Mitochondrial Gene Expression Is Regulated at the Level of Transcription during Early Embryogenesis of Xenopus laevis*

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Mitochondrial transcription in the early Xenopus laevis embryo resumes several hours after active mtDNA replication, effectively decoupling mtDNA transcription and replication. This developmental feature makes Xenopus embryogenesis an appealing model system to investigate the regulation of mitochondrial transcription. Studies reported here refine our understanding of the timing, magnitude, and mechanism of this transcriptional induction event. Northern analyses of six mitochondrial mRNAs (normalized to mtDNA) reveal that transcript levels remain basal between fertilization and gastrulation and then undergo a coordinate induction, culminating in a 20–28-fold increase over egg levels by 48 h of development. Measurement of mitochondrial run-on transcription rates demonstrates a good correlation between transcription rates and transcript levels, showing that transcription itself is the primary determinant of transcript abundance. Experimental increases in mitochondrial ATP and energy charge also correlate with patterns of transcript levels and transcription rates, suggesting that developmental changes in the biochemical composition of the mitochondrial matrix could be regulating transcriptional activity. Consistent with this idea, transcriptional run-on rates in mitochondria of early embryos can be stimulated by the addition of tricarboxylic acid cycle intermediates to the run-on reaction. However, mitochondria of later stages do not show this response to the addition of metabolite. In combination, these data suggest that mitochondrial transcription is under metabolic regulation during early Xenopus embryogenesis.

The Xenopus laevis egg stockpiles mitochondria and mitochondrial DNA along with other organelles and macromolecules during its year-long development (1). This active period of mitochondrial biogenesis ceases with the completion of egg development and does not resume until relatively late in embryogenesis, as measured by mtDNA synthesis, cytochrome oxidase activity, and mitochondrial protein accumulation (2). However, active mitochondrial transcription resumes much earlier, as indicated by total mitochondrial RNA synthesis (2–4) and mRNA accumulation (5). This apparent decoupling of mitochondrial transcription from mtDNA replication makes this model very useful for studying the nuances of transcriptional regulation of mitochondrial genes.

The precise timing of the resumption of mitochondrial transcription in the developing embryo remains controversial. Initial studies reported a low rate of mitochondrial rRNA synthesis up to the gastrula stage (10 h postfertilization), with the RNA content per embryo doubling by 96 h of development (2). Microinjection of [32P]GTP into fertilized eggs detected active mitochondrial RNA synthesis only after the midblastula transition (3). Measurement of mtRNA synthesis rates during the terminal stages of oogenesis and fertilization showed that synthesis rates remained essentially unchanged by the events associated with fertilization (6). In contrast, direct measurements of mitochondrial RNA steady-state levels during early embryogenesis showed that transcript levels decreased 5–10-fold in a coordinate fashion within a few hours after fertilization, remaining depressed up to the late neurula stage (24 h of development) and increasing thereafter (5). Based on these results, a fertilization-induced shutdown of mitochondrial gene expression during early embryogenesis was proposed.

Despite this uncertainty regarding the precise status of mitochondrial gene expression during early Xenopus embryogenesis, this model offers the opportunity to investigate mitochondrial gene regulation paradigms other than that of modulating gene dosage (mtDNA). Using mtDNA levels at each stage to normalize transcript levels, we determine stage- and gene-specific transcriptional rates. The results reveal that mitochondrial gene expression during early embryogenesis is regulated primarily by transcription. The data also suggest that the developmental cue(s) directing these changes is likely to be related directly or indirectly to the changing biochemical composition of the mitochondrial matrix during embryogenesis.

EXPERIMENTAL PROCEDURES

Plasmid Clones and Riboprobes—Gene-specific DNA templates for making hybridization probes and targets were constructed by cloning polymerase chain reaction-amplified fragments into the HindIII site of pBS1 (+/−) phagemid (Stratagene, La Jolla, CA). Sense or antisense riboprobes were synthesized from linearized clones using T3/T7 RNA polymerase kits (Stratagene). The clones and their corresponding positions in the frog genome (7) are as follows: pBSND1, nucleotides 4849–5715; pBSCTb, nucleotides 16417–17245; pBSCOII, nucleotides 9168–9790; pBSND4, nucleotides 12409–13571; pBSND6, nucleotides 16110–15680.

