained of supernatant after the final centrifugation step, and allowed to equilibrate at 0°C in the purged glovebag for at least 30 min. The mitochondria were then incubated on ice for 30 min before the start of the assays. All anoxic assays were performed in their entirety inside the nitrogen-purged glovebag.

The effect of anoxia on mitochondrial transcription

To test the effect of anoxia on mitochondrial run-on transcription assays, we exposed both the embryos (*in vivo*) and their isolated mitochondria (*in vitro*) to anoxic incubations. This approach was taken to ensure that incubation conditions reflected as closely as possible the intracellular milieu of an animal undergoing anoxic exposure. We therefore performed all steps of mitochondrial isolation anoxically and at low temperature. After dechorionation and an 8 h developmental incubation, embryos were placed in nitrogen-bubbled 0.25 mol l^{-1} NaCl for 4 h. Embryos were then chilled on ice, transferred anaerobically to a nitrogen-purged glovebox, blotted dry, and 10 g of tissue was then homogenized with homogenization buffer made anoxic as described above. All subsequent steps of the isolation procedure were performed in the glovebox, except for centrifugation runs, which were performed after transferring the preparation into gas-impermeable centrifuge tubes with screw-caps (Oak Ridge 3119-0500). The isolated mitochondria were then stored on ice in anoxic FHB at the appropriate pH (6.4 or 7.9) until use. It is appropriate to note that mitochondria stored on ice for 9 h after isolation showed no decrease in transcription relative to mitochondria assayed 45 min after the same isolation (data not shown).

Transcriptional run-on assays were performed essentially as described previously (Eads and Hand, 1999). Briefly, assays were initiated by adding 50 μmol l^{-1} each of ATP, CTP and GTP, 100 μmol l^{-1} UTP and 3.7 MBq [α-^{32}P]UTP (222 TBq $mmol^{-1}$, 370 MBq ml^{-1}; Perkin-Elmer, Wellesley, MA, USA) to 80 μl of mitochondria diluted with FHB and 10 units of RNasin to a final volume of 210 μl. Assuming a negligible contribution of endogenous UTP from the mitochondrial preparation, the final specific radioactivity of UTP was 176.5 GBq mol^{-1}. The transcription reaction was allowed to proceed at 30°C, and 30 μl aliquots were applied to glass-fiber filters (GF/C; Whatman) at the indicated time-points. Transcription was measured as the incorporation of [α-^{32}P]UTP into trichloroacetic acid-insoluble RNA counted by liquid scintillation (Eads and Hand, 1999). Mitochondrial protein content was measured by a modified Lowry assay (Peterson, 1977). Statistical analysis was carried out using the unpaired Student's *t*-test or analysis of variance (ANOVA) using Statview software (SAS Inc., Cary, NC, USA). Values are presented as means ± S.E.M.

Measurement of transcriptional initiation for mitochondria with a nuclease protection assay

The contribution of *in vitro* (or *de novo*) transcriptional initiation to the run-on assays described in Eads and Hand (1999) were measured using a methodology adapted from Gaines and Attardi (1984). The main site for heavy-chain transcription initiation in *A. franciscana* mitochondria is immediately upstream (5′) from the 12S rRNA (Carroduegas and Vallejo, 1997), so 5′ and 3′ fragments of this gene were chosen for measuring new initiation (see Fig. 1 for a schematic rationale). Primers were chosen to PCR-amplify a fragment of approximately 300 bp at the 5′ end and 110 bp at the 3′ end of the 12S rRNA (Table 1) using *A. franciscana* mtDNA. These fragments were gel-purified and ligated into pGem-T vectors (Promega, Madison, WI, USA) according to the manufacturer's instructions. Orientation of inserts was established by restriction digest; restriction sites were chosen using the program available from SUNY Geneseo Biology at http://darwin.bio.geneseo.edu/~yin/WebGene/RE.htm. The clones yielding antisense RNA were used for synthesis reactions, and antisense RNA was produced with Ambion's Megascript kit (Ambion, Austin, TX, USA). These unlabeled antisense riboprobes were used in the nuclease protection assays described below.

Mitochondria were given *in organello* treatments as outlined earlier. However, for treatment with anoxia *in vivo*, hydrated embryos were dechorionated, given an 8 h developmental

Table 1. *Locations and sequences of primers used in ribonuclease protection assays and primer extension reactions*

Primer name	Genomic location*	Sequence†
RPA antisense clones		
12S 5′ forward	13 996-13 977	5′ AGTTTGATTCTAGCTAGTTT (H)
12S 5′ reverse	13 715-13 696	5′ AGGGTATCTAATCCTAGTTC (L)
12S 3′ forward	13 399-13 380	5′ GTCTATCAACCTGAAGGAGG (H)
12S 3′ reverse	13 309-13 290	5′ TTTCAAGATACACCTTCCGG (L)
Footprinting		
LSP 1	15 664-15 683	5′ AAGAATTATATGTTTGATAA (H)
LEU 1	12 125-12 144	5′ GTAAGCCAGGTTAGTTTCTA (H)
HSP 1	14 061-14 080	5′ CTTTGTCCGTTACTATTACT (H)
HSP 2	13 890-13 909	5′ ACTAGCTAGAATCAAACTTT (L)
HSP 3	14 171-14 190	5′ TCAAAGCAGTCTCCCCGGCG (H)
HSP 4	14 250-14 269	5′ ATTTAAATGTGGAAAAATAC (H)
HSP 5	13 921-13 940	5′ TCACTGCACTTCTAACCTAT (L)

*Numbered according to Valverde et al. (1994a). Accession number NC 001620.
†H and L denote heavy and light chain, respectively.

