Transcriptional requirements of the distal heavy-strand promoter of mtDNA

Ornella Zollo[a], Valeria Tiranti[b], and Neal Sondheimer[a,c,1]

[a]Division of Child Rehabilitation, The Children's Hospital of Philadelphia and [c]Department of Pediatrics, University of Pennsylvania, Philadelphia, PA 19104; and [b]Division of Molecular Neurogenetics, Foundation Neurological Institute "C. Besta", 20126 Milan, Italy

Edited* by Douglas C. Wallace, Center for Mitochondrial and Epigenomic Medicine, Children's Hospital of Philadelphia, Philadelphia, PA, and approved March 6, 2012 (received for review November 12, 2011)

The heavy strand of mtDNA contains two promoters with nonoverlapping functions. The role of the minor heavy-strand promoter (HSP2) is controversial, because the promoter has been difficult to activate in an in vitro system. We have isolated HSP2 by excluding its interaction with the more powerful HSP1 promoter, and we find that it is transcribed efficiently by recombinant mtRNA polymerase and mitochondrial transcription factor B2. The mitochondrial transcription factor A is not required for initiation, but it has the ability to alternatively activate and repress the HSP2 transcriptional unit depending on the ratio between mitochondrial transcription factor A and other transcription factors. The positioning of transcriptional initiation agrees with our current understanding of HSP2 activity in vivo. Serial deletion of HSP2 shows that only proximal sequences are required. Several mutations, including the disruption of a polycytosine track upstream of the HSP2 initiation site, influence transcriptional activity. Transcription from HSP2 is also observed when HeLa cell mitochondrial extract is used as the source of mitochondrial polymerase, and this transcription is maintained when HSP2 is provided in proper spacing and context to the HSP1 promoter. Studies of the linked heavy-strand promoters show that they are differentially regulated by ATP dosage. We conclude that HSP2 is transcribed and has features that allow it to regulate mitochondrial mRNA synthesis.

mitochondrial biology | organelle

The mitochondrial genome (mtDNA) encodes 13 proteins that are constituents of the electron transport chain and ATP synthase. Transcription of mtDNA proceeds bidirectionally, radiating out from the noncoding D-loop. The light-strand promoter (LSP) is required for the synthesis of ND6 mRNA and eight of the mitochondrial tRNAs (1). Transcription from LSP also primes the origin of heavy-strand replication, which is the first step in the synthesis of mtDNA (2).

The heavy strand encodes 12 mRNAs, both mitochondrial rRNAs and the remaining tRNAs. Intracellular levels of mature rRNAs are in great excess to the mRNAs (3). Although it was initially proposed that this imbalance was caused by the existence of a single initiation site, with most primary transcripts terminating before reaching the protein coding region, later studies used a combination of ribonucleotide incorporation assays and capping of precursor transcripts to suggest the existence of two distinct heavy-strand initiation sites in close proximity (4–6). The first heavy-strand promoter (HSP1), which lies fully within the D-loop, is responsible for the synthesis of a transcript composed of the 12S and 16S rRNAs and intervening tRNAs (Fig. 1*A*). The minor or distal heavy-strand promoter (HSP2) initiates within the sequence of the mitochondrial tRNA for phenylalanine. HSP2-initiated transcription produces the remaining mitochondrial mRNAs by processing nearly the full length of the mtDNA.

Studies of HSP2 promoter function are complicated by its unusual position, both within a tRNA-coding gene sequence and with its first transcribed base in close proximity to the 5′ end of the 12S rRNA. The 5′ end of the transcript generated from HSP2 in cells was precisely mapped to C646 using primer extension and S1 digest of nascent transcripts from HeLa cells (7). However, recent studies that examined the minimal set of proteins required for mitochondrial transcription in vitro were unable to identify any transcription arising from HSP2 (8, 9). To resolve this apparent conflict, we investigated transcription from HSP2 in vitro using recombinant proteins and HeLa cell mitochondrial extracts. We find that the promoter is active, responsive to mitochondrial transcription factor A (TFAM) dosage, and sensitive to mutations in upstream sequence.