Frogs, Eggs, Embryos, and Mitochondria—Sexually mature male and female X. laevis frogs were purchased from Xenopus I, Ann Arbor, MI. Fertilized embryos were obtained using in vitro fertilization procedures (8, 9). Briefly, female frogs were injected with 700–1,000 units of human chorionic gonadotropin (Sigma) into the peritoneal cavity 10 h before eggs were required. Fertilization was done in batches of 300–400 eggs in 1 × F-1 (41.25 mM NaCl, 1.25 mM KCl, 0.25 mM CaCl2, 0.0625

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1 The abbreviations used are: pBS, pBluescript; ND1, ND4, and ND6, NADH dehydrogenase 1, 4, and 6, respectively; CVTb, cytochrome b; COII, cytochrome oxidase II; MOPS, 4-morpholinepropanesulfonic acid; HPLC, high performance liquid chromatography; ATPase 6, ATP synthetase 6; LSP, light strand promoter; HSP, heavy strand promoter.
mm MgCl₂, 0.5 mM Na₂HPO₄, 2.5 mM HEPES, pH 7.8), using sperm obtained from mated testes. During the first 8 h of growth, the 1 × F-1 buffer was replaced with 0.2 × F-1 buffer in the Petri dishes. Afterward, the whole Petri dish was immersed in a large dish filled with 2–3 liters of 0.2 × F-1 buffer until hatchling. Developmental stages were identified according to Nieuwkoop–Faber tables (10). Eggs and embryos were dejellied by gently swirling in 2% cysteine solution in a glass Dounce homogenizer with a type B Teflon pestle. The homogenate was spun twice at 1,500 × g for 10 min in a JA20 rotor, and mitochondria were pelleted at ~9,000 × g. Mitochondria were washed and repelleted twice using MSE buffer in Eppendorf tubes at 9,000 × g for 2 min.

Isolation of Mitochondrial Nucleic Acids—For total RNA isolation, 100 eggs or embryos were thawed in 10 ml of lysis buffer (4 mM guanidine thiocyanate, 100 mM sodium acetate, pH 5.0, 5 mM EDTA), incubated for 20 min at room temperature to facilitate dissociation of nucleoprotein complexes, extracted three or four times with phenol:chloroform:isoamyl alcohol (25:24:1), and ethanol precipitated. The RNA samples were run in 1.2% MOPS (pH 7.0, 5% sodium acetate, 0.1 mM EDTA) formaldehyde-agarose gels; blotted on nylon N² membranes (Amersham Pharmacia Biotech) with 10 SSC; prehybridized in 6× SSPE, 50% formamide, 5% Denhardt’s solution, 0.5% SDS, 200 μg/ml RNA at 60°C; and hybridized overnight at 60°C in a fresh aliquot of the same buffer with radiolabeled riboprobes (50–100 ng/ml). Blots were washed in 40 mM Na+, 1% SDS, 1 mM EDTA at 60°C for 1.5 h with three or four changes, and the blots were exposed to film and PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). In experiments with total RNA, 2 μg of total RNA was electrophoresed per lane. For measuring RNA levels/unit of mitochondrial genome, mtDNA levels in total nucleic acid samples were quantitated by dot blotting. Samples were incubated with 3 μl of RNAse mixture (Ambion, Inc., Austin, TX) at 37°C for 15 min to degrade RNA; denatured in 0.4M NaOH at 96°C for 10 min; adjusted to 1 μl volume of 100 mM Tris, pH 7.2, 50 mM NaCl, 1 mM MgCl₂, 2.5 mM sodium acetate, 2 m M MOPS, pH 7.0, 1 mM EDTA (12) with mtDNA-specific riboprobes at 60°C. Blots were washed as above and quantitated. Mitochondrial DNA-normalized samples were then run in formaldehyde-agarose gels for Northern analysis as described above, and RNA levels were reported per unit of mitochondrial genome.

