Regulatory features of transcription in isolated mitochondria from Artemia franciscana embryos

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Eads, Brian D., and Steven C. Hand. Regulatory features of transcription in isolated mitochondria from Artemia franciscana embryos. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1588–R1597, 1999.—Optimal conditions were developed for an in organello transcriptional run-on assay using mitochondria isolated from Artemia franciscana embryos to investigate potential regulatory features of RNA synthesis under conditions of anoxia-induced quiescence. Transcription is not dependent on oxidative phosphorylation for maximal activity when exogenous ATP is available. bona fide transcription products, as assessed by hybridization with specific mitochondrial cDNAs from A. franciscana, are produced in an inhibitor-sensitive manner. Transcription rate measured at pH 7.9 is reduced 80% when pH is lowered to 6.3, a pH range that mimics the in vivo change seen on exposure of embryos to anoxia. The proton sensitivity of mitochondrial RNA synthesis may provide a mechanism to depress this significant energy expenditure during quiescence. The influence of nucleotide concentration on kinetics is complicated by an interdependence among nucleotide species. ATP inhibition observed at subsaturating UTP concentrations is relieved when UTP is at saturating, physiologically relevant levels. Taken together, these data suggest that local (versus nuclear mediated) control is important in dictating mitochondrial transcription during rapid modulations in gene expression, such as those observed under anoxia-induced quiescence.

organellar gene expression; quiescence; transcriptional run-on assay; pH regulation

The ability of an animal to downregulate metabolism and enter a quiescent state in response to environmental stress is dependent on the depression of both catabolic and anabolic processes (see Ref. 13). During anoxia-induced quiescence in embryos of the brine shrimp Artemia franciscana, arrest of protein synthesis has been documented at the level of the intact embryo (7, 18), cell lysates (19), and isolated mitochondria (23, 24). Considering the survival time under anoxia for these gastrula-stage embryos is on the order of years (6), one might also anticipate that seemingly minor sources of ATP turnover would be sharply curtailed to achieve the degree of metabolic arrest required. A recent estimate for the energetic cost of DNA transcription plus replication in mammalian cells is roughly 10% of the standard metabolic rate (4), and embryos of A. franciscana do not undergo DNA replication at this developmental stage (27). Thus from energetic arguments alone, we predict that transcription must be shut down under anoxia in A. franciscana embryos. Additionally, the ontogenetic increase in mRNA levels observed during development is arrested during anoxia, and message levels in the cytoplasm and mitochondria are stabilized (16, 18). The most parsimonious explanation for the observed constancy of mRNA pools under anoxia is simultaneous arrest of transcription and mRNA degradation. Because changes in transcription and mRNA degradation can be followed in organello (8, 29), we chose to begin an evaluation of transcription in A. franciscana embryos by following this process in mitochondria. On the basis of the development of an optimized transcriptional run-on assay, we report herein characteristics of RNA synthesis in isolated mitochondria and assess how physiological factors relevant to anoxia-induced quiescence may affect gene expression. The reversible state of metabolic quiescence induced by anoxia in A. franciscana embryos is associated with several important physiological events. Within the first hour of anoxia, intracellular pH (pH) drops from 7.7–7.9 (aerobic value) to ≈6.8 (3, 25) and a concomitant decrease occurs in ATP levels (32). Heat dissipation is also depressed to exceedingly low levels (14, 20).

The acidification of pH that accompanies anoxia has been implicated in the arrest of metabolic processes in the cytoplasm and protein synthesis in the mitochondria (for a review, see Ref. 15). Exposure of embryos to elevated CO2 in the presence of oxygen, termed aerobic acidosis, acidifies pH1 and elicits an arrest of catabolic and anabolic processes in A. franciscana embryos analogously to anoxia. For example, the ontogenetic increase in mRNA for mitochondrially encoded subunit 1 of cytochrome oxidase is blocked by both anoxia and aerobic acidosis (16). Additionally, mitochondrial protein synthesis shows an 80% reduction in [3H]leucine incorporation when extramitochondrial pH is lowered from 7.8 to 6.7 (22). This extramitochondrial acidification promotes a change in matrix pH of equal or greater magnitude. Experiments with nigericin (an H+/K+ ionophore) demonstrate that mitochondrial protein synthesis is responding directly to the change in matrix pH (24). Thus a role for pH1 in the potential arrest of mitochondrial transcription is plausible as well. To test this possibility, measurements of transcriptional capacity were made at various extramitochondrial pH values, for which the corresponding matrix pH value is known. Changes in other physiological parameters, such as ATP concentration and ionic composition, were also evaluated to determine their effect on mitochondrial transcription.
Mitochondrial transcription in brine shrimp embryos