Results

Requirements for HSP2 Transcription. The mitochondrial heavy-strand promoters are closely spaced, and the isolation of HSP2 was required for visualization of its activity. When templates containing both the HSP1 and HSP2 promoters (spanning nucleotides 460–801) were transcribed by recombinant mtRNA polymerase (POLRMT), TFAM, and mitochondrial transcription factor B2 (TFB2M), we observed products corresponding to runoff transcription from HSP1 alone (Fig. 1*B*). After truncating the promoter so that it lacked HSP1 (nucleotides 583–801), we observed species corresponding to runoff transcription from the predicted HSP2 promoter at nucleotide 646. The difference between the relative activity of the two promoters was striking—HSP1 was greater than 100-fold more highly expressed under identical conditions.

Next, we evaluated different combinations of recombinant proteins to determine which were required for HSP2 activity. Although a requirement for TFAM in mitochondrial transcription was previously assumed, it has been suggested more recently that TFAM is dispensable for HSP1 transcription by POLRMT and TFB2M (9). Template-dependent transcriptional activity was seen when the three proteins were combined in equimolar ratio but was equally robust in reactions where TFAM was excluded (Fig. 1*C*). No transcription was observed when either POLRMT or TFB2M was excluded.

To determine the span of the functional HSP2 promoter, we created templates with truncations upstream of the site of initiation. A construct (nucleotides 623–801) extending 20 nt upstream had greatly reduced activity compared with the larger nucleotides 583–801 template. As expected, a deletion that removed the predicted start site (nucleotides 663–801) was inactive (Fig. 1*D*). This finding suggests that the promoter is enhanced by sequence at some distance from the start site but that only a short sequence spanning ~20 nt upstream is absolutely required for initiation.

Author contributions: N.S. designed research; O.Z. and N.S. performed research; V.T. contributed new reagents/analytic tools; O.Z. and N.S. analyzed data; and O.Z. and N.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]To whom correspondence should be addressed. E-mail: sondheimer@email.chop.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1118594109/-/DCSupplemental.

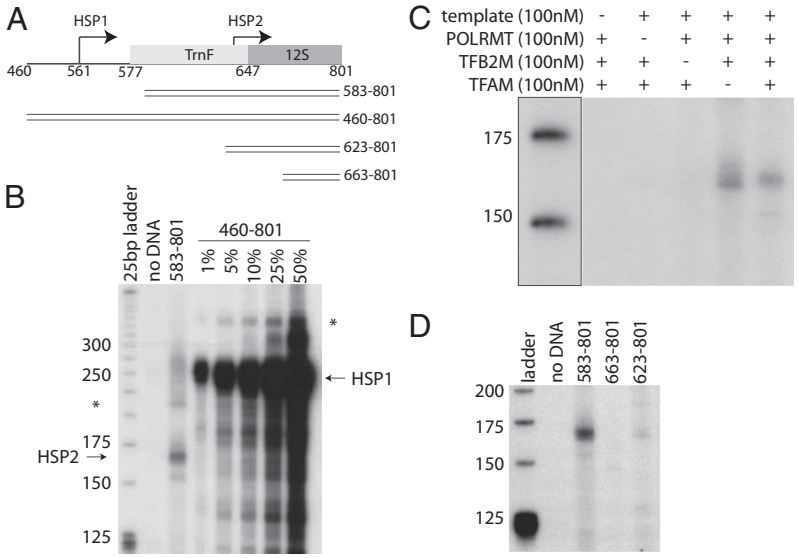

Fig. 1. In vitro transcription of HSP2 using purified transcription factors. (*A*) HSP2 and constructs used in this study. (*B*) Transcription of a 100-nM template containing HSP2 alone (nucleotides 583–801) by recombinant POLRMT, TFAM, and TFB2M (100 nM each) generated a runoff product of the expected size. Transcription using an equimolar template containing both promoters (nucleotides 460–801) produced strong products from HSP1 transcription but no detectible species from HSP2. To facilitate comparison between the promoters, the products of the HSP1-driven transcription were diluted before loading as shown. *RNA matching the size of input DNA is typically produced by POLRMT exposed to linear templates because of a nonspecific opening and transcription from DNA ends. (*C*) Combinations of recombinant transcription factors at 100 nM were used to transcribe HSP2. (*D*) In vitro transcription assays were performed using several truncations of the predicted HSP2 promoter.