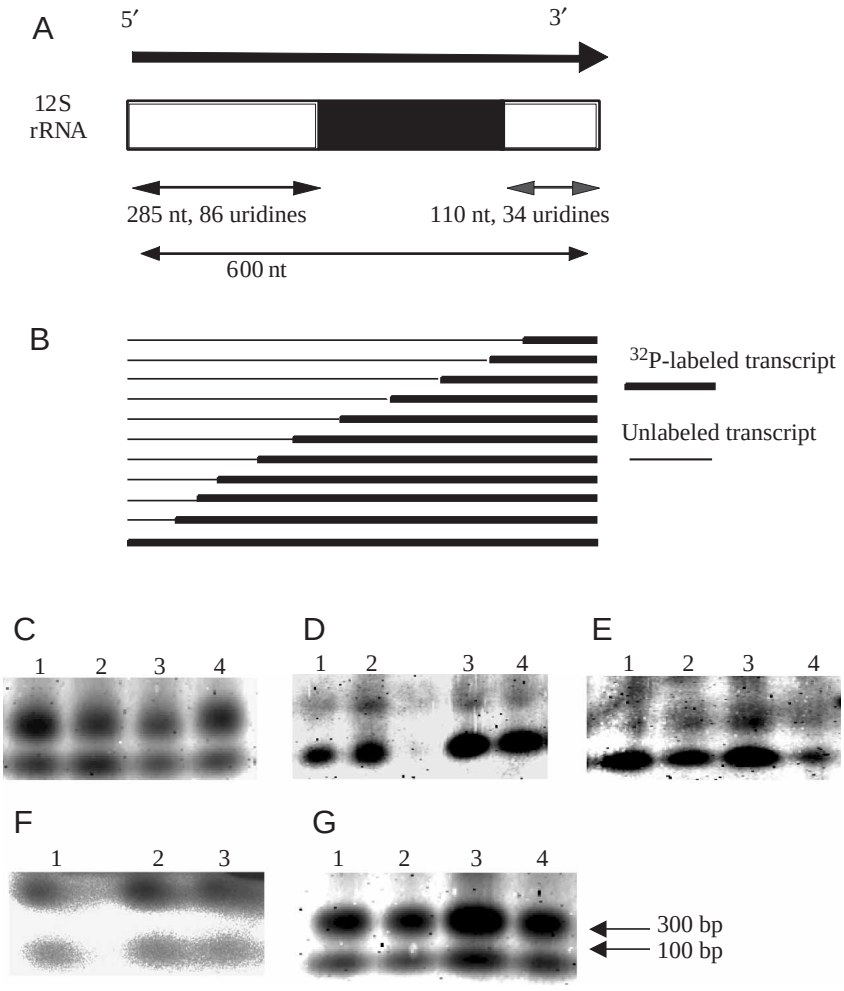

Fig. 1. Schematic of the rationale and methodology used to calculate the contribution of *in vitro* (*de novo*) initiation to mitochondrial transcription in *Artemia franciscana* (after Gaines and Attardi, 1984). Transcription initiation site of the H strand lies 5′ to the 12S rRNA (Carrodeguas and Vallejo, 1997). (A) Unfilled boxes represent 12S rRNA fragments labeled *in organello* with [^{32}P]UTP and protected from nuclease digestion by antisense riboprobes. The filled box represents the unprotected portion of the 12S rRNA not measured by the assay. The sizes and UTP contents of the fragments are shown, and RNA polymerase distributions are assumed to be random, which would give the labeling patterns illustrated in (B). Under the assumption of no *in vitro* initiation, the radioactivity in the 5′ fragment would be a function of the length of this labeled, protected piece multiplied by the number of uridines it contains. Similarly, the radioactivity in the 3′ fragment would be a function of the average length of the labeled fragment plus the length of the 12S rRNA upstream of it (which accounts for the polymerases upstream at the time that label is added) multiplied by the number of uridines. However, if all labeling is due to *de novo* initiation, the labeling of 5′ and 3′ fragments would occur simply in proportion to their U content (86 and 34, respectively). Using these assumptions, the proportion of labeling in the protected fragments of 12S rRNA due to *de novo* initiation or elongation can be calculated by solving the following two equations: (1) radioactivity in 5′ fragment=0.55*X*+2.52*Y* and (2) radioactivity in 3′ fragment=*X*+*Y*, where *X* represents counts due to elongation, *Y* represents counts due to *de novo* initiation, 2.52 is the ratio of 86:34 uridines, and 0.55 is calculated from the equation: 5′ radioactivity/3′ radioactivity= (285/2)×86U/(110/2+600)×34U=0.55. In other words, the ratio of 2.52*Y* to 0.55*X* gives the ratio of radioactivity from initiation to that from elongation *in vitro* in the 5′ fragment. (C–G) Transcriptional initiation decreases under conditions of acidic pH for *A. franciscana* mitochondria. 12S rRNA products from ribonuclease protection assays were labeled *in organello*, separated by gel electrophoresis and quantified with a phosphorimager. Panel C, control mitochondria (normoxia, pH 7.9); panel D, normoxia at pH 6.4; panel E, *in vitro* anoxia at pH 6.4; panel F, *in vitro* anoxia at pH 7.9; and panel G, *in vivo* anoxia assayed aerobically at pH 7.9. Lanes are experimental replicates. Arrows indicate migration distance of standards.

incubation and then transferred to media bubbled with 100% nitrogen for 4 h. Embryos were blotted dry, homogenized aerobically, and mitochondria were isolated as described previously (Eads and Hand, 1999). To start the assay, 180 µl of mitochondria in FHB were incubated at 30°C with 50 µmol l^{-1} each of rATP, rCTP and rGTP, 10 units of RNasin, 70 mmol l^{-1} dithiothreitol and 3.7 MBq of [α-^{32}P]UTP in a final volume of 200 µl. After 30 min, 1 ml of a lysis buffer, containing 4.5 mol l^{-1} guanidinium thiocyanate, 50 mmol l^{-1} EDTA, 25 mmol l^{-1} Tris-HCl, 100 mmol l^{-1} β-mercaptoethanol, 0.2% antifoam A (Sigma) and 2% *N*-laurelsarcosine, was added to the mitochondrial mixture. After thorough vortexing, the sample was centrifuged at 10 000 ***g*** (4°C) for 5 min, and an equal volume of ice-cold 100% isopropanol was added to the supernatant. The sample was chilled at –20°C for 2 h, centrifuged at 14 000 ***g*** for 30 min (4°C) and washed with 70% isopropanol. Due to low specific activity of the labeled RNA, the entire nucleic acid pellet (approximately 10^8 d.p.m.) was taken up in a final volume of 30 µl of hybridization buffer, which contained 5 mol l^{-1} guanidinium thiocyanate, 5 mmol l^{-1} EDTA (pH 7.0) and 3–5 µg unlabeled antisense RNA. The hybridization reaction was incubated for at least 6 h at 37°C. Control reactions containing no unlabeled probe or no labeled sample were run in parallel.

Due to the large amounts of labeled, unhybridized RNA, the samples were divided into three aliquots for nuclease digestion. Each 10 µl aliquot was added to 240 µl of buffer containing 450 mmol l^{-1} NaCl, 25 mmol l^{-1} Tris (pH 8.0) and 5 mmol l^{-1} EDTA with 15 µl of nuclease cocktail (Ambion) and incubated at 37°C for 2 h. A control with no nuclease cocktail was also incubated in parallel. 250 µl of K buffer, containing 0.3%

sarkosyl, 20 μg proteinase K and 1 mmol l^{-1} $CaCl_2$ (final concentrations), was then added and incubated for 1 h at 37°C. The samples were then phenol–chloroform extracted twice (phenol pH 4.0), extracted once with chloroform–isoamyl alcohol (24:1) and ethanol precipitated with 10 μg yeast tRNA. Pellets were washed in 90% ethanol, dried and solubilized in 8 μl 100% deionized formamide. At this point, pellets from the same hybridization were generally recombined, although in some cases they were run separately. After denaturing for 1 min at 80°C and quick chilling, samples were run with 1 μl loading dyes (6×; Promega) in 8% polyacrylamide/8 mol l^{-1} urea gels in 1× TBE buffer (89 mmol l^{-1} Tris base, 89 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA) at 600–700 V for approximately 1 h. Gels were dried and imaged using a Molecular Dynamics phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). Molecular size markers (Roche, Indianapolis, IN, USA) were run in parallel and separately stained in ethidium bromide for size determination.

Footprinting of mitochondrial DNA by methylation interference

Isolated, intact mitochondria from aerobically incubated embryos were given *in organello* treatments as described above and subjected to dimethylsulfide (DMS) methylation. Briefly, approximately 4 μg mitochondrial protein were incubated with 50 μmol l^{-1} rNTPs (200 μl final volume) in FHB at 30°C for 20 min. Fresh DMS (2% in water; Acros Chemical, Geel, Belgium) was added to a final concentration of 0.05–0.2% and incubated for 3 min at 30°C. Some aliquots received no DMS *in organello* but were used for DMS treatment *in vitro* after extraction of DNA ('naked' DNA). 0.5 ml of ice-cold phosphate-buffered saline (PBS: 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 4.3 mmol l^{-1} Na_2PO_4, 1.4 mmol l^{-1} KH_2PO_4) was then added, and samples were vortexed and centrifuged at 10 000 ***g*** (4°C) for 1 min. The wash was repeated twice with 0.9 ml PBS, and the mitochondrial pellet was resuspended in 400 μl of buffer containing 10 mmol l^{-1} Tris (pH 7.5), 0.2 mol l^{-1} NaCl, 0.1% sodium dodecyl sulfate (SDS) and 0.1 mg ml^{-1} proteinase K. Samples were vortexed and incubated at room temperature for 1 h, followed by extraction with an equal volume of phenol (pH 7.9). The aqueous phase was extracted twice with phenol–chloroform and twice with chloroform–isoamyl alcohol. 50 μl of a DMS stop buffer containing 1.5 mol l^{-1} sodium acetate (pH 7.0), 1 mol l^{-1} β-mercaptoethanol and 0.1 mg ml^{-1} yeast tRNA was then added to the supernatant, followed by addition of 625 μl of ice-cold 100% isopropanol. After 2 h at –20°C, the samples were centrifuged at 14 000 ***g*** for 30 min (4°C) and washed in 70% isopropanol. The pellet was resuspended in 100 μl of 1× TE buffer (10 mmol l^{-1} Tris, pH 7.5, 1 mmol l^{-1} EDTA), reprecipitated with ethanol and resuspended in 100 μl of 1 mol l^{-1} piperidine (Acros Chemical).

Control DNA given no DMS *in organello* was resuspended in 100 μl of water instead of 1 mol l^{-1} piperidine. Aliquots of 20–40 μg nucleic acid were incubated in 200 μl of 1× TE buffer and 0.05–0.2% DMS (final concentrations) at 37°C for 2 min, then added to 50 μl of DMS stop buffer. Samples were added to 780 μl of ice-cold 100% ethanol, held at –20°C for 2 h and centrifuged at 14 000 ***g*** for 30 min. After washing in 70% ethanol, pellets were solubilized in 1 mol l^{-1} piperidine.