Materials and Methods

Embryo incubation and mitochondrial isolation. Dehydrated, encysted gastrulae of A. franciscana (Optima grade) were purchased from Sanders Brine Shrimp (Ogdem, UT) and stored at -20°C until use. All chemicals were reagent grade or higher and purchased either from Sigma (St. Louis, MO) or Mallinckrodt (Chesterfield, MO), unless otherwise noted. Embryos were hydrated in tap water at 0°C for at least 4 h before incubation and then 20 g were incubated at room temperature in 650 ml of 0.25 M NaCl on a rotary shaker at 1,100 rpm for 8 h (22). After this developmental incubation, the embryos were dechorionated in antiformin solution (1% hypochlorite from bleach, 60 mM NaCO3, and 0.4 M NaOH) followed by a rinse in 1% thiosulfate and multiple washings in ice-cold 0.25 M NaCl, as previously described (22). After the embryos were filtered and blotted dry, 10 g were added to 40 ml of ice-cold homogenization buffer (HB). The HB consisted of (in mM) 500 sucrose (Pfanstiehl, Waukegan, IL), 150 KCl, 20 HEPES, 1.0 EGTA, and 0.5% (wt/vol) fatty acid-free BSA adjusted to pH 7.5 with KOH at room temperature. Embryos were homogenized in a Thomas Teflon-glass homogenizer at 500–600 rpm using three passes. The homogenate was centrifuged at 2,900 g (4°C) for 10 min, and the resulting supernatant was collected and centrifuged at 9,000 g for 15 min (4°C). The resulting pellet was resuspended in 35 ml of HB and centrifuged again for 15 min at 9,000 g (4°C). The final pellet was resuspended in 2.5 ml HB (mitochondrial protein concentration of 12 mg/ml) and kept on ice until use. Mitochondrial protein was quantified according to Peterson (28) using BSA as a standard.

Mitochondrial respirometry. To verify that isolated mitochondria were intact and functionally coupled, the respiratory control ratio (RCR) was assessed. Methods were essentially as described in Ref. 25. Briefly, O2 consumption during state 3 and state 4 respiration was measured at 25°C using Strathkelvin model 1302 oxygen electrodes coupled to model 781 oxygen meters, and data were collected digitally with Datascan V software from Sable Systems (Las Vegas, NV). The volume of the respirometry chamber (Strathkelvin RC350) was 1.5 ml. Initially, 30 µl of diluted mitochondrial suspension (150–180 µg mitochondrial protein) were added to 1.47 ml of respiration medium (RM). The RM contained (in mM) 500 sucrose, 150 KCl, 20 HEPES, 10 K2HPO4, 3 MgCl2, 1 EGTA, and 1 µM rotenone and 0.5% (wt/vol) fatty acid-free BSA. After O2 consumption stabilized (2–3 min), 15 µl of 0.5 M sodium succinate prepared in 20 mM HEPES (pH 7.5) were added, and respiration rate was recorded for 3–4 min. Then 45 µl of 5 mM ADP in 20 mM HEPES (pH 7.5) were added, and state 3 respiration was measured. ADP concentrations of stock solutions were determined spectrophotometrically from optical density at 254 nm (millimolar extinction coefficient = 15.4). The RCR was calculated as state 3 respiration divided by state 4 using DatGraf software (Ororos, Innsbruck, Austria). Corrections for back-diffusion and electrode response time were performed as in Ref. 25.