Confirmation of the Site of Initiation by S1 Analysis. An alignment of the initiation sites for the mitochondrial promoters HSP1 and LSP highlights several similarities. Transcription at these sites initiates at adenosine residues after a short cytosine-rich stretch (Fig. 2*A*). A similar motif is found at the site of HSP2 initiation. Determination of the 5′ end of HSP2 transcripts using RNA obtained from tissues or intact mitochondria is complicated by the proximity of the promoter to the 5′ end of the mature 12S rRNA, which is present in vast excess. The examination of transcripts produced using recombinant POLRMT, TFB2M, and TFAM allows us to avoid contamination from newly synthesized rRNA.

We performed S1 analysis of RNA transcribed from the HSP2-containing template. The mapping identified a triplet of bands spanning potential initiation points from 642 to 644 nt (Fig. 2*B*). These mapped 5′ ends were in reasonable agreement with those ends previously determined using RNA from HeLa cells (C646) (7). To confirm the detected initiation point, we performed 5′ amplification of cDNA ends, which found that most ends mapped at A644 (Fig. 2*C*).

Mutagenesis of HSP2. In previous random mutagenesis studies of the HSP1 and LSP promoter, the disruption of a short upstream cytosine-rich sequence interfered with promoter function (10). Similarly, the mutation of an upstream polycytosine motif near the site of HSP2 initiation reduced activity (Fig. 3*A*, 637–640T template). To confirm that this finding was not an artifact originating from the use of recombinant proteins, we repeated this study using transcription driven by partially purified HeLa cell mitochondrial extracts and obtained a similar response to the mutation of the polycytosine tract (Fig. 3*B*).

Because we had observed reduced transcription of a 5′-truncated HSP2 construct, we examined the possibility that sequences farther upstream regulate expression. We created a series of mutants where a poly-T sequence was inserted in place of sequential 10-bp stretches of the promoter. Limited transcription was seen from a construct where nucleotides 611–620 were replaced with a poly-T sequence. Again, the effect was seen in transcription reactions driven by either recombinant proteins or mitochondrial extracts.

Next, we analyzed mutations of individual bases between the polycytosine tract and the coding sequence of 12S. The T642G mutation potently induced transcription by recombinant transcription factors (Fig. 3*C*). Other substitutions near the site of initiation caused modest but reproducible alterations in RNA synthesis. Because substitutions may cause initiation at ectopic sites, we used S1 nuclease analysis to examine the size of the transcript produced from the T642G template and found that it was identical to the size of the transcript produced from WT DNA (Fig. S1). We next examined the effect of these mutations on transcription driven by mitochondrial extracts. Surprisingly, the T642G mutation caused little change in transcription by mitochondrial extracts, making the significance of this position in vivo unclear (Fig. S2*A*).

A unique feature of HSP2 is that its sequence overlaps the coding region for the mitochondrial phenylalaninyl tRNA (MTTF). As a result, mutations in MTTF coding sequence may interfere with mitochondrial function through disruptions in translation, transcription, or both. We examined the effect of the m.642T > C mutation that was described in an adult patient with mitochondrial disease (11). There was no obvious defect in transcription of an m.642T > C template by either mitochondrial extracts or recombinant proteins (Fig. 3*C* and Fig. S2*A*). Furthermore, RT-PCR analysis of cDNA generated from patient muscle did not reflect the failure of HSP2 transcription, which would impair the expression of most mitochondrial mRNAs (Fig. S2 *B* and *C*). Instead, Northern blotting of tRNAs confirmed low levels of MTTF, consistent with destabilization of the tRNA.