All samples (*in organello*-treated and naked DNA) were incubated at 90°C for 30 min, then quick-chilled at –80°C for 15 min before drying in a vacuum concentrator. Pellets were resuspended in 300 μl of water and dried twice more to remove residual piperidine. Piperidine-cleaved samples were used as template in primer-extension assays using Taq polymerase (Promega). Briefly, oligonucleotide primers (10–20 pmol) were 5′ end-labeled using T4 polynucleotide kinase (New England Biotech, Beverly, MA, USA) and 1.85–3.0 MBq [γ-^{32}P]ATP (222 TBq $mmol^{-1}$, 370 MBq ml^{-1}; Perkin Elmer), purified over G-25 Sephadex spin-columns, phenol–chloroform extracted and ethanol precipitated with 2.5 μg yeast RNA. For primer annealing and extension reactions, 0.5–1 μg of DNA was used in 50 μl reactions containing 1× PCR buffer (Taq polymerase A; Promega), 0.1 mmol l^{-1} dNTPs, 1.5 mmol l^{-1} $MgCl_2$, 5 units of Taq and 1 pmol of oligonucleotide primer (see Table 1 for primer identification). Samples were covered with mineral oil and cycled 15–20 times [94°C for 1 min, primer annealing temperature (49–62°C) for 1.5 min, 72°C for 2 min] using an MJ Research thermal cycler (MJ Research, Waltham, MA, USA).

Samples were phenol–chloroform extracted and ethanol precipitated in the presence of 20 mmol l^{-1} sodium acetate and 10 μg yeast RNA, centrifuged at 14,000 ***g*** for 30 min (4°C) and washed with 70% ethanol. After drying, pellets were resuspended in 2 μl of 100% deionized formamide plus 1 μl loading dye, denatured at 95°C for 2 min, quick chilled and electrophoresed on 8% polyacrylamide/7 mol l^{-1} urea sequencing gels. Gels were run at 1800 V, 50 W and 30 mA until xylene cyanol had reached the bottom of the gel. They were then dried and exposed on a Phoshorimager cassette (Molecular Dynamics). Bands were quantified using ImageQuant software (Molecular Dynamics), and lanes were normalized using bands outside the footprinted region to account for loading differences. Following the convention used in other reports on *in organello* footprinting (Cantatore et al., 1995; Roberti, 1999), bands showing >30% difference were considered footprinted. In this study, we designate the bases visualized for the *in organello* DMS-treated mitochondria as 'overmethylated' or 'undermethylated' by using naked DNA as the reference. Either result can indicate the presence of proteins *in organello*, as a consequence of protein-induced changes in DNA topology that allow more or less access to methylating agent.

Results

Mitochondrial transcription is decreased by anoxia and low pH

In response to anoxic incubation of embryos *in vivo* for 4 h followed by anoxic mitochondrial isolation and transcription assay at pH 6.4, a significant decrease of 77% in overall UTP

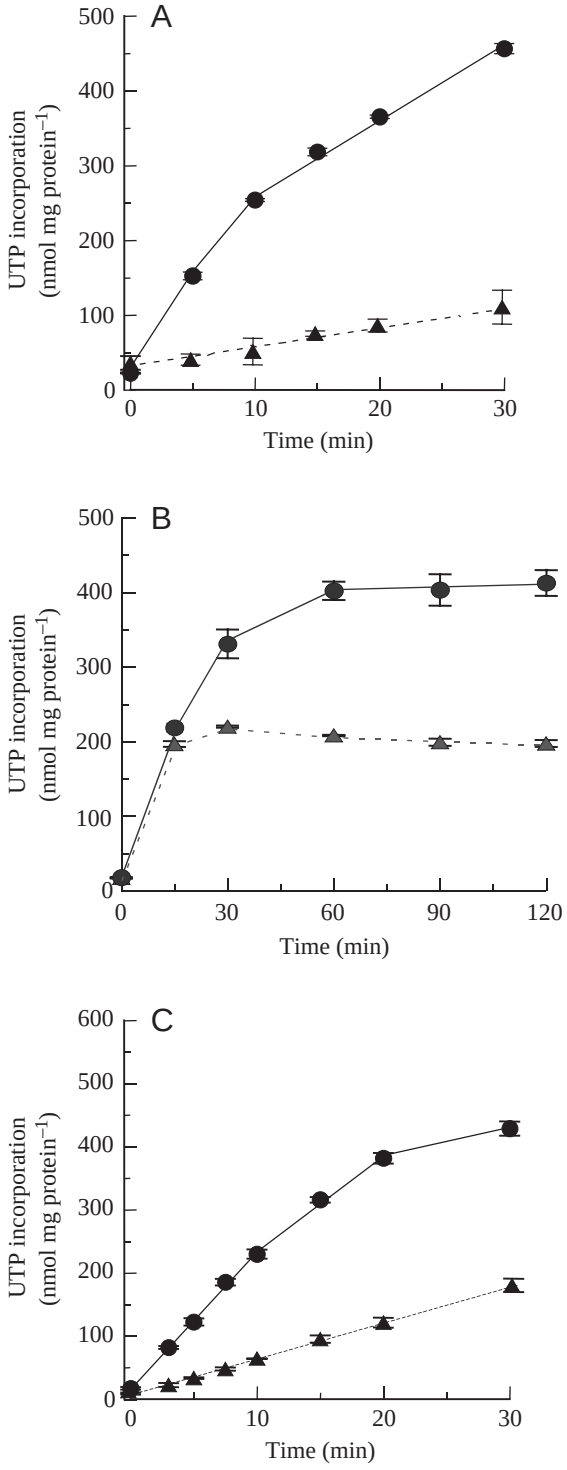

Fig. 2. Transcription by mitochondria isolated from *Artemia franciscana* embryos is depressed by anoxia and low pH. (A) Control mitochondria (normoxia, pH 7.9; circles) are compared with organelles isolated under anoxia from anoxic embryos and assayed anoxically at pH 6.4 (triangles). (B) Control mitochondria (circles) are compared with organelles isolated under anoxia from anoxic embryos and assayed anoxically at pH 7.9 (triangles). (C) Control mitochondria (circles) are compared with normoxic mitochondria incubated at pH 6.4 (triangles). Note different timescales along the *x*-axis. All values are means ± S.E.M. (N=3).

incorporation was seen after 30 min relative to normoxic, pH 7.9 controls (P<0.01; Fig. 2A, Table 2). This experimental incubation most closely reflects prevailing cellular conditions during anoxia-induced quiescence in *A. franciscana* and, when combined with the oxygen-free isolation, provides our most reliable estimate of the degree of transcriptional arrest in this state. Linear portions of the plot were used to calculate rates of RNA synthesis, which showed an 89% decrease from 3197 nmol UTP mg protein^{-1} h^{-1} under control conditions to 316 nmol UTP mg protein^{-1} h^{-1} at pH 6.4 (Table 2). The extended linearity of the incorporation at pH 6.4 is probably related to the low synthesis rate; the faster incorporation observed in controls becomes non-linear sooner. However, simply increasing the nucleotide concentration beyond the optimized level to avoid possible precursor limitation was not feasible due to nucleotide inhibition at higher concentrations (Eads and Hand, 1999). When the pH of the anoxic assay was raised to 7.9, overall incorporation at 30 min doubled relative to the anoxic incubation at pH 6.4 (Fig. 2B). This increase reflects qualitatively the increase in new initiation seen under anoxia *in vitro* at pH 7.9 (see below). Mitochondrial [^{32}P]UTP incorporation under anoxia at pH 7.9 was 52% lower at the end of the assay than normoxic controls at pH 7.9, which suggests a direct effect of oxygen deprivation either *in vivo*, *in vitro* or both. Interestingly, transcription rates at 15 min under anoxia were indistinguishable from controls (Table 2), and incorporation thereafter did not increase (Fig. 2B). The basis of the different patterns of incorporation of [^{32}P]UTP at low and high pH is not currently known but is almost certainly partly due to decreased initiation at low pH (see below).

In order to explore the effect of low pH independent of the effect of anoxia, we repeated the low pH assay under normoxia. The results shown in Fig. 2C reveal that overall [^{32}P]UTP incorporation by mitochondria incubated at pH 6.4 is significantly lower (59% decrease; P<0.02) than in mitochondria incubated at pH 7.9. Additionally, transcription rate is decreased by 74% to 574 nmol UTP mg protein^{-1} h^{-1} (Table 2). Mitochondria held at pH 6.3 for 1 h and then assayed for transcription showed no difference in [^{32}P]UTP incorporation relative to mitochondria held at pH 7.0 or 7.9 for 1 h and then assayed similarly (data not shown). Thus, exposure to low pH *in vitro* for this time period apparently does not promote irreversible effects on mitochondrial transcription.

Transcriptional initiation decreases under low pH in organello

Ribonuclease protection assays of mitochondrial RNA labeled *in organello* showed that under control conditions (normoxia, pH 7.9), *in vitro* transcriptional initiation contributes approximately 77% to the measured [^{32}P]UTP incorporation (Fig. 1; Table 3). This value is indistinguishable from new initiation under conditions of anoxia at pH 7.9 (78.8%). Exposure of intact embryos to anoxia *in vivo* followed by normoxic isolation and incubation did not promote a

Table 2. *Transcriptional rates and total UTP incorporation decrease in isolated mitochondria from* Artemia franciscana *embryos when incubated under anoxia or pH 6.4*

Treatments	Transcription rate (with all initiation)*	[^{32}P]UTP incorporation (%)†	*N*
Normoxia, pH 7.9	3197±94	100	9
Anoxia *in vivo* and *in vitro*, pH 6.4	361±108	23	3
Anoxia *in vivo* and *in vitro*, pH 7.9	3015±54	48	6
Normoxia, pH 6.4	837±45	41	6

*Transcription rates were calculated across the linear portions of run-on assays. Data are expressed as means ± S.E.M. Units for rates are nmol UTP incorporated mg mitochondrial protein^{-1} h^{-1}.