Mitochondrial transcription assays. All procedures involving RNA were carried out under RNase-free conditions. Solutions (except those containing Tris) were treated with 0.2% diethylpyrocarbonate (DEPC) and autoclaved, plastics kept sterile or autoclaved, and all glassware baked at 250°C for at least 3 h. To evaluate transcriptional capacity, 26.7 µl of mitochondrial suspension were added to 33.3 µl of reaction buffer, which was HB fortified with 6 mM MgCl2 and 20 mM K2HPO4. This mixture was then transferred to a 30°C water bath and incubated for 5 min before 9.8 µl of a prewarmed nucleotide mix were added to initiate the reaction. The nucleotide mix contained nucleoside triphosphates (initially 0.3 mM ATP, 0.6 mM CTP, 0.6 mM GTP, 47.6 µM UTP; Promega, Madison, WI), 70 mM dithiothreitol in 10 mM acetate buffer (pH 5.2), 10 units of RNasin (Promega), and 1.7 µCi/ml α-[32P]UTP (3,000 Ci/mmol, 10 mCi/ml; New England Nuclear, Boston, MA). The final specific radioactivity of UTP was 1.05 Ci/mol, which assumed a negligible contribution of endogenous UTP from the mitochondrial preparation. For time course experiments, reaction volumes were scaled up threefold to allow for repetitive sampling. The transcription reaction was allowed to proceed at 30°C, and 30-µl aliquots were removed at the indicated time points and streaked onto Whatman GF/C filters (24 mm). The reaction was quenched immediately by transferring the filters to 500 ml of ice-cold 10% TCA containing 1% pyrophosphate. After soaking the filters for at least 1 h, they were given two consecutive washes in 5% TCA containing 0.5% pyrophosphate at room temperature (10 min each). The filters were washed three times in 95% ethanol (10 min each) and allowed to air dry. Each filter was then placed in 2 ml of Scintiverse II scintillation cocktail (Fisher, Pittsburgh, PA), and radioactivity was counted with an LKB model 1209 liquid scintillation counter. Statistical analysis was carried out using the unpaired Student’s t-test.

To determine the effect of pH on transcription, 20 mM HEPES buffer was used between pH 6.3 and 7.0 and 50 mM Tris was used from 7.1 to 8.3 to span the desired pH range. No buffer-specific effects on transcription were observed, as assessed by comparing the two buffers at a common pH (P > 0.05; data not shown). pH values were checked at the end of each assay to verify stability of pH.

RNA extraction and electrophoresis. Run-off reactions were performed with [32P]UTP (elevated final specific radioactivity 3,000 Ci/mmol) or without any tracer. Reactions were halted after 30 min by adding 2 ml of RNA extraction buffer (4.5 M guanidinium thiocyanate, 50 mM EDTA, 25 mM Tris-HCl, 0.1 M β-mercaptoethanol, 0.2% antifoam A, and 2% Na-laurelsarsosine). After thorough vortexing, 220 µl of 2 M sodium acetate (pH 4.0) were added, followed by 2 ml of water-equilibrated phenol (pH 4.0) and 400 µl of chloroform-isooamil alcohol (23:1 ratio). The room temperature samples were chilled on ice for 10 min and centrifuged at 2,500 g for 20 min (4°C). One volume of isopropanol was added to the supernatant, and the mixture was vortexed. The sample was then stored at -20°C for 3 h before centrifugation for 45 min at 20,000 g (4°C). The recovered pellet was washed with 3 ml of 70% isopropanol and recentrifuged. The washed pellet was suspended in 900 µl of RNA extraction buffer and vortexed until solubilized. The RNA was precipitated by adding 900 µl of isopropanol and chilling on ice for 10 min. The sample was centrifuged at 14,000 g for 30 min (4°C) and then washed in 70% ethanol, followed by centrifugation at 14,000 g for 15 min (4°C). RNA samples were stored in 100 µl of DEPC-treated H2O at -80°C or used immediately for Southern blotting (see Verification of bona fide mitochondrial transcriptions). A260/A280 ratios were ≥1.6 for all preparations. A typical RNA extraction of a mitochondrial reaction mixture yielded 10–20 µg RNA.

Total mitochondrial RNA (1–3 µg if unlabeled, 0.1–1 µg if labeled) was denatured in four volumes of 98% deionized formamide, heated at 75°C for 10 min and cooled on ice for 5 min before loading onto gels. Size fractionation by electrophoresis was performed on 6% polyacrylamide-8 M urea gels using TBE, which contained (in mM) 90 Tris, 90 boric acid, 2.5 EDTA (pH 8.0) in the gels; 1× TBE (pH 8.3) served as the electrode buffer. The gel was electrophoresed (200 V, 6 h) and then stained in ethidium bromide (0.05%) or visualized on a gel documentation system.
Verification of bona fide mitochondrial transcripts. A Southern blot of unlabeled cDNA probes for two mitochondrially encoded mRNAs was hybridized to purified, radiolabeled mitochondrial RNA from five pooled transcription reactions. Genomic fragments for cytochrome b (cyt b) and the NADH dehydrogenase subunit 1 (ND1) were amplified from DNA extracted from A. franciscana mitochondria (described in Ref. 16). Oligonucleotide primers were designed on the basis of the published mitochondrial genome of A. franciscana (33). For cyt b, the primers were 5'-GCC AAC ATT TCT ATT TGA TGA-3' (forward) and 5'-TCA TCC CGG TTG GAT ATG GGC-3' (reverse); the ND1 primers were 5'-AAG ATT TTG GGT TAC ATT CAG-3' (forward) and 5'-TAT GTA TTC TGT ATT GAA TCC-3' (reverse). After PCR, the products were gel purified and ligated into pGEM-T vector (Promega). The vector was used to transform competent cells (XL-1, a JM 109 F' tet resistant strain; Epicurian Coli cells, Stratagene, La Jolla, CA). The cloned gene fragments (ND1, 575 bp; cyt b, 743 bp) were identified by sequence analysis using the dideoxy-termination method with a Sequenase kit (US Biological, Cleveland, OH). Actin mRNA, was prepared as previously described (16). The cDNA fragments were excised from plasmids using restriction endonucleases and purified by agarose gel electrophoresis. After isolating the cDNA from agarose (GeneClean, B10101, Vista, CA), DNA concentration was determined spectrophotometrically, and DNA was stored in water at −80°C.