Regulation of HSP2 Transcription by TFAM Dosage. The ratio between TFAM and POLRMT/TFB2M influences transcription from the HSP1 and LSP promoters (9). Therefore, we examined the effect of varying TFAM levels at HSP2. Although TFAM is not required, changing the ratio between TFAM and POLRMT/TFB2M affected HSP2 activity (Fig. 4). Initially, added TFAM increased the synthesis of RNA, but it occurred only at

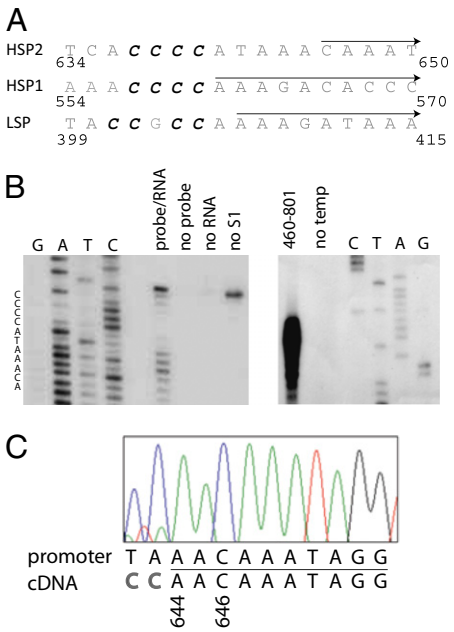

Fig. 2. Localization of the HSP2 start site. (*A*) Alignment of the mitochondrial promoters. The polycytosine tract preceding transcription is highlighted in bold, and the primary transcript is underlined. The HSP2 start site as drawn is taken from the work by Martin et al. (7). (*B*) S1 analysis of RNA produced by recombinant TFAM, TFB2, and POLRMT acting on the nucleotides 583–801 template (*Left*) produced bands corresponding to ends between positions 642 and 644. For convenience, the sequencing ladder shown reads the nontemplate strand and has the same 5′ end as the S1 probe. A small amount of fully protected probe is also seen because of synthesis of full-length RNA from linear templates. We examined RNA produced from the nucleotides 460–801 template (*Right*) using an S1 probe complementary to nucleotides 554–607. S1 cleavage of this probe gave a 47-nt product matching the expected size of the HSP1 transcript. (*C*) RACE sequencing from RNA synthesized by recombinant proteins transcribing nucleotides 583–801. Sequences inserted by the reverse transcriptase (polycytosine) predominate upstream of A644, indicating that 5′ ends begin predominantly at this point.

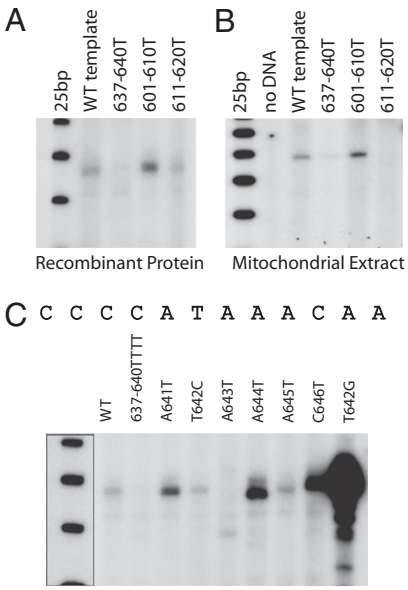

Fig. 3. Mutagenesis of HSP2. (*A*) A series of mutation constructs were created in which blocks of upstream nucleotides where replaced by poly-T tracts. (*B*) Transcription by HeLa cell mitochondrial extracts (10 μg) in the presence of a 100-nM nucleotides 460–801 template with the specified poly-T replacement mutations. (*C*) A series of HSP2 promoter mutants were created in which one base at a time was mutated from the WT sequence. The ability of HSP2 mutants to support transcription was investigated using recombinant proteins as described previously. The WT sequence is noted above the autoradiogram.

substoichiometric levels. Transcription was most active with the addition of 10 nM TFAM (a ratio of 1:10 with POLRMT). Increasing TFAM to equimolar levels eliminated the TFAM-dependent stimulation, and the addition of excess TFAM was inhibitory.

Influence of ATP Concentration on HSP1 and HSP2 Transcription. Previous studies have documented the down-regulation of HSP1- and LSP-initiated transcription at high concentrations of ATP (12). To examine the effect of ATP concentration on the linked human heavy-strand promoters, we used a template containing the HSP1 and HSP2 promoters in their proper spacing. As noted previously, this template was unable to produce detectable transcripts from HSP2 when activated by recombinant POLRMT, TFAM, and TFB2M—only HSP1 was observed. However, HeLa cell mitochondrial extracts produced species corresponding to the initiation of both promoters. As expected, no products were produced in the absence of ATP (Fig. 5). Initially, increasing ATP concentration tended to activate both promoters. However, as ATP concentration rose through 1 mM, HSP2 transcription became increasingly favored, and HSP1 was repressed.