Mitochondrial Run-on Transcription—The incubation conditions for the run-on transcription assays were modifications of reported protocols (13, 14). Mitochondria from eggs and staged embryos were isolated in MSE buffer as above, washed once with run-on wash buffer (40 mM Tris, 7.2, 50 mM NaCl, 10% glycerol, 5 mM MgCl₂, 1 mg/ml bovine serum albumin), and incubated in run-on buffer (40 mM Tris, pH 7.2, 50 mM NaCl, 10% glycerol, 5 mM MgCl₂, 2 mg/ml bovine serum albumin) with 50 μCi of [α-¹³C]UTP at 30°C for 30 min. Mitochondria were then pelleted; lysed in 50 mM Tris, pH 7.5, 200 mM NaCl, 25 mM EDTA, 1% SDS, and the clarified crude lysate was used to probe unlabeled riboprobe target panels with 32P-labeled riboprobes (Fig. 1). All transcripts shown in Fig. 1 and Table I were quantitated by densitometry and computerized using a PhosphorImager (Molecular Dynamics). Protein pellets obtained after trichloroacetic acid precipitation were resuspended in 10 mM Tris, pH 8.5, 0.5% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and the clarified supernatant was used for protein estimation (16).

RESULTS

To clarify the status of mitochondrial gene expression during early Xenopus development, mitochondrial transcript levels were measured initially exactly as reported previously (5). Identical amounts of total RNA (2 μg) from unfertilized eggs and staged embryos were separated by electrophoresis on 1.2% formaldehyde-agarose gels, blotted to nylon membranes, and probed with six gene- and strand-specific riboprobes (Fig. 1). All transcripts showed a 1.5–2-fold decrease in steady-state levels by 14 h of development relative to that of the unfertilized egg, returned to levels found in egg by 20 h in the case of NADH 1, cytochrome b, cytochrome oxidase II, and ATP synthetase 6 (Fig. 1 and Table I) and increased marginally thereafter. The levels of NADH 4 and 6 continued to be low even in the tadpole stage (50 h of development). The 12 S and 16 S rRNA levels decreased only marginally during this window of development (data not shown). Overall, these results are only in partial agreement with the patterns of mitochondrial transcript levels reported in an earlier study (5).

One reason for this discrepancy could be the decision to normalize steady-state mitochondrial RNA to total egg or embryonic RNA. In addition to variation among animals, there are clear and dramatic differences in nuclear transcription during early Xenopus embryogenesis after the midblastula transition.
Regulation of Mitochondrial Gene Expression

Total egg or embryonic RNA was subjected to Northern analysis as shown in Fig. 1, and the results of three experiments were used to calculate the steady-state levels of the six mitochondrial transcripts. Each transcript level is expressed relative to the level measured in the unfertilized egg.

![Figure 2](image)

**FIG. 2.** Steady-state levels of mitochondrial RNA/unit of mitochondrial genome during early development. Panel A, Northern analysis of mtDNA-normalized RNA samples from eggs and embryos at 6 h, 9 h, 20 h, 30 h, 48 h, and 7 days postfertilization with the indicated gene-specific riboprobes. Panel B, equal volumes of the samples used above were RNase treated, alkali denatured, and probed on dot blots to quantitate the amount of mtDNA in that sample. Digestion with DNase I completely eliminated this signal (not shown).

### Table I

| Transcript | Egg | 5 h | 14 h | 20 h | 30 h | 38 h | 50 h |
|------------|-----|-----|-----|-----|-----|-----|-----|
| ND1        | 1.0 | 1 ± 0.13 | 0.84 ± 0.13 | 1.3 ± 0.004 | 1.44 ± 0.2 | 1.3 ± 0.16 | 1 ± 0.3 |
| ND4        | 1.0 | 0.75 ± 0.13 | 0.55 ± 0.18 | 0.9 ± 0.11 | 0.84 ± 0.18 | 0.9 ± 0.14 | 0.76 ± 0.23 |
| ND6        | 1.0 | 1.18 ± 0.07 | 0.77 ± 0.08 | 0.67 ± 0.03 | 1 ± 0.12 | 0.46 ± 0.05 | 0.44 ± 0.08 |
| CYTb       | 1.0 | 0.8 ± 0.07 | 0.42 ± 0.03 | 1 ± 0.05 | 0.92 ± 0.07 | 1.23 ± 0.24 | 1.01 ± 0.26 |
| COII       | 1.0 | 1.03 ± 0.27 | 0.9 ± 0.34 | 1.11 ± 0.15 | 1.5 ± 0.53 | 1.23 ± 0.04 | 1.1 ± 0.15 |
| ATPase 6b  | 1.0 | 0.6 | 0.4 | 1.0 | 1.1 | 1.5 | 1.4 |

**a** Hours postfertilization.

**b** ATPase 6 levels were measured only once at each stage.

(17). Therefore, using total RNA to normalize mitochondrial transcripts will lead to a progressive reduction in apparent mtRNA levels during embryogenesis. We therefore sought an alternative normalization approach for mtRNA analysis.