A Southern blot was performed by combining the three unlabeled cDNA fragments (400 ng each) and boiling the DNA for 10 min, quick-coding on ice, and electrophoresing through a 1.5% agarose gel (80 V, 3 h). The gel contained 1× TAE (40 mM Tris-acetate, 2 mM disodium EDTA, pH 8.5), which also served as the running buffer. After electrophoresis the gel was denatured in 1.5 M NaCl and 0.5 M NaOH for 30 min at room temperature. The gel was washed three times with water (10 min each), soaked for 30 min in neutralization buffer (1.5 M NaCl, 0.5 M Tris·HCl, pH 7.5, 0.1 M EDTA), and then capillary blotted onto a prewetted nylon membrane (GeneScreen Plus, DuPont, Boston, MA) overnight. After air drying, the membrane was ultraviolet cross-linked for 3.5 min and then capillary blotted onto a prefilted nylon membrane (GeneScreen Plus, DuPont, Boston, MA). DNA concentration was determined spectrophotometrically, and DNA was stored in water at −80°C.

RESULTS

Transcription in isolated mitochondria. Mitochondria used for run-on assays exhibited an average RCR of 5.32 ± 0.44 (mean ± SE, n = 25 independent preparations). Those with an RCR ≤4.0 were not used because of a correlated decrease in transcriptional capacity (data not shown). Transcripts of ~20 bases and longer are recovered for counting by acid precipita-

![Fig. 1. Time course of [32P]UTP incorporation by mitochondria isolated from gastrula-stage Artemia franciscana and incubated at 10 mg/ml medium B. Data are means ± SE for 3 separate determinations at each time point. Linear segments were fitted by eye.](http://ajpregu.physiology.org/)

Table 1. Composition of medium used for transcription assays

<table>
<thead>
<tr>
<th>Component</th>
<th>Medium A</th>
<th>Medium B</th>
</tr>
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<tbody>
<tr>
<td>KCl, mM</td>
<td>150</td>
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<tr>
<td>MgCl₂, mM</td>
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<td>3.5</td>
</tr>
<tr>
<td>UTP, mM</td>
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</tr>
<tr>
<td>GTP, mM</td>
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<td>0.05</td>
</tr>
<tr>
<td>ATP, mM</td>
<td>0.30</td>
<td>0.60</td>
</tr>
<tr>
<td>Mitochondrial protein, mg/ml</td>
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<td>10</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Medium A is initial formulation and medium B is optimized mixture developed during course of study. Only experimentally varied components are listed.
assay with the optimized conditions developed during this study.

Insensitivity of transcription to oxidative phosphorylation. Conducting run-on assays under conditions that promote oxidative phosphorylation (i.e., in the presence of ADP and succinate) did not influence the measured \[^{32P}\]UTP incorporation (Fig. 2) compared with assay conditions with the same final concentration of ATP but without oxidative phosphorylation. Specifically, the addition of 1 mM ADP plus 5 mM succinate to run-on assays initially containing 0.3 mM ATP fostered rapid oxidative phosphorylation; all ADP was quickly converted to ATP, thus yielding 1.3 mM total ATP. Incorporation measured for this treatment was quite similar to run-on assays in the absence of oxidative phosphorylation, in which 1.3 mM ATP was added exogenously. Both of these assay conditions promoted lower rates and total incorporation than did assays with 0.3 mM ATP because of the inhibitory effect of ATP on incorporation (see below).