Discussion

In this study, we have examined features of the minor heavy-strand promoter (HSP2). We found that the simultaneous and linked presence of HSP1 suppressed HSP2 transcription by recombinant TFB2M, POLRMT, and TFAM. Even when studied in isolation, HSP2 was more than 100-fold more weakly transcribed that HSP1 (Fig. 1*B*). Other investigators have not observed transcription from HSP2 in vitro, most notably in a recent study where specific attempts were made to identify products from an isolated promoter (8). This apparent discrepancy may be because of the use of high fixed ratios of TFAM/POLRMT in recombinant transcription reactions, which we found to repress HSP2 activity.

In the presence of the full complement of mitochondrial matrix proteins, transcription from both of the naturally linked promoters was observed, more closely mimicking natural expression. This finding indicates that additional activators or coactivators regulate the balance between the heavy-strand promoters or are required for full HSP2 activation. Several candidates for this role exist, including LRPPRC, which activates transcription and stabilizes existing mRNAs (13–15).

Mapping of transcripts produced from oligonucleotide templates using either HeLa cell mitochondrial extracts (7) or recombinant protein shows that the initiation point lies within the coding sequence for the tRNA for phenylalanine (MTTF) and very close to the first nucleotide of 12S. Identification of the exact 5′ end of this transcript from in vivo-produced RNA is

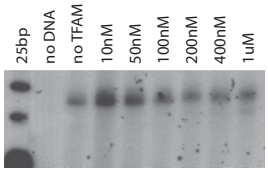

Fig. 4. HSP2 transcription responds to TFAM dosage. Transcription reactions were performed using the indicated concentration of TFAM. Template, POLRMT, and TFB2M were added at 100 nM.

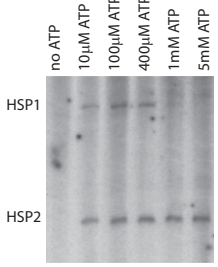

Fig. 5. Heavy-strand promoters have differential responses to ATP concentration. Transcription reactions were performed using the nucleotides 460–801 template containing both the HSP1 and HSP2 promoters and HeLa cell mitochondrial extracts. All components were held constant except for the level of ATP, which varied up to 5 mM.

complicated by the proximity of 12S. We suggest, based on our evidence, that the first nucleotide may have some degree of flexibility and that initiation of transcription at 646C may not be the sole product of HSP2 transcription. The predominant 5′ end from the in vitro reactions in this study mapped to 644A.

Mutational analysis of HSP2 shows the effect of mutations upstream of the first nucleotide. The mutation of a polycytosine tract (nucleotides 637–640) preceding the point of initiation reduces transcription. The response to this mutation is similar to the effect of previously examined mutations of cytosine-rich sequence in the HSP1 and LSP promoter and suggests a common mechanism of promoter recognition (10). Previous studies of HSP1 and LSP suggest that POLRMT plays the predominant role in promoter recognition, and the polycytosine tract seems to be important for its recognition of promoters (16, 17).

Other substitutions within the promoter had complex effects that were dependent on both the source of transcriptional activity and the inserted nucleotide. The T642G mutation potently activated HSP2 transcription, making the promoter many fold stronger than WT. However, this effect was only seen using recombinant protein. HeLa cell mitochondrial extracts were not perceptibly activated by the mutation. This finding points to a potential weakness of studying transcription using recombinant proteins, which are present in high concentration and exclude the influence of other unrecognized coactivators or transcription factors.

A different mutation of the same nucleotide (m.642T > C) has been identified in a patient with features of mitochondrial disease, including intellectual impairment, myopathy, and external ophthalmoplegia (11). Because the HSP2 promoter overlaps the *MTTF* gene, it was possible that the mutation caused mitochondrial impairment by interfering with transcription rather than by direct effects on the tRNA. However, the m.642T > C mutation caused only a modest reduction in mitochondrial transcription in vitro, and Northern blot analysis showed that the mutation compromised the levels of MTTF. We conclude that m.642T > C impairs MTTF stability by its disruption of Watson–Crick pairing within the acceptor stem. A similar evaluation of reported MTTF mutations for effects on transcription may be warranted.