†Expressed as percentage incorporation relative to controls (normoxia, pH 7.9) over the 30 min assay.

Table 3. *Transcriptional initiation in isolated mitochondria from* A. franciscana *embryos is decreased by low pH*

Treatment	5′ (×10^3 d.p.m.)	3′ (×10^3 d.p.m.)	Initiation per elongation*	Percent new initiation (%)†	*N*
Control (normoxic, pH 7.9)	399±23	267±35	7.6	76.8±8.8	9
Normoxia, pH 6.4	89±0.4	10±0.7	0.91	31.3±5.2‡	7
In vitro anoxia, pH 6.4	16±2.4	19±0.4	0.96	32.9±5.6‡	4
In vitro anoxia, pH 7.9	36±1.9	20±1.0	7.5	78.8±4.1	3
In vivo anoxia	170±7.0	82±4.7	15	88.6±1.6	4

*The ratio of 2.52*Y* to 0.55*X* gives the d.p.m. due to initiation and elongation in the 5′ fragment, where *X* and *Y* are calculated as in the legend of Fig. 1. Note that specific activity in the 5′ fragment from elongation is half that due to initiation, so initiation per elongation is halved to calculate percent new initiation. The number of separate determinations (*N*) for percent new initiation is also shown.

†Reported as mean ± S.E.M.

‡Significant difference relative to control (P<0.05).

significant difference in new initiation relative to controls (Table 3). However, a major depression is seen in the proportion of transcription due to new initiation under conditions of low pH (pH 6.4), either aerobically or anoxically (32%; Table 3). In contrast to the original method developed by Gaines and Attardi (1984), we used both 5′ and 3′ antisense probes in the same incubations rather than separately, and therefore controlled a major source of error. Standard errors were in the order of ≤11% (Table 3).

Identification of protein–DNA contacts in the H-strand and L-strand promoter regions

The technique of methylation interference and primer extension was used to map regions of mtDNA bound by proteins, typically polymerases and transcription factors (see Ghivizzani et al., 1994; Micol et al., 1997; Enríquez et al., 1999). Using this method, the presence of proteins was deduced along regular intervals in the control region of the mitochondrial genome upstream of the 12S rRNA, which

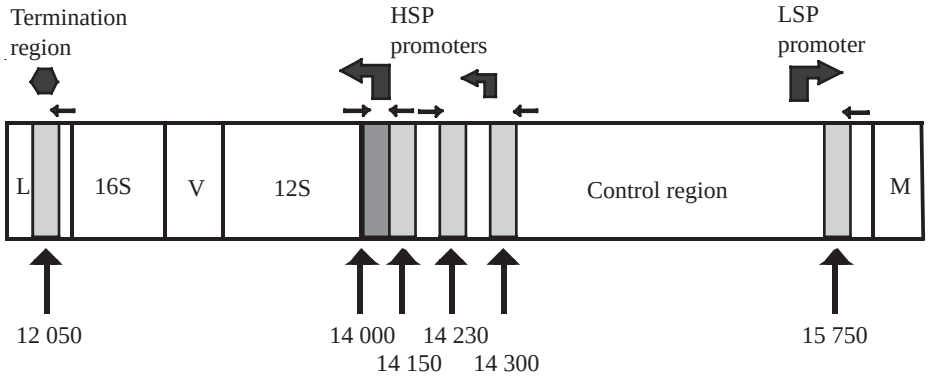

Fig. 3. Regions of the mitochondrial genome of *Artemia franciscana* footprinted with dimethylsulfide (DMS). Light grey boxes are segments where DNA–protein contacts were localized (indicated by arrows below). The dark grey box depicts the putative H-strand initiation region (HSP) where no contacts were seen. tRNA (L, tRNA$_{leu}$; V, tRNA$_{val}$; M, tRNA$_{met}$) and rRNAs (12S and 16S) are identified. Approximate primer positions are noted by small arrows (above). The locations of the strong H-strand promoter (HSP; larger left-facing arrow), the weak HSP (smaller left-facing arrow), the L-strand promoter (LSP; right-facing arrow) and the termination region (octagon) are given. Drawing is not to scale.

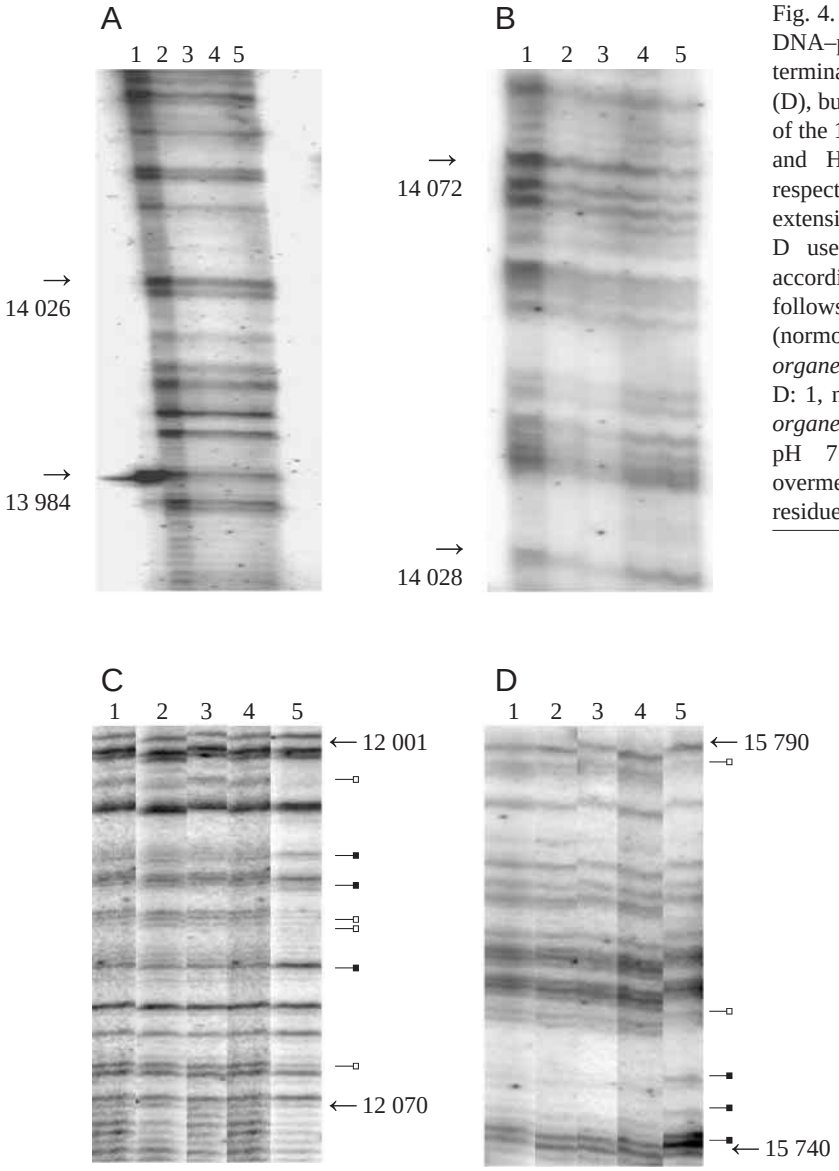

Fig. 4. Footprinting of *A. franciscana* mitochondria reveals DNA–protein contacts. Footprints were detected in the termination region within $tRNA_{leu}$ (C) and the control region (D), but no protein binding was detected immediately upstream of the 12S rRNA (A and B). For panels A and B, primers HSP1 and HSP2 were used for the heavy and light chains, respectively (see Table 1 for details). Panel C depicts primer extension of the light chain using the primer LEU 1, and panel D used the primer LSP 1 (genome positions numbered according to Valverde et al., 1994a). Lane assignments are as follows: panels A and B, 1, naked DNA; 2, *in organello* control (normoxia, pH 7.9); 3, *in organello* anoxia, pH 7.9; 4, *in organello* anoxia, pH 6.4; 5, normoxia, pH 6.4. Panels C and D: 1, normoxia, pH 6.4; 2, *in organello* anoxia, pH 6.4; 3, *in organello* anoxia, pH 7.9; 4, *in organello* control (normoxia, pH 7.9); 5, naked DNA. Relative to naked DNA, overmethylated residues are filled squares and undermethylated residues are unfilled squares.