Transcription inhibitor studies. The capacity of several inhibitors to depress mitochondrial transcription is shown in Table 2. The most potent inhibitor was actinomycin D, which inhibited mitochondrial transcription by 85% at a final concentration of 100 µg/ml. A. franciscana mitochondria were relatively insensitive to high concentrations of both cordycepin and rifampycin. For example, 500 µg/ml cordycepin (as either 3'-deoxyadenosine or 3'-dATP) decreased nucleotide incorporation by 59%, whereas the same concentration of rifampycin decreased transcription by 24%. To test the possibility that less effective inhibitors had low permeability and were excluded from the matrix, mitochondria were lysed either osmotically or mechanically and then given inhibitor treatment. The inhibitory effects of 500 µg/ml cordycepin showed only a small enhancement of inhibition in lysed mitochondria (14% increase; data not shown). The result suggests that the low sensitivity of mitochondrial transcription to cordycepin is not due to matrix exclusion of the drug but is likely a feature of the RNA polymerase. Performing transcription assays at 0°C inhibited transcription by >96% (Table 2).

Mitochondrial protein concentration and ionic strength. Transcription rate, expressed per milligram mitochondrial protein, is moderately sensitive to the mitochondrial concentration in the assay. Maximal transcription rates were obtained at a mitochondrial density of ~10 mg mitochondrial protein per milliliter (Fig. 3). Transcription at lower concentrations (2–4 mg/ml) was 70% of maximum, whereas higher concentrations (12–15 mg/ml) supported transcription at 90% of optimum. The effects of potassium and magnesium (added as chloride salts) on transcription were tested over a range of physiological values (see Ref. 12). Potassium concentrations of 90–120 mM supported maximal incorporation rates (Fig. 4), whereas maximal incorporation occurred at ~3.5 mM magnesium (Fig. 5). The ionic optima for both K\(^+\) and Mg\(^{2+}\) were qualitatively and quantitatively similar for the 12- and 30-min assays (data not shown).

Nucleotide concentrations. To monitor the effects of UTP on transcription, total UTP concentration was varied from 10 to 200 µM while the specific radioactivity was held constant at 1.05 Ci/mol (Fig. 6). At concentrations >100 µM, incorporation reached a plateau and did not increase appreciably. This pattern agrees with an earlier report for rat liver mitochondria (5) that shows a similar optimum. The quantitative and qualitative natures of the relationship between transcription and UTP concentration were very similar for both the 12- and 30-min assays (data not shown). As shown in Fig. 7A, the effect of exogenously added CTP on transcription rate was small; the lowest rate (at 1.5

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**Table 2. Effect of inhibitors on mitochondrial transcription rate in isolated A. franciscana mitochondria**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration, µg/ml</th>
<th>%Inhibition</th>
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</thead>
<tbody>
<tr>
<td>Cordycepin</td>
<td>25</td>
<td>20.8 ± 1.3</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>25</td>
<td>11.1 ± 1.4</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>25</td>
<td>63.2 ± 1.2</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>25</td>
<td>10.7 ± 1.9</td>
</tr>
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</table>

Values are means ± one SE for 3 separate determinations. Mitochondria were preincubated with inhibitors for 20 min at 30°C before the assay. Inhibition is expressed relative to controls without inhibitor.
mM) was 87% of the optimal rate observed with no exogenous CTP. The trend of higher incorporation rates at lower nucleotide concentrations was also seen for GTP, which supported maximal transcription rates at 25–50 µM and was inhibitory at higher concentrations (Fig. 7B). It is appropriate to note that this pattern was observed at both 50 µM UTP (i.e., the initial concentration used for nucleotide studies) and 110 µM UTP (the final, optimized concentration); similarly, the CTP inhibition at 1 mM was also present at the optimized UTP concentration but to an even greater degree (data not shown).

Results from the 12-min assays showed that ATP was strongly inhibitory to transcription when assays were conducted in the presence of 50 µM UTP (Fig. 8A). In fact, maximum incorporation was achieved without exogenously added ATP, and this rate was twofold higher than that seen with 0.8 mM ATP. It is appropriate to note that measurable matrix ATP is present in A. franciscana mitochondria even in the absence of extramitochondrial ATP or substrates for oxidative phosphorylation (23). However, when assays were performed with 110 µM UTP rather than 50 µM UTP, the ATP optimum was 0.6 mM (Fig. 8B). In addition, transcription at 2 mM ATP was substantially higher.
than rates observed without exogenous ATP. The intramitochondrial UTP concentration for *A. franciscana* is not known at present, but matrix UTP data for HeLa cell mitochondria (11) suggest that our ATP profile obtained with saturating UTP is the more physiologically relevant (see DISCUSSION). The observation that the influence of ATP on the in organello transcription rate depends on the UTP concentration has not been previously reported.