The role of TFAM in mitochondrial transcription is complex. We observed that activation of HSP2 required only TFB2M and POLRMT. When TFAM was titrated into the transcription reactions, the effects were biphasic. A modest stimulation was observed at low ratios of TFAM to POLRMT, but the principal effect at higher concentrations was to suppress transcription. TFAM may exert its repressive effects in the in vitro system by nonspecific interactions with the template (18). The enhancement of transcription at low TFAM concentrations may be mediated by specific binding of correctly oriented TFAM within the promoter, but the significance of this observation should be viewed carefully. TFAM is not known to be present at levels substoichiometric to template (19), and the use of a linear template may complicate the evaluation of a protein that bends DNA during activation (20–22).

These findings are best considered in the light of the disputed requirement for TFAM at HSP1 and LSP (8, 9). TFAM had initially been classified as a core transcription factor (23), but more recent evidence suggests a similarly biphasic role in the modulation of HSP1 and LSP, with activation and repression predominant at different TFAM concentrations (9). This finding has given rise to the suggestion that TFAM acts to modulate mitochondrial transcription, increasing the availability of rRNAs at low concentration, increasing mRNA and mtDNA replication at moderate concentration, and shutting both transcription and replication down at high concentration (24, 25).

Transcription from the human HSP1 and LSP is affected by ATP concentration (12, 16), an effect that has also been observed at mitochondrial promoters in *Saccharomyces cerevisiae* (26). We found that increasing ATP concentration up to 100 μM stimulated both HSP1 and HSP2, which is in agreement with studies previously performed using recombinant proteins at HSP1 (16). Increasing the level of ATP beyond this point tended to suppress transcription at HSP1 while preserving HSP2 transcription. The levels at which a decline in HSP1 transcription is seen are within the range of expected matrix ATP concentration, suggesting the relevance of this process to physiological regulation (27). The loss of HSP1 expression at ATP concentrations greater than 1 mM has been previously observed in transcription driven by HeLa cell mitochondrial extract (12). We speculate that the reduction in HSP1 expression could represent a mechanism for the down-regulation of mitochondrial translation at points when energy supply is replete and a near-term increase in electron transport activity is not required. HSP2 is not similarly controlled, providing a point for the differential regulation of this promoter compared with LSP and HSP1. The mechanisms governing these relationships will require additional study.

The mitochondrial transcriptional program is remarkably efficient, producing 13 mRNAs and sufficient rRNAs and tRNAs for translation from a small number of primary transcripts. HSP2 has proven least amenable to study because of its proximity to HSP1 and the first base of the 12S rDNA. Difficulties in the analysis of HSP2 in vitro have cast some doubts on its existence. However, our data and the data simultaneously observed in the work by Lodeiro et al. (25) show that either recombinant proteins or mitochondrial extracts can activate HSP2. In combination with evidence from metabolic labeling of primary transcripts, this finding confirms the role of HSP2 in driving mitochondrial gene expression.

The use of separate heavy-strand promoters for rRNA and mRNA synthesis has logical appeal, but many questions remain. The full complement of regulatory mechanisms that balance promoter choice is not known. TFAM and ATP levels may regulate this process, but it is likely that other transcription factors or coactivators are required. The improving understanding of mitochondrial transcription will contribute to our ability to understand its importance in health and disease.

Materials and Methods

Cloning. Human mtDNA fragments were cloned into pCR 2.1 (Invitrogen) with flanking EcoRV sites. Mutations were generated with the Gene Tailor System (Invitrogen) and confirmed by sequencing.

Recombinant Proteins and HeLa Cell Mitochondrial Extract. Purified recombinant POLRMT, h-mtTFA, and h-mtTFB2 (Enzymax) were produced as described (16). HeLa cell mitochondrial extracts used in transcriptional assays were produced as previously described (13). Mitochondrial extracts were fractionated on a diethylaminoethyl-Sephacel column, and the fraction eluting with 0.3 M KCl was used for in vitro transcription.