previously had been indirectly inferred (Carrodeguas and Vallejo, 1997) to contain promoters for H-strand transcriptional initiation (see Fig. 3 for a schematic representation). As shown in Fig. 3, the primers HSP 3, HSP 4 and HSP 5 (see Table 1) revealed differences in methylation between control (naked) DNA and DNA methylated *in organello*, consistent with protein binding that increased or decreased the availability of methylation sites on the DNA. As shown in Figs 4–7, we found differences in methylation at a number of sites in the following genomic regions: 12 030–12 065, 14 125–14 138, 14 220–14 240, 14 270–14 300, 14 335–14 355 and 15 740–15 760. Primers HSP 1 and HSP 2, designed to probe the region immediately upstream from the 12S rRNA (12S rRNA starts at 14 000; see Table 1 for primer identification), were initially used. A previous report of transcriptional initiation in *A. franciscana* provided evidence that this area probably contained a strong promoter (Carrodeguas and Vallejo, 1997). Protein binding was located in the region 75–95 bp upstream (5′) to the 12S rRNA using HSP 5 (Figs 6C, 7C). Specifically, bases 14 074, 14 080, 14 083, 14 087 and 14 093 (numbering after Valverde et al., 1994a) were all undermethylated *in organello*. We were unable to find interference patterns further downstream, in an area predicted to contain the strong H-strand promoter (Fig. 4A,B). Several primers were used to amplify both strands and detect any methylation

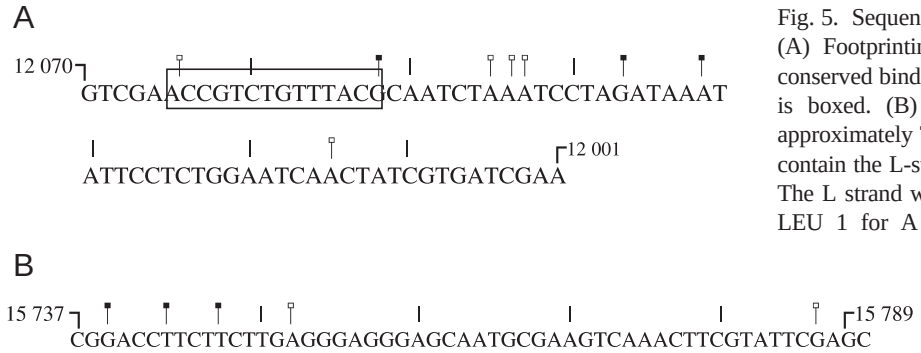

Fig. 5. Sequences of footprinted regions observed in Fig. 4. (A) Footprinting in the termination region of $tRNA_{leu}$. The conserved binding sequence of mTERF (Valverde et al., 1994b) is boxed. (B) The sequence in the control region located approximately 75 nt 5′ of the $tRNA_{met}$ gene in an area thought to contain the L-strand promoter (Carrodeguas and Vallejo, 1997). The L strand was footprinted in both cases. Primers used were LEU 1 for A and LSP 1 for B (see Table 1 for primers). Genomic positions are indicated. Relative to naked DNA, overmethylated residues are filled squares and undermethylated residues are unfilled squares.

differences, but these experiments did not reveal protein binding (data not shown).

Although a footprint was not localized within 75 bp of the 12S rRNA near the putative stronger promoter (14 000–14 075), the area from 14 125–14 355 showed four discrete footprinted areas in a region thought to contain a weaker H-strand promoter. Methylation differences are depicted in Figs 6B and 7B at sites 14 269, 14 281, 14 291, 14 297, 14 304, 14 335, 14 344, 14 354 and 14 357 that could indicate protein binding immediately upstream of the putative H-strand promoter (at 14 250; Carrodeguas and Vallejo, 1997). Also, the region from 14 200 to 14 240 shows several highly undermethylated guanines relative to naked DNA (Figs 6A, 7A), and from 14 120 to 14 173 there is marked overmethylation of both adenines and guanines (Fig. 6A). The size of these footprints is compatible with the typical footprint of mitochondrial transcription factor A (Ghivizzani et al., 1994; Cantatore et al., 1995). The region between 15 739 and 15 763 corresponds to a sequence hypothesized to contain the L-strand promoter (Carrodeguas and Vallejo, 1997) and has overmethylated residues at 15 739, 15 743 and 15 747 and undermethylations at 15 752 and 15 787 (Figs 4D, 5B). The area between 12 030 and 12 065 contains a sequence in the $tRNA_{leu}$ gene believed to bind the mitochondrial transcription factor TERM (previously known as mTERF; Valverde et al., 1994b) and has several methylation differences (Figs 4C, 5A). To our knowledge, this is the first evidence that the 12 030–12 065 sequence is protein-bound in *A. franciscana*. The identity of this protein awaits a direct demonstration.

Interestingly, experimental incubations *in organello* that reduced the frequency of *de novo* initiation did not cause changes in footprinting patterns relative to the controls (Figs 4, 6). Based on these observations, we speculate that the depressed initiation seen at low pH could be triggered by processes such as covalent modification or protein–protein assembly at promoter sites that might not be detected by methylation interference.

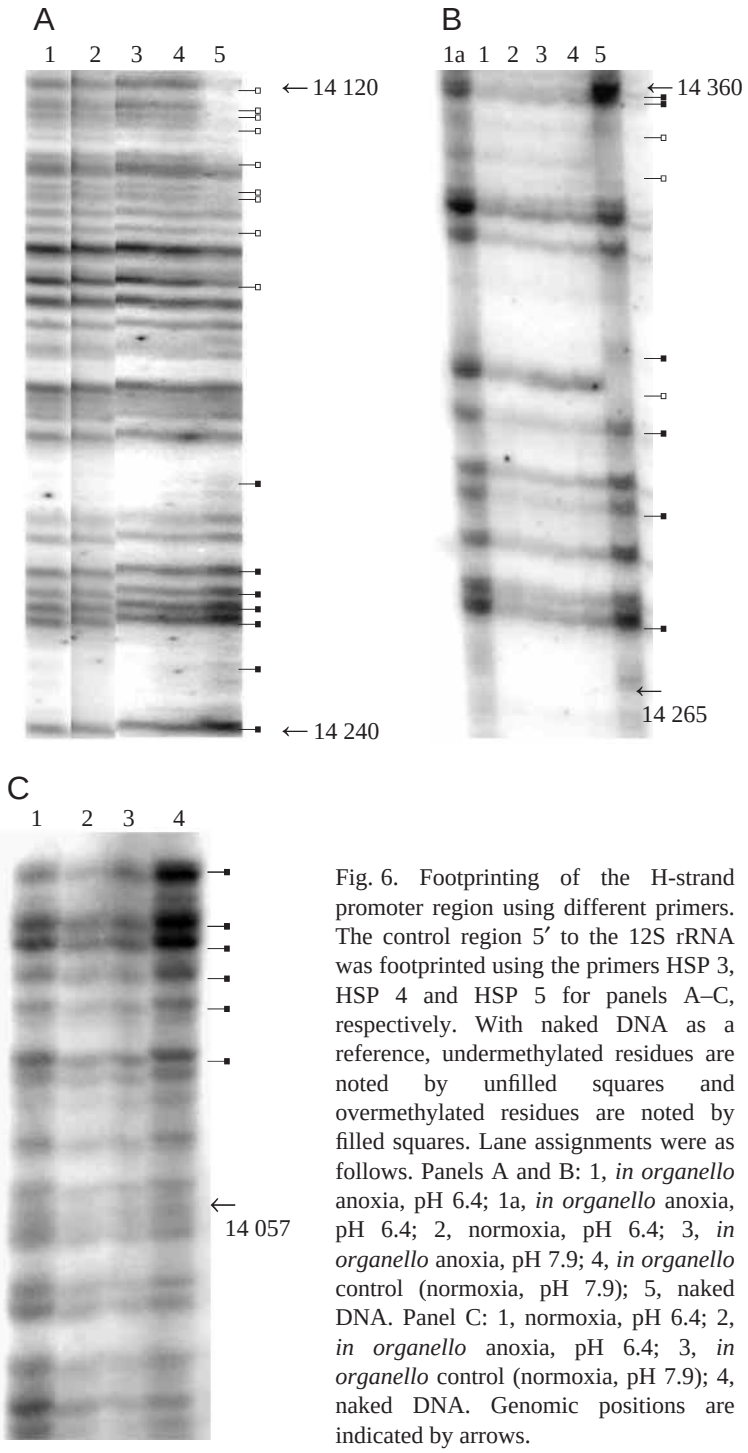

Fig. 6. Footprinting of the H-strand promoter region using different primers. The control region 5′ to the 12S rRNA was footprinted using the primers HSP 3, HSP 4 and HSP 5 for panels A–C, respectively. With naked DNA as a reference, undermethylated residues are noted by unfilled squares and overmethylated residues are noted by filled squares. Lane assignments were as follows. Panels A and B: 1, *in organello* anoxia, pH 6.4; 1a, *in organello* anoxia, pH 6.4; 2, normoxia, pH 6.4; 3, *in organello* anoxia, pH 7.9; 4, *in organello* control (normoxia, pH 7.9); 5, naked DNA. Panel C: 1, normoxia, pH 6.4; 2, *in organello* anoxia, pH 6.4; 3, *in organello* control (normoxia, pH 7.9); 4, naked DNA. Genomic positions are indicated by arrows.