Inhibition of transcription at acidic pH. A range of extramitochondrial pH values from 8.2 to 6.3 was used to assess the effect of protons on transcription rate (Fig. 9). These values encompass the physiological range observed for *A. franciscana* embryos [pH 7.7–7.9 under normoxic conditions to pH 6.3 after 24 h of anoxia (3, 25)]. The corresponding matrix values for *A. franciscana* mitochondria have been documented across this same range (24). Transcription exhibited a maximum rate at pH 7.9, which represents a matrix pH of 8.4. When assayed at the lowest pH value observed under anoxia, transcription was inhibited by 81% relative to the optimum. The distinct shoulder in the pH profile observed between 7.2 and 7.5 is quite reproducible. The pattern may reflect different pH optima for the transcription of the heavy versus light strands of mitochondrial DNA. This situation has been previously documented for mitochondria from HeLa cells (11).

In organello transcription products. When total RNA was isolated from mitochondria after run-on assays and separated on 6% polyacrylamide-8 M urea gels, several distinct bands could be visualized with ethidium bromide and by autoradiography (Fig. 10, A and B). The sizes of RNA products ranged from 300 to 1,700 nucleotides, which correlates approximately with sizes of genes known to be encoded by the *A. franciscana* mitochondrial genome (33). Additionally, Southern blot analysis was used to establish whether or not the transcripts synthesized during run-on assays were of mitochondrial origin (Fig. 10, C and D). Radiolabeled RNA from the run-on assays clearly did not hybridize with the unlabeled cDNA probe for nuclearly encoded actin, which served as a negative control. In contrast, radiolabeled RNA did react with cDNA probes for mitochondrial genes (ND1 and cyt b, prepared from the mitochondrial genome of *A. franciscana*). Because of the long exposure time on the PhosphorImager needed to verify that hybridization did not occur with the actin probe, mRNA hybridizing with the mitochondrial probes did not fully resolve as two bands. Nevertheless, the results indicate that the transcripts are synthesized from the mitochondrial genome.

![Fig. 6. Influence of exogenous UTP on transcription rate in mitochondria isolated from *A. franciscana* embryos. Specific activity was held constant at 1.05 Ci/mol UTP for all UTP concentrations. No exogenous ATP or CTP was added. All data are means ± SE for 3 separate determinations at each nucleotide concentration.](http://ajpregu.physiology.org/)

![Fig. 7. Dependence of exogenous CTP (A) and GTP (B) on transcription rate in *A. franciscana* mitochondria. Mitochondria were incubated in medium B with 50 µM UTP but without ATP. All data are means ± SE for 3 separate determinations at each nucleotide concentration.](http://ajpregu.physiology.org/)
DISCUSSION

In this study we have developed an in organello transcriptional run-on assay for mitochondria isolated from A. franciscana embryos to facilitate investigation of regulatory properties that may modulate transcriptional capacity under conditions of anoxia-induced quiescence. Optimized for maximal rates of UTP incorporation, the mitochondria used in this assay produce bona fide transcriptional products in an inhibitor-sensitive manner. To our knowledge, this is the first transcriptional run-on system developed for invertebrate mitochondria. High respiratory control ratio, one indicator of mitochondrial integrity and acceptor control, is generally a predictor of high transcription rates for these mitochondria. However, experiments with oxidizable substrate, phosphate acceptor, and electron transport chain inhibitor indicate that oxidative phosphorylation per se is not essential during the course of the assay to achieve maximal transcription, so long as exogenous ATP is supplied. The influence of nucleotide concentrations on transcriptional rate is more complex than previously appreciated, in that interdependence is observed among some nucleotide species. Particularly noteworthy is the observation that the strong ATP inhibition observed at subsaturating concentrations of UTP is diminished when UTP concentration is increased to support maximal transcription rate. The pH dependency of in organello transcription indicates pronounced proton sensitivity across the physiologically relevant range for these embryos. The extramitochondrial pH optimum is 7.9, which matches the in vivo pH of embryos under normoxic conditions (7.7–7.9; Refs. 3, 25). Transcription is inhibited >80% by lowering extramitochondrial pH to 6.3, the lowest value measured for

Fig. 8. Dependence of transcriptional rate on ATP concentration for A. franciscana mitochondria. A: effect of ATP at subsaturating (50 µM) UTP levels. Inset depicts identical experiment except that duration of assay was increased from 12 to 30 min. B: effect of ATP at saturating UTP levels. In all cases, medium B was used with no CTP or GTP present. All data are means ± SE for 3 separate determinations at each ATP concentration.
anoxic embryos in vivo (3). Thus it appears that pH is one factor that may serve to depress mitochondrial transcription during anoxia-induced quiescence.