In Vitro Transcription Assay. In vitro transcription reactions were performed with 100 nM digested template, 10 mM Hepes, 1 mM DTT, 10 mM $MgCl_2$, 100 μg/mL BSA, 100 mM NaCl, 400 μM ATP, GTP, CTP, 40 μM UTP, 0.3 μCi/μL (α-32P) UTP, and recombinant proteins at the concentrations indicated. After incubation at 32 °C for 30 min, the reaction was stopped by adding 200 μL 10 mM Tris·HCl (pH 8), 0.2 M NaCl, 1 mM EDTA, 0.5% SDS, and 100 μg/mL proteinase K for 45 min at 42 °C. The transcription products were recovered by phenol extraction and precipitated with ethanol. The pellets were resuspended in 6 μL gel-loading buffer (98% formamide, 10 mM EDTA (pH 8), 0.025% xylene cyanol FF, 0.025% bromophenol blue) and heated at 95 °C, and the transcription products were analyzed on 8% polyacrylamide/7 M urea gel.

For transcription with HeLa cell mitochondrial extract, we used 20 μL affinity-purified proteins fraction and 400 μM ATP, GTP, UTP, 10 μM CTP, and 1.8 μCi/μL (α-32P) CTP. All other conditions were as described above. When evaluating HSP2 transcription driven by mitochondrial extracts, the larger nucleotides 460–801 template was used to allow the visualization of concurrent HSP1 transcription.

Northern Blot. Muscle tissue RNA (1 μg) from patient and nondiseased controls was loaded on 15% polyacrylamide gel and transferred to a membrane (Hybond-N+; Amersham). The membrane was incubated with a hybridization solution (LXpressHyb; Clontech) containing 1×10^6 cpm/μL 5′-(γ-32P) ATP-labeled oligonucleotides as previously described (28). For the cytochrome c oxidase subunit I expression, the RNA was separated on a 1.2% agarose/formaldehyde gel and hybridized overnight at 42 °C with a DNA probe labeled with a DECAprime II Kit (Ambion).

S1 Analysis. S1 analysis was performed using oligonucleotide probes as previously described (29). The HSP2 probe spanned nucleotides 694–635. After overnight hybridization with target RNA, S1 (100 U) was added for 1 h at 32 °C. Products were analyzed on a 12% acrylamide/urea gel.

5′ RACE. RNA from transcription reactions was annealed to a primer spanning nucleotides 801–785 (30). After extension and polyadenylation, cDNA was amplified using a poly-T primer, and nested reverse primers (nucleotides 801–785 and 728–708) and products were sequenced.

ACKNOWLEDGMENTS. Craig Cameron, Fernanda Lodeiro, and Brett Kaufman read drafts of the manuscript. Eleonara Lamantea provided technical assistance. Support for this work was provided by National Institutes of Health Grant K08-HD58022 (to N.S.) as well as the Children's Hospital of Philadelphia Pediatric Development Fund.