A better and more logical reference for normalizing mitochondrial RNA levels is the mitochondrial DNA itself because mtDNA is the transcriptional template, and its concentration per embryo remains essentially unchanged during the first 48 h of development (2). We therefore isolated DNA and RNA simultaneously from purified mitochondria of distinct developmental stages. Equal proportions of the DNA/RNA mixture from each developmental stage were treated with RNase to remove RNA, alkali denatured, and blotted to quantitate the level of mtDNA in each sample (Fig. 2B). In control experiments, DNase I digestion of these samples before alkali denaturation and blotting completely eliminated the signal (not shown), confirming that the values obtained represented mtDNA exclusively, without RNA carryover.

RNA samples normalized for mtDNA were then run on formaldehyde-agarose gels, blotted, and hybridized with six gene-specific riboprobes (Fig. 2A), and the steady-state level of each transcript was expressed relative to the level found in the unfertilized egg. The results from two unrelated frogs are presented in Table II. Steady-state levels of all transcripts remained very near levels found in the egg until 9 h of development, increased slightly (1.5–4-fold) by 20 h, increased moderately (6–8-fold) by 30 h, and increased dramatically (13–60-fold in frog 1 and 17–23-fold in frog 2) by 48 h of development. The 96-h embryos displayed even higher steady-state transcript levels, 21–100-fold over egg. By 7 days postfertilization (5-day-old tadpole), however, steady-state levels dropped to levels found in the 30-h embryo, 2–8-fold over egg. It is apparent from Table II that there is substantial animal-to-animal variation in the magnitude of transcript levels. However, the pattern of induction of mitochondrial gene expression during embryogenesis is similar.

**Transcription Rates Correlate with Transcript Levels**—To investigate whether the developmental control of mitochondrial gene expression is regulated at the level of transcription, mitochondrial run-on transcription assays were performed using isolated organelles from unfertilized eggs and embryonic stages. To detect transcription separately from the LSP and HSP was 2-fold higher than levels found in the unfertilized egg by 5 h, 4–5-fold higher by 14 h, and 4–6-fold
higher by 20 h of development (Fig. 4A). The 28-h embryo showed the largest increase in transcriptional activity, with transcription rates from the LSP and HSP being 25-fold and 91-fold higher than levels in the egg (Fig. 4A), respectively. Overall transcription was ~70-fold higher than the level in eggs (Fig. 4B). Transcription in the 48-h embryo, although 2-fold less than the 28-h stage, was still elevated significantly, being 15-fold and 44-fold higher than the level in eggs for LSP and HSP, respectively (Fig. 4A).

A comparison of the overall transcription rates with steady-state transcript levels is presented schematically in Fig. 5. The resumption of transcription in the developing embryo (hatched bars in Fig. 5) correlates well with the steady-state accumulation of transcripts (line in Fig. 5) because increased rates of transcription precede the appearance of increased RNA levels by a few hours. Transcript rates of the 14-h and 20-h embryos were 5–6-fold higher than in eggs. Transcript levels were 2–4-fold higher by 20 h and 6–8-fold higher by 30 h than the level in eggs. The high transcriptional rate measured in the 28-h embryo (~70-fold over egg) is also consistent with the substantial transcript accumulation observed by 48 h (13–60-fold over egg) of development, assuming unaltered RNA turnover during this period.