Discussion

We have examined mechanisms of transcription under conditions promoting anoxic quiescence in *A. franciscana* using mitochondria isolated from gastrula-stage embryos and find that both molecular oxygen and pH acidification are able to decrease transcription *in vitro*. Results from this study also suggest that the acidification of pHi during anoxic quiescence decreases initiation of mitochondrial transcription. Ribonuclease protection assays demonstrated that new initiation of transcription contributed substantially to measured nucleotide incorporation *in vitro* under control conditions (approximately 78% at pH 7.8, normoxia), but at pH 6.4, the contribution was reduced to 32% of transcription under either anoxic or normoxic conditions. Thus, in order to minimize the amount of transcriptional initiation that could be considered an artifact of mitochondrial isolation and incubation, we chose to assay run-on transcription anoxically at low pH (6.4) and, furthermore, to incubate the embryos under anoxia *in vivo* and isolate the mitochondria anoxically. These conditions should reflect most closely the situation *in vivo* during anoxia-

induced quiescence in *A. franciscana*. A decrease in transcription of 77% was measured under these conditions compared with controls. Low pH (6.4) in the presence of oxygen decreased transcription to a greater extent than anoxia at control pH. We hypothesized that the proteins responsible for initiating transcription would bind promoter sequences less effectively under lower pH conditions. *In organello* footprinting identified protein binding to H and L strands in regions projected to be important as promoter sites and for termination. However, footprinting showed that protein dissociation from mtDNA at acidic pH is unlikely to be a mechanism responsible for this decreased initiation.

The energetic cost of even low levels of mitochondrial transcription during long-term anoxia is likely to be unsustainable for *A. franciscana* embryos. DNA replication and transcription are estimated to require approximately 10% of the energy budget of a mammalian cell (Buttgereit and Brand, 1996; Rolfe and Browne, 1995), a value that may be a bit high for gastrula-stage embryos of *A. franciscana*, because little DNA replication occurs across pre-emergence development (Olson and Clegg, 1977). The 89% decrease elicited in mitochondrial transcription rate in our study does not constitute full arrest, which could reflect imperfect simulations of *in vivo* conditions in our assays. The addition of optimal levels of exogenous NTPs during the assays (Eads and Hand, 1999) could represent, for example, ATP concentrations that are above physiological concentrations, given that anoxic *A. franciscana* embryos experience a decline of over 80% in ATP during the first hour of exposure (Stocco et al., 1972; Anchordoguy and Hand, 1994). Also, the increased stability of mRNA under conditions of both anoxia and low pH (B. D. Eads and S. C. Hand, unpublished data) implies that transcripts should accumulate faster in mitochondria incubated under these conditions than under normoxia and pH 7.9, which would artificially underestimate the degree of transcriptional depression.

Gaines and Attardi (1984) noted a decrease in the relative labeling of certain RNAs in isolated mitochondria across a pH range similar to the one reported in this study. These authors speculated that changes in RNA stability and/or processing occurred at low pH in a way that appeared to be transcript-specific, such that fewer long transcripts and more short transcripts accumulated. Our study of mRNA decay (B. D. Eads and S. C. Hand, unpublished data) under the same conditions examined in the present study indicated that both anoxia and low pH are able to extend half-life to varying and mRNA-specific degrees, although we did not evaluate rRNA stability in that work. Further investigation on the pH-based regulation of rRNA metabolism in *A. franciscana* mitochondria is likely to be a fruitful avenue for studying transcription in these animals.

Studies of nuclear transcriptional events during periods of oxygen limitation have received attention in mammalian systems during hypoxia and have revealed a fundamental role for the transcription factor HIF-1 (Semenza, 1998). The transcriptional responses of anoxia-tolerant vertebrates such as the wood frog *Rana sylvatica* and the crucian carp *Carassius carassius* have also been studied. Both anoxia and freezing induce an increase in five mitochondrial tRNAs in the wood frog (Cai and Storey, 1997), although the physiological significance remains obscure. Likewise, crucian carp do not decrease RNA synthesis in response to long-term anoxia, even though protein synthesis is downregulated (Smith et al., 1999). Anoxia tolerance has been studied in numerous invertebrates (reviewed in Grieshaber et al., 1994), but data regarding transcriptional changes have only recently begun to accumulate. For example, a number of genes are differentially expressed during exposure of *Drosophila melanogaster* to anoxia (Haddad, 2000), but this species possesses poor anoxia tolerance overall. Upregulation of several genes has been observed in the yeast *Saccharomyces cerevisiae* during anoxia (reviewed in Zitomer and Lowry, 1992; Kwast et al., 1998). In the above cases, differential gene expression may serve to re-program

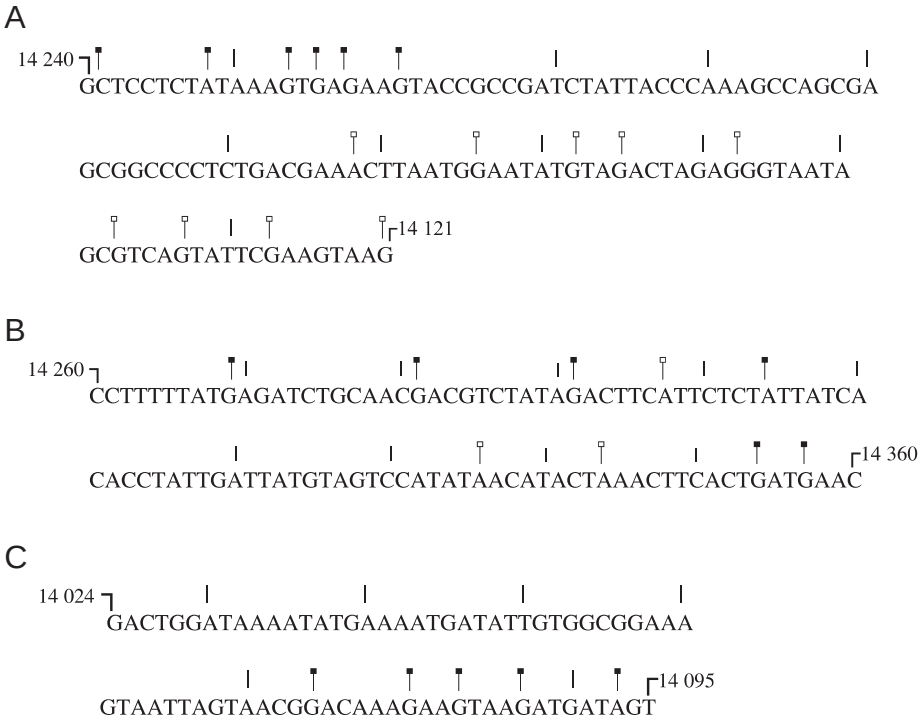

Fig. 7. Sequences of footprinted regions in the putative H-strand promoter. With naked DNA as a reference, undermethylated residues are noted by unfilled squares and overmethylated residues are noted by filled squares. Genomic positions are indicated. Panels A, B and C correspond to the gels A, B and C shown in Fig. 6.

metabolism for survival under oxygen limitation. Especially in vertebrates, tolerance is generally limited to a few days or weeks unless temperature is lowered. In *A. franciscana*, by comparison, the ability to survive anoxia at room temperature for years (cf. Clegg, 1997) apparently requires such deep metabolic depression that differential gene expression is no longer energetically feasible.