1. Anderson S, et al. (1981) Sequence and organization of the human mitochondrial genome. *Nature* 290:457–465.
2. Chang DD, Clayton DA (1985) Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc Natl Acad Sci USA* 82:351–355.
3. Gelfand R, Attardi G (1981) Synthesis and turnover of mitochondrial ribonucleic acid in HeLa cells: The mature ribosomal and messenger ribonucleic acid species are metabolically unstable. *Mol Cell Biol* 1:497–511.
4. Montoya J, Gaines GL, Attardi G (1983) The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. *Cell* 34:151–159.
5. Montoya J, Christianson T, Levens D, Rabinowitz M, Attardi G (1982) Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA. *Proc Natl Acad Sci USA* 79:7195–7199.
6. Yoza BK, Bogenhagen DF (1984) Identification and in vitro capping of a primary transcript of human mitochondrial DNA. *J Biol Chem* 259:3909–3915.
7. Martin M, Cho J, Cesare AJ, Griffith JD, Attardi G (2005) Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis. *Cell* 123:1227–1240.
8. Litonin D, et al. (2010) Human mitochondrial transcription revisited: Only TFAM and TFB2M are required for transcription of the mitochondrial genes in vitro. *J Biol Chem* 285:18129–18133.
9. Shutt TE, Lodeiro MF, Cotney J, Cameron CE, Shadel GS (2010) Core human mitochondrial transcription apparatus is a regulated two-component system in vitro. *Proc Natl Acad Sci USA* 107:12133–12138.
10. Hixson JE, Clayton DA (1985) Initiation of transcription from each of the two human mitochondrial promoters requires unique nucleotides at the transcriptional start sites. *Proc Natl Acad Sci USA* 82:2660–2664.
11. Valente L, et al. (2009) Identification of novel mutations in five patients with mitochondrial encephalomyopathy. *Biochim Biophys Acta* 1787:491–501.
12. Enríquez JA, Fernández-Silva P, Pérez-Martos A, López-Pérez MJ, 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.
13. Sondheimer N, Fang JK, Polyak E, Falk MJ, Avadhani NG (2010) Leucine-rich pentatricopeptide-repeat containing protein regulates mitochondrial transcription. *Biochemistry* 49:7467–7473.
14. Liu L, et al. (2011) LRP130 protein remodels mitochondria and stimulates fatty acid oxidation. *J Biol Chem* 286:41253–41264.
15. Xu F, Morin C, Mitchell G, Ackerley C, Robinson BH (2004) The role of the LRPPRC (leucine-rich pentatricopeptide repeat cassette) gene in cytochrome oxidase assembly: Mutation causes lowered levels of COX (cytochrome c oxidase) I and COX III mRNA. *Biochem J* 382:331–336.
16. Lodeiro MF, et al. (2010) Identification of multiple rate-limiting steps during the human mitochondrial transcription cycle in vitro. *J Biol Chem* 285:16387–16402.
17. Gaspari M, Falkenberg M, Larsson NG, Gustafsson CM (2004) The mitochondrial RNA polymerase contributes critically to promoter specificity in mammalian cells. *EMBO J* 23:4606–4614.
18. Kaufman BA, et al. (2007) The mitochondrial transcription factor TFAM coordinates the assembly of multiple DNA molecules into nucleoid-like structures. *Mol Biol Cell* 18:3225–3236.
19. Alam TI, et al. (2003) Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res* 31:1640–1645.
20. Fisher RP, Lisowsky T, Parisi MA, Clayton DA (1992) DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. *J Biol Chem* 267:3358–3367.
21. Malarkey CS, Bestwick M, Kuhlwilm JE, Shadel GS, Churchill ME (2012) Transcriptional activation by mitochondrial transcription factor A involves preferential distortion of promoter DNA. *Nucleic Acids Res* 40:614–624.
22. Ngo HB, Kaiser JT, Chan DC (2011) The mitochondrial transcription and packaging factor Tfam imposes a U-turn on mitochondrial DNA. *Nat Struct Mol Biol* 18:1290–1296.
23. Fisher RP, Clayton DA (1988) Purification and characterization of human mitochondrial transcription factor 1. *Mol Cell Biol* 8:3496–3509.
24. Shutt TE, Bestwick M, Shadel GS (2011) The core human mitochondrial transcription initiation complex: It only takes two to tango. *Transcription* 2:55–59.
25. Lodeiro, et al. (2012) *Proc Natl Acad Sci USA* 109:6513–6518.
26. Amiott EA, Jaehning JA (2006) Mitochondrial transcription is regulated via an ATP "sensing" mechanism that couples RNA abundance to respiration. *Mol Cell* 22:329–338.
27. Metelkin E, Demin O, Kovács Z, Chinopoulos C (2009) Modeling of ATP-ADP steady-state exchange rate mediated by the adenine nucleotide translocase in isolated mitochondria. *FEBS J* 276:6942–6955.
28. Glatz C, D'Aco K, Smith S, Sondheimer N (2011) Mutation in the mitochondrial tRNA(Val) causes mitochondrial encephalopathy, lactic acidosis and stroke-like episodes. *Mitochondrion* 11:615–619.
29. Greene JM, Struhl K (2001) S1 analysis of messenger RNA using single-stranded DNA probes. *Curr Protoc Mol Biol*, 10.1002/0471142727.mb0406s45.
30. Dorit RL, Ohara O (2001) cDNA amplification using one-sided (anchored) PCR. *Curr Protoc Mol Biol*, 10.1002/0471142735.im1024s08.