Transcription Rates Correlate with the Energy Status of Mitochondria—To estimate the stage-specific metabolic status of mitochondria, the adenine nucleotide content of double sucrose gradient-purified mitochondria from unfertilized eggs and embryos was measured by reversed phase, ion-paired, HPLC. These studies revealed a 2–3-fold increase in mitochondrial ATP levels in the 30- and 44-h embryo over those of earlier stages (from 650–850 pmol/mg mitochondrial protein to 1,400–2,500 pmol/mg mitochondrial protein). The energy charge (ATP/ADP + AMP) also increased proportionally (from 0.12–0.17 to 0.33–0.4). In contrast, total cellular (egg or embryonic) ATP levels remained constant at ~2 mM during this time. This selective increase in mitochondrial ATP levels in later developmental stages suggests an induction or shift of mitochondrial metabolic activity during embryogenesis.

To test whether the induction of mitochondrial transcription in the 30-h embryo is related to the stimulation of mitochondrial metabolic activity, metabolic intermediates were added to mitochondrial run-on transcription reactions derived from 20-h embryos (a relatively un-induced stage for mitochondrial transcription) (Table III). Equal aliquots (equivalent to 100 embryos) of mitochondria prepared from 20-h embryos were incubated in basic buffer (condition 1); basic buffer + ADP (condition 2); basic buffer + α-ketoglutaric acid (condition 3); basic buffer + sodium phosphate, ADP, and α-ketoglutaric acid (condition 4); or basic buffer + ATP (condition 5). The overall mitochondrial run-on transcription rate showed a dramatic induction (8–22-fold over basic buffer) with the addition of all of the substrates needed for endogenous production of ATP in the mitochondria (condition 4 in Table III). The addition of exogenous ATP (condition 5) or low level production of endogenous ATP by the addition of the citric acid cycle intermediate α-ketoglutaric acid (condition 3) caused a reproducible 5–7-fold stimulation over basic buffer. The addition of ADP alone (condition 2) or ADP + phosphate (not shown) resulted in only a 2–3-fold enhancement in transcription rate. The addition of other trichloroacetic acid cycle intermediates pyruvate, malate, or glutamate also produced 6–10-fold stimulation of transcriptional activity (data not shown). Similar experiments performed with mitochondria from 48-h embryos (a transcriptionally active stage for mitochondria) in basic buffer with sodium phosphate, ADP, and α-ketoglutaric acid showed almost no stimulation of transcription rates (less than 1.5-fold; data not shown) compared with those seen with early embryos. This further suggests that changes in mitochondrial metabolism during development have profound effects on the mitochondrial transcriptional machinery. These results also suggest that transcriptionally quiescent mitochondria of the 20-h embryo are fully competent for high level transcription and are possibly awaiting a developmentally induced change in the mitochondrial microenvironment for induction of transcription to occur.

To explore further the specificity of the processes stimulating mitochondrial transcription, the mitochondrial drugs antimycin A and atractyloside, which block oxidative phosphorylation and ATP synthesis, were tested for their effect on mitochondrial transcription (Table III). Each inhibitor was added along with ADP, phosphate, and α-ketoglutaric acid (antimycin A, condition 6; atractyloside, condition 7) in the run-on reactions derived from 20-h embryonic mitochondria. Both drugs inhibited the transcription-inducing effects of substrate addition substantially (compare condition 4 with condition 6 or 7). The addition of cyanide, a specific inhibitor of cytochrome oxidase, also produced a similar inhibition (data not shown).
DISCUSSION

Aside from a simple response to mitochondrial genome content, mechanisms regulating vertebrate mitochondrial gene expression remain largely unstudied. Early embryogenesis of *X. laevis* provides an experimental window on this problem because mitochondrial transcription precedes resumption of mtDNA synthesis by at least 24 h. The timing, magnitude, and nature of this early transcriptional regulation were studied by measuring steady-state levels of six mRNAs and the two mitochondrial rRNAs between fertilization and 72 h of development. All mtRNA levels remained relatively unchanged between fertilization and gastrulation (10 h postfertilization) but increased thereafter in a coordinate fashion and culminated in a dramatic 13–60-fold (by 48 h) or 21–100-fold (by 96 h) increase over levels found in unfertilized eggs (Fig. 2 and Table II). That this coordinate increase of steady-state mtRNA levels is caused by an induction of mitochondrial transcription was substantiated by measurements of mitochondrial run-on transcription rates. Transcription rates in eggs and embryos remained at basal levels between fertilization and gastrulation and then showed moderate 2–3-fold increases in the 14-h and 20-h embryos and a dramatic 70-fold induction in the 28-h embryo (Figs. 3 and 4). The parallel behavior of mitochondrial transcription rates and transcript levels leads to the pivotal mechanistic question: what developmental stimuli regulate...
relative to egg levels). The line graph represents the pattern of steady-state levels of mitochondrial transcripts (expressed relative to egg levels). The bar graph represents overall mitochondrial run-on transcription rates (also expressed relative to egg levels) measured in mitochondria from each developmental stage.