Isolated mitochondria exhibit a substantial degree of local (*versus* nuclear-mediated) control over transcription that can be exploited to study mechanisms of initiation. This feature is quite useful in the present context because transcriptional studies of *A. franciscana* embryos cannot be performed *in vivo* due to a virtually impermeable cyst wall. The downregulation of initiation by pH is a mechanism that promotes a 67% decrease in transcription in mitochondria isolated from *A. franciscana* and hypothetically may be responsible *in vivo* for low mitochondrial transcription during anoxia-induced quiescence. Other, as yet undiscovered, mechanisms may operate in concert to depress fully this step in gene expression. *In vitro* initiation of mitochondrial transcription has been known for some time (Gaines and Attardi, 1984), but its effect during *in organello* assays has generally been ignored. Studies of *in organello* transcription have focused on the acute influence of exogenous factors such as ATP (Gaines et al., 1987; Enríquez et al., 1996), thyroid hormone (Enríquez et al., 1999) or mitochondrial transcription factor A (TFAM, formerly mTFA; Montoya et al., 1997) on overall UTP incorporation or on mtDNA–protein interactions as they relate to transcription. These studies and others (Eads and Hand, 1999; Micol et al., 1997) indicate a substantial degree of autonomy regarding mitochondrial transcription when removed from nuclear inputs. The possibility remains that nucleo-cytoplasmic regulation may impact endogenous mitochondrial control, particularly over extended time periods. However, faithful reproduction of *in vivo* patterns is a hallmark of transcription in isolated mitochondria (Gaines and Attardi, 1984; Enríquez et al., 1996), and the acute and profound effect of *in vitro* treatments underscores the importance of autonomous regulation.

To examine the decreased initiation at low pH, the approach selected was to footprint transcriptional promoter regions in isolated mitochondria by methylation interference. Footprinting in mitochondria is, in principle, similar to nuclear footprinting, although there is a key difference. Mitochondrial transcription proteins are, in general, much smaller than their nuclear counterparts (e.g. the 105 kDa mtRNA polymerase of *A. franciscana* is comparable with a single subunit of RNA polymerase II), reflected by smaller regions of DNA contact and less striking differences in footprint patterns (see Ghivizzani et al., 1994; Cantatore et al., 1995; Micol et al., 1997; Enríquez et al., 1999; Roberti et al., 1999). The agreement between footprinting *in organello* and *in vivo* (Ghivizzanni et al., 1994; Micol et al., 1997) indicates that this technique can be used reliably to localize transcriptionally important proteins. Previous reports have correlated differences in promoter occupancy of the mitochondrial H strand with changes in transcription (Micol et al., 1997; Enríquez et al., 1999). For example, in HeLa cells, mRNA and rRNA production responded differentially to exogenous ATP (Gaines et al., 1987), and this response was directly correlated with changes in protein binding in the promoter region of the H strand (Micol et al., 1997). Similarly, upregulation of mitochondrial transcription promoted *in organello* by thyroid hormone was reflected by changes in the pattern of footprinting in the H-strand promoter (Enríquez et al., 1999). Thus, we expected to find a difference in footprinting patterns between incubations at low and high pH that would correlate with the decrease in transcriptional initiation. The inability to document such an association has several possible explanations.

Although models of transcriptional initiation exist for mitochondria of several species (see Tracy and Stern, 1995), it has not been shown whether *A. franciscana* mitochondria require a DNA-binding protein such as a TFAM homolog, a dissociable specificity factor like mitochondrial transcription factor B (mTFB; inferred from Santiago and Vallejo, 1998) or both. Our data are consistent with a role for TFAM in *A. franciscana* mitochondria. Mitochondria from rat liver (Cantatore et al., 1995) and human placenta (Ghivizzani et al., 1994) show footprinting in regions corresponding to TFAM-binding domains, including transcriptional promoter regions, and TFAM remains bound to promoters during mitochondrial transcription (Taanman, 1999). The protein binding we documented in *A. franciscana* occurred at intervals similar to those in the studies just mentioned, and the location of footprints in the control region spans the area containing two putative H-strand promoters. However, because these patterns did not change across experimental incubations, the acute effect of pH that we observed on transcriptional initiation is compatible with covalent modification. While no sequence data is available for the *A. franciscana* mtRNA polymerase, phosphorylation of the enzyme or a specificity protein could control initiation, by analogy to the Pol II carboxy-terminal domain (Uptain et al., 1997). Several residues in the conserved carboxy-terminal domains of TFAM are potential sites of phosphorylation (Goto, 2001) as well.

Interestingly, we were unable to detect a footprint in the stronger H-strand promoter of the *A. franciscana* mtDNA (Carrodeguas and Vallejo, 1997), while the weaker H-strand and L-strand promoters did indicate protein binding. Perhaps only a single H-strand promoter exists. In mapping transcription start sites, Carrodeguas and Vallejo (1997) used *in vitro* capping of mitochondrial RNA, a process that does not work equally well for all RNAs (cf. Levens et al., 1981). A 5′ leader sequence for the 12S rRNA that had initiated from the ‘weaker’ promoter at position 14 250 would be expected under the single promoter hypothesis. The leader could be processed co-transcriptionally to yield an uncapped or rapidly degraded 5′ fragment and a nascent, capped rRNA. Alternatively, it is possible that transcriptional initiation at the strong H-strand promoter does not depend on TFAM binding but rather on an mTFB-type protein. In this case, a footprint would not be expected because mTFB associates with mtRNA polymerase

rather than binding directly to DNA like TFAM does (cf. Prieto-Martín et al., 2001). However, because reconstituted transcription depends critically on high levels of TFAM as well as mTFB (Falkenberg et al., 2002), we consider the latter scenario unlikely.

Finally, the footprint found of mTERF in the $tRNA_{leu}$ gene at positions within and near a conserved sequence for rDNA transcription (Valverde et al., 1994b) indicates that *in organello* this sequence is bound by the termination factor. Presumably, regulated binding at this site in transcriptionally active mitochondria controls the formation of mRNAs in the H strand (see Fig. 4). Our study shows no differences among *in organello* treatments regarding mTERF occupancy, similar to previous work in HeLa cells reporting no effect of ATP concentration or ethidium bromide (Micol et al., 1997).

In summary, anoxia and low pH are able to decrease mitochondrial transcription rate to 11% of controls, consistent with metabolic arrest by *A. franciscana* embryos during anoxia. *De novo* initiation contributes significantly to transcription in isolated mitochondria, and at low pH the input of new initiation decreases by over 50%. This depression of initiation is potentially a significant regulator of RNA synthesis *in vivo* in mitochondria of *A. franciscana* embryos. Several sites of protein binding were discovered that correspond to the transcription termination region downstream of the ribosomal RNAs. Protein binding was also observed in areas of the control region previously indicated to contain transcriptional promoters. No differences were found in patterns of methylation interference based on treatments of the isolated mitochondria such as incubation at low pH. Thus, the regulation of transcriptional initiation in *A. franciscana* mitochondria apparently involves other mechanisms in addition to protein–DNA binding.

This project was supported by National Science Foundation grant IBN-9723746 to S.C.H. We thank Dr Douglas Crawford (University of Missouri, Kansas City) for scientific advice.

References

Anchordoguy, T. J. and Hand, S. C. (1994). Acute blockage of the ubiquitin-mediated proteolytic pathway during invertebrate quiescence. *Am. J. Physiol.* **267**, R895-R900.

Buttgereit, F. and Brand, M. (1995). A hierarchy of ATP-consuming processes in mammalian cells. *Biochem. J.* **312**, 163-167.

Cai, Q. and Storey, K. B. (1997). A novel RNA species from the turtle mitochondrial genome: induction and regulation of transcription and processing under anoxic and freezing stresses. *Genome* **40**, 534-543.

Cantatore, P., Daddabbo, L., Fracasso, F. and Gadaleta, M. N. (1995). Identification by *in organello* footprinting of protein contact sites and of single-stranded DNA sequences in the regulatory region of rat mitochondrial DNA. Protein binding sites and single-stranded DNA regions in isolated rat liver mitochondria. *J. Biol. Chem.* **270**, 25020-25027.

Carrodeguas, J. A. and Vallejo, C. G. (1997). Mitochondrial transcription initiation in the crustacean *Artemia franciscana*. *Eur. J. Biochem.* **250**, 514-523.

Clegg, J. S. (1997). Embryos of *Artemia franciscana* survive four years of continuous anoxia: the case for complete metabolic rate depression. *J. Exp. Biol.* **200**, 467-475.

Clegg, J. S. (2001). Cryptobiosis – a peculiar state of biological organization. *Comp. Biochem. Physiol. B* **128**, 613-624.

Clegg, J. S. and Jackson, S. A. (1989). Aspects of anaerobic metabolism of *Artemia* cysts. In *The Cellular and Molecular Biology of Artemia Development* (ed. A. H. Warner, J. Bagshaw and T. MacRae), pp. 1-15. New York: Plenum Press.

Eads, B. D. and Hand, S. C. (1999). Regulatory features of transcription in isolated mitochondria from *Artemia franciscana* embryos. *Am. J. Physiol.* **277**, R1588-R1597.

Enríquez, J., Fernández-Silva, P., Pérez-Martos, A., López-Pérez, M. and Montoya, J. (1996). The synthesis of mRNA in isolated mitochondria can be maintained for several hours and is inhibited by high levels of ATP. *Eur. J. Biochem.* **237**, 601-610.