Oxidative phosphorylation per se appears to have little influence on transcription in mitochondria isolated from A. franciscana embryos based on two pieces of evidence. First, addition of antimycin A at a concentration that fully inhibits state 3 and state 4 respiration (see Ref. 24) has only a small effect on transcription if ATP is available (10% inhibition; Table 2). Similar results have been obtained in HeLa cells (11) and rat liver (8). Furthermore, addition of 1 mM exogenous ATP to transcription assays yielded the same $\text{[}^{32}\text{P}\text{]}\text{UTP}$ incorporation rate as 1 mM ADP plus 5 mM succinate for A. franciscana mitochondria; both additions were inhibitory relative to assays with 0.3 mM ATP (Fig. 2). Because both treatments also elevate the matrix ATP in these mitochondria, likely by transport via an ATP(ADP)-Mg$^{2+}$/Pi exchanger (see Ref. 22), it is reasonable to suggest that the higher ATP levels intrinsically inhibit transcription. Studies with isolated mitochondria from HeLa cells found that 1 mM ATP or 1 mM ADP plus 1 mM pyruvate were able to stimulate transcription 100-fold relative to no ATP (11). This large increase in labeling was not seen in isolated rat liver mitochondria, for which $\text{[}^{32}\text{P}\text{]}\text{UTP}$ incorporation was increased by about twofold with 1 mM ADP plus 2.5 mM malate or with 1 mM exogenous ATP (8). Taken together, these results illustrate that mitochondrial transcription is responsive to mitochondrial ATP concentration rather than to oxidative phosphorylation or associated processes, such as electron transport.

The response of mitochondrial transcription to exogenous nucleotide concentration was initially studied at a subsaturating UTP concentration of 50 µM UTP (Table 1), which revealed slight inhibition by CTP as concentration was raised from 0 to 1 mM and more marked inhibition by elevated GTP. By comparison, 1 mM ATP decreased transcription by 62% over the 12-min assay and 66% after 30 min, relative to the maximum. Inhibition by exogenous ATP seemed counterintuitive to us, because the result implied that the decrease in ATP concentration observed during anoxia-induced quiescence in A. franciscana embryos would favor increased transcription during a quiescent state. However, when the effect of ATP was reevaluated at saturating UTP concentration (≈100 µM), the inhibition of transcription by 1 mM ATP was markedly attenuated. In fact, as shown in Fig. 8B, transcription was higher with 2 mM ATP than with zero exogenous ATP. In transcriptional run-on assays using mitochondria isolated from either rat liver (5, 8) or HeLa cells (10, 11), transcription was sharply inhibited above 1-2 mM ATP. These studies concluded that $\text{[}^{32}\text{P}\text{]}\text{UTP}$ access to the matrix was not impaired at high ATP and thus was not the cause of reduced transcription. However,
the ATP inhibition for these mammalian cells was observed under conditions far below UTP saturation (i.e., at picomolar concentrations). If the relief of ATP inhibition by saturating UTP found in our study is a general feature of mitochondrial transcription, then it is possible that transcriptional inhibition at high ATP levels would be minimal in mammalian mitochondria, similar to the case for A. franciscana. Importantly in this context, the physiological UTP concentration in the mitochondrial matrix for HeLa cells has been estimated to be 215 µM (11), i.e., a level that apparently would be saturating for transcription.

Our studies with transcription inhibitors revealed insensitivity to high concentrations of both rifampicin, an inhibitor specific for bacterial RNA polymerase, and to cordycepin, a 3'-OH adenosine analog (Table 2). On the basis of similarly low inhibitions by cordycepin with lysed mitochondria, the limited effects of these inhibitor treatments were apparently due to insensitivity of the polymerase rather than exclusion from the matrix. Resistance to rifampicin has also been noted for RNA polymerase purified from A. franciscana mitochondria (30). We found both cordycepin and cordycepin triphosphate to be equally ineffective with isolated mitochondria. In contrast, actinomycin D at 100 µg/ml reduced transcription in mitochondria by 85% relative to untreated controls.