Table III

| Reaction condition                           | Relative transcription rate |
|---------------------------------------------|----------------------------|
| 1. Basic buffer                             | 1.0                        |
| 2. Basic + 100 μM ADP                       | 2.5–3.0                    |
| 3. Basic + 1 mM α-ketoglutaric acid         | 6.0–7.0                    |
| 4. Basic + 100 μM ADP + 1 mM α-ketoglutaric acid + 10 mM sodium phosphate | 8.0–22.0 |
| 5. Basic + 1 mM ATP                         | 5.0–7.0                    |
| 6. Conditions of 4 + 10 μg/ml antimycin A   | <2.0                       |
| 7. Conditions of 4 + 500 μM atractyloside   | <1.5                       |

this resumption of active mitochondrial transcription in the embryo?

In one scenario, the developmental cue could be a nuclear encoded factor that is imported into mitochondria, stimulating transcription directly or indirectly, perhaps by inactivating inhibitors of transcription. Alternatively, stimulation of mitochondrial metabolic activities such as oxidative phosphorylation or substrate biosynthesis could indirectly activate transcription. The fact that transcription rates in mitochondria isolated from the 20-h embryo in which mitochondrial transcription is uninduced could be stimulated by the addition of trichloroacetic acid cycle intermediates such as α-ketoglutarate (Table III), malate, pyruvate, or glutamate, unlike mitochondria isolated from the 48-h tadpole in which mitochondrial transcription is already maximally induced, lends credence to the metabolite induction scenario. The lower ATP content and energy charge ratios of stages before 30 h are also consistent with this view. Finally, the marked inhibitory effects of antimycin A and atractyloside on transcription rates (Table III) demonstrate the exquisite sensitivity of mitochondrial transcription, either directly or indirectly, to perturbations of energy metabolism.

Metabolic studies of carbon flow during amphibian development have revealed developmental shifts in substrate utilization which may be relevant to the transcriptional induction we report. Between the first embryonic cleavage and gastrulation, embryos use amino acids from yolk in preference to carbohydrates from stored glycogen as the primary carbon source (18), with mitochondrial glutamate oxidation playing a major role (19, 20). Glycolysis is not active during early embryonic cleavage even though the glycolytic machinery is intact (21–23). Fertilization also triggers transient increases in cytoplasmic calcium (24–26) which are likely to have profound effects on mitochondrial physiology because calcium regulates oxidative metabolism through effects on several matrix dehydrogenases (27–30). It is also possible that changes in mitochondrial ultrastructure could act as either the cause or effect of these metabolic changes during development. Evidence in support of this comes from electron micrographs of mature mouse eggs, showing mitochondria to be condensed, electron-dense structures. In contrast, mitochondria of four- to eight-cell embryos are swollen and structurally distinct, with numerous transversely arranged cristae (31, 32). These embryos exhibit a coincident rise in oxygen consumption and cyanide-sensitive ATP synthesis (33, 34). In the developing mouse embryo, mitochondrial transcription is active from the two- to four-cell embryonic stage onward, as indicated by a 25–50-fold increase in rRNA, cytochrome oxidase I, and cytochrome oxidase II mRNA levels (35). Such de novo transcription appears to play an essential role in mitochondrial differentiation during cleavage because inhibitors of mitochondrial RNA and protein syntheses also block the normal growth and differentiation of mitochondrial cristae (32). A similar mechanism of mitochondrial differentiation from an “embryonic” to “adult” state is likely to exist during X. laevis embryogenesis in concert with the changes in the metabolic steady states of the cell. Results reported here provide a mechanistic link between mitochondrial metabolism and mitochondrial transcriptional regulation during early embryogenesis. Unraveling the precise details of this link should help further understanding of the intricacies of cellular-mitochondrial communication.

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Regulation of Mitochondrial Gene Expression

6271

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