Enríquez, J. A., Fernández-Silva, P., Garrido-Pérez, N., López-Pérez, M. J., Pérez-Martos, A. and Montoya, J. (1999). Direct regulation of mitochondrial RNA synthesis by thyroid hormone. *Mol. Cell. Biol.* **19**, 657-670.

Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.-G. and Gustafsson, C. M. (2002). Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat. Genet.* **31**, 289-294.

Gaines, G. and Attardi, G. (1984). Highly efficient RNA-synthesizing system that uses isolated human mitochondria: new initiation events and in vivo-like processing patterns. *Mol. Cell. Biol.* **4**, 1605-1617.

Gaines, G., Rossi, C. and Attardi, G. (1987). Markedly different ATP requirements for rRNA synthesis and mtDNA light strand transcription versus mRNA synthesis in isolated human mitochondria. *J. Biol. Chem.* **262**, 1907-1915.

Ghivizzani, S. C., Madsen, C. S. and Hauswirth, W. W. (1993). *In organello* footprinting. Analysis of protein binding at regulatory regions in bovine mitochondrial DNA. *J. Biol. Chem.* **268**, 8675-8682.

Ghivizzani, S. C., Madsen, C. S., Nelen, M. R., Ammini, C. V. and Hauswirth W. W. (1994). *In organello* footprint analysis of human mitochondrial DNA: human mitochondrial transcription factor A interactions at the origin of replication. *Mol. Cell. Biol.* **14**, 7717-7730.

Grieshaber, M. K., Hardewig, I., Kreutzer, U. and Pörtner, H.-O. (1994). Physiological and metabolic responses to hypoxia in invertebrates. *Rev. Physiol. Biochem. Pharmacol.* **125**, 43-147.

Goto, A., Matsushima, Y., Kadowaki, T. and Kitagawa, Y. (2001). *Drosophila* mitochondrial transcription factor A (d-TFAM) is dispensable for the transcription of mitochondrial DNA in Kc167 cells. *Biochem. J.* **354**, 243-248.

Haddad, G. G. (2000). Enhancing our understanding of the molecular responses to hypoxia in mammals using *Drosophila melanogaster*. *J. Appl. Physiol.* **88**, 1481-1487.

Hand, S. C. (1998). Quiescence in *Artemia franciscana* embryos: reversible arrest of metabolism and gene expression at low oxygen levels. *J. Exp. Biol.* **201**, 1233-1242.

Hand, S. C., Podrabsky, J. E., Eads, B. D. and van Breukelen, F. (2001). Interrupted development in aquatic organisms: ecological context and physiological mechanisms. In *Animal Developmental Ecology* (ed. D. Atkinson and M. Thorndyke), pp. 219-234. Oxford, UK: BIOS Publishing.

Hardewig, I., Anchordoguy, T. J., Crawford, D. L. and Hand, S. C. (1996). Profiles of nuclear and mitochondrial encoded mRNAs in developing and quiescent embryos of *Artemia franciscana*. *Mol. Cell. Biochem.* **158**, 139-147.

Hochachka, P. W., Lutz, P. L., Sick, T., Rosenthal, M. and van den Thillart, G. (ed.) (1993). *Surviving Hypoxia. Mechanisms of Control and Adaptation*. Boca Raton, FL: CRC Press.

Hofmann, G. E. and Hand, S. C. (1994). Global arrest of translation during invertebrate quiescence. *Proc. Natl. Acad. Sci. USA* **91**, 8492-8496.

Kwast, K. E., Burke, P. V. and Poyton, R. O. (1998). Oxygen sensing and the transcriptional regulation of oxygen-responsive genes. *J. Exp. Biol.* **201**, 1177-1195.

Kwast, K. E. and Hand, S. C. (1996a). Oxygen and pH regulation of protein synthesis in mitochondria from *Artemia franciscana* embryos. *Biochem. J.* **313**, 207-213.

Kwast, K. E. and Hand, S. C. (1996b). Depression of mitochondrial protein synthesis during anoxia: contributions of oxygen limitation, matrix acidification, and redox state. *J. Biol. Chem.* **271**, 7313-7319.

Kwast, K. E., Shapiro, J. I., Rees, B. B. and Hand, S. C. (1995). Oxidative phosphorylation and the realkalinization of intracellular pH during recovery from anoxia in *Artemia franciscana*. *Biochem. Biophys. Acta* **1232**, 5-12.

Levens, D. L., Ticho, B., Ackerman, E. and Rabinowitz, M. (1981). Transcriptional initiation and 5′ termini of yeast mitochondrial RNA. *J. Biol. Chem.* **256**, 5226-5232.

Micol, V., Fernández-Silva, P. and Attardi, G. (1997). Functional analysis

of *in vivo* and *in organello* footprinting of HeLa cell mitochondrial DNA in relationship to ATP and ethidium bromide effects on transcription. *J. Biol. Chem.* **272**, 18896-18904.

Montoya, J., Perez-Martos, A., Garstka, H. L. and Wiesner, R. J. (1997). Regulation of mitochondrial transcription by mitochondrial transcription factor A. *Mol. Cell. Biochem.* **174**, 227-230.

Olson, C. S. and Clegg, J. S. (1976). Nuclear numbers in encysted dormant embryos of different Artemia populations. *Experientia* **32**, 864-870.

Peterson, G. (1977). A simplification of the protein assay method of Lowry et al., which is generally more applicable. *Anal. Biochem.* **83**, 346-356.

Prieto-Martín, A., Montoya, J. and Martínez-Azorín, F. (2001). A study on the human mitochondrial RNA polymerase activity points to existence of a transcription factor B-like protein. *FEBS Lett.* **503**, 51-55.

Roberti, M., Loguercio-Polosa, P., Musicco, C., Milella, F., Qureshi, S. A., Gadaleta, M. N., Jacobs, H. T. and Cantatore, P. (1999). *In vivo* mitochondrial DNA-protein interactions in sea urchin eggs and embryos. *Curr. Genet.* **34**, 449-458.

Rolfe, D. F. S. and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in animals. *Physiol. Rev.* **77**, 731-758.

Santiago, J. and Vallejo, C. G. (1998). Identification of a mitochondrial RNA polymerase in the crustacean Artemia fransiscana. *Arch. Biochem. Biophys.* **353**, 276-284.

Semenza, G. (1998). Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. *Curr. Opin. Genet. Dev.* **8**, 588-594.

Smith, R. W., Houlihan, D. F., Nilsson, G. E. and Alexandre, J. (1999). Tissue-specific changes in RNA synthesis *in vivo* during anoxia in crucian carp. *Am. J. Physiol.* **277**, R690-R697.

Somero, G. N. (ed.) (1998). The biology of oxygen: evolutionary, physiological and molecular aspects. *J. Exp. Biol.* **201**, 1043-1254.

Stallcup, M. R., Ring, J. and Yamamoto, K. R. (1978). Synthesis of mouse mammary tumor virus ribonucleic acid in isolated nuclei from cultured mammary tumor cells. *Biochemistry* **17**, 1515-1521.

Stocco, D. M., Beers, P. C. and Warner, A. H. (1972). Effect of anoxia on nucleotide metabolism in encysted embryos of the brine shrimp. *Dev. Biol.* **27**, 479-493.

Taanman, J.-W. (1999). The mitochondrial genome: structure, transcription, translation and replication. *Biochim. Biophys. Acta* **1410**, 103-123.

Tracy, R. L. and Stern, D. B. (1995). Mitochondrial transcription initiation: promoter structures and RNA polymerases. *Curr. Genet.* **28**, 205-216.

Uptain, S. M., Kane, C. M. and Chamberlain, M. J. (1997). Basic mechanisms of transcript elongation and its regulation. *Annu. Rev. Biochem.* **66**, 117-172.

Valverde, J. R., Batuecas, B., Moratilla, C., Marco, R. and Garesse, R. (1994a). The complete mitochondrial DNA sequence of the crustacean Artemia franciscana. *J. Mol. Evol.* **39**, 400-408.

Valverde, J. R., Marco, R. and Garesse, R. (1994b). A conserved heptamer motif for ribosomal RNA transcription termination in animal mitochondria. *Proc. Natl. Acad. Sci. USA* **91**, 5368-5371.

van Breukelen, F., Maier, R. and Hand, S. C. (2000). Depression of nuclear transcription and extension of mRNA half-life under anoxia in *Artemia franciscana* embryos. *J. Exp. Biol.* **203**, 1123-1130.

Warner, A. H. and Clegg, J. S. (2001). Diguanosine nucleotide metabolism and the survival of Artemia embryos during years of continuous anoxia. *Eur. J. Biochem.* **268**, 1568-1676.

Zitomer, R. S. and Lowry, C. V. (1992). Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **56**, 1-11.