Mitochondrial protein concentration and ionic strength were found to influence transcription in A. franciscana mitochondria (Table 1). Optimal protein concentration was higher than that reported for transcription in mitochondria from rat liver (2 mg/ml; 5), HeLa cells (0.8 mg/ml; 10), or yeast (1.0 mg/ml; 29). Transcription may increase as the protein concentration is raised due to enhanced mitochondrial stability; the decline in rate seen at protein levels beyond the optimum may be due to substrate limitation. Optimal magnesium concentration in these mitochondria (3.5 mM) is close to the 5 mM optimum reported for yeast (29) and HeLa cell (10) mitochondria. By comparison, the potassium optimum of 120 mM is much higher than the 10 mM optimum for yeast mitochondria (29). However, our data are in close agreement with the K⁺ and Mg²⁺ optima for protein synthesis in A. franciscana mitochondria (22).

A pronounced effect of pH on transcription is demonstrated here across the physiological range for A. franciscana embryos. The acute drop in pH, observed during 1 h of anoxia, from 7.7 to 6.7 (3, 25), suggests that pH decline alone could be expected to decrease transcription by 63% in these mitochondria. Due to further acidification in pH, after 2 h anoxia (3), the inhibition could increase to over 80%. The observed depression in transcription at low pH cannot be explained by a pH effect on matrix energy status, because intramitochondrial purine levels do not change as a function of extramitochondrial pH (23). Rather, matrix proton concentration per se is likely influencing transcription directly, as was demonstrated for protein synthesis in these mitochondria using nigericin (24). These data indicated that the pH shift may exert a significant downregulation of mitochondrial gene expression under anoxia, as a consequence of simultaneous inhibition of transcription and translation. However, it is also appropriate to note that our preliminary data indicate that the removal of molecular O₂ can also decrease transcription in the absence of a change in pH. This observation implies that during entry into anoxia-induced quiescence, RNA synthesis in A. franciscana mitochondria responds to multiple factors. A similar pattern holds for protein synthesis in A. franciscana mitochondria, where removal of molecular O₂ also depresses its rate (23, 24).

Perspectives

Implications of this study are relevant to the broader issue of nuclear-mediated versus local regulation of mitochondrial gene expression. Nuclear regulation of mitochondrial transcription primarily involves mitochondrial transcription factor A (mtTFA; 9), a nuclear-encoded protein that stimulates polycistronic transcription of the mitochondrial genome in vitro (31). The promoter of the mtTFA gene is activated in turn by nuclear respiratory factor 1 (34). Thus, the availability of mtTFA to the mitochondrion would be sufficient in principle to control mitochondrial transcription, at least for slow cellular responses. However, we suggest such a system is not sufficiently responsive to explain the rapid and acute modulation of gene expression in A. franciscana embryos under anoxia (minutes to hours). Faithful transcription by isolated rat liver mitochondria for up to 6 h (8) suggests that mtTFA is provisioned in large excess, which is inconsistent with speedy regulation via changes in mtTFA levels. Parallel arguments have been made for local control of translation in mitochondria from A. franciscana embryos (23, 24). Rapid cessation of gene expression when confronted with anoxia may be critical for preserving cellular energy stores in these embryos.

Consequently, we suggest that A. franciscana embryos display a degree of local control over mitochondrial transcription during anoxia via the influence of acidic pH, depletion of molecular oxygen per se (Eads and Hand, unpublished data), and perhaps restricted ATP. As mentioned above, the acidification of pH in these embryos occurs within minutes of anoxic exposure and is likely to depress transcription based on the data presented here. The general concept of autonomous (versus nuclear mediated) control of mitochondrial transcription described for hepatocytes (8) is very consistent with our view, although we find their proposed model for feedback inhibition by high ATP not fully concordant with existing data. First, ATP inhibition appears substantially attenuated when UTP levels are saturating, at least for A. franciscana mitochondria. Second, ATP in mammalian cells is regulated at constant levels for the most part (see Ref. 17), and, typically, changes are seen only under extreme or pathological conditions that are associated with reduced viability. It is questionable whether hepatocyte mitochondria under moderate hypoxic challenge would experience drops in ATP concentration sufficiently large...
to influence transcription (26). Third, the premise that transcription would be upregulated during marked ATP limitation runs counter to classical views that energy-consuming processes are downregulated by lowered adenylate status (e.g., Ref. 1). Long-term, moderate hypoxia apparently can stimulate mitochondrial gene expression (26), but more severe treatments of mammalian cells in which ATP is likely to be compromised (anoxia, severe hypoxia), clearly depress mitochondrial gene expression (e.g., Ref. 21). During anaerobic quiescence in A. franciscana embryos, the depression of cytoplasmic ATP levels is profound and could impact matrix ATP.

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