Identification of NAD$^+$ capped mRNAs in *Saccharomyces cerevisiae*

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Contributed by Roy Parker, December 5, 2016 (sent for review November 17, 2016; reviewed by Jens Lykke-Andersen and David Tollervey)

RNAs besides tRNA and rRNA contain chemical modifications, including the recently described 5′ nicotinamide-adenine dinucleotide (NAD$^+$) RNA in bacteria. Whether 5′ NAD-RNA exists in eukaryotes remains unknown. We demonstrate that 5′ NAD-RNA is found on subsets of nuclear and mitochondrial encoded mRNAs in *Saccharomyces cerevisiae*. NAD-mRNA appears to be produced cotranscriptionally because NAD-RNA is also found on pre-mRNAs, and only on mitochondrial transcripts that are not 5′ end processed. These results define an additional 5′ RNA cap structure in eukaryotes and raise the possibility that this 5′ NAD$^+$ cap could modulate RNA stability and translation on specific subclasses of mRNAs.

NAD-RNA | RNA modification | mitochondria | transcription

The number and prevalence of known chemical modifications on mRNAs have dramatically increased in the past several years (1). Quantification of these modification events suggests they occur in many RNAs (2, 3). Importantly, several of these modifications have functional consequences (4–6). For example, the presence of a single N^6-methyladenosine within the 5′ UTR of an mRNA increases translation initiation (4). In addition, the methylation status of cytosine residues within the 3′ UTR of the p16(INK4) human mRNA affects mRNA stability (6). Due to the increasing sensitivity of RNA sequencing (RNA-Seq) and small-molecule mass spectrometry, it is reasonable to hypothesize that many novel chemical modifications within mRNAs remain to be discovered.

One modification recently identified in bacteria is 5′ nicotinamide-adenine dinucleotide (NAD$^+$)–linked RNA (7, 8). Because canonical bacterial RNAs contain a 5′ triphosphate terminus, addition of NAD$^+$ to the 5′ end of RNAs represents a rudimentary "capping" mechanism, perhaps designed to impart specific properties for these RNAs by granting them a more structurally complex 5′ end. Consistent with this idea, NAD$^+$ addition to RNAs appears to occur during transcription initiation (9), as opposed to the more complex eukaryote 5′ capping, which occurs after transcription has commenced (10). Because the NAD$^+$ modification defines the 5′ end of NAD-RNAs, this modification can affect RNA stability in *Escherichia coli* (7, 9).

For decades, 5′ end classification and study of eukaryotic mRNAs have been restricted to canonical 7-methylguanosine (m^{7}G) "caps" and their methylated variants (11). The m^{7}G cap modulates numerous facets of mRNA metabolism, including stability (12, 13), translation (14, 15), and export (16). The importance of this modification is underscored by the substantial cellular machinery dedicated to its addition and removal (17, 18). Thus, mRNAs containing noncanonical 5′ termini may have distinct properties and be subject to alternative metabolic events.

Described herein is the identification of NAD-RNAs in the eukaryote *Saccharomyces cerevisiae*. Examples of NAD-RNAs in *S. cerevisiae* include nuclear encoded mRNAs for ribosomal proteins, as well as some mitochondrial encoded transcripts. Our data suggest that the NAD$^+$ moiety is added during initiation in both nuclear and mitochondrial transcription. Identification of 5′ NAD-RNA in eukaryotes advances the catalog and understanding of chemically modified mRNAs. Moreover, given the functional importance of 5′ end RNA modifications, this study beckons a renewed understanding of caps in eukaryotes.

Results

Yeast Cells Contain Specific NAD$^+$-Modified mRNAs. To examine if NAD-RNA exists in yeast, we used the approach wherein NAD-RNAs (structurally depicted in Fig. 1*A*) are identified by the reaction of the NAD$^+$ moiety with ADP ribosylcyclase (ADPRC), thereby allowing biotin incorporation and purification of modified RNAs on streptavidin (7). Comparison of in vitro synthesized uncapped and 5′ NAD-RNA demonstrated that this method was specific for the purification of NAD-RNA (Fig. S1). Using this method, we detected enrichment of NAD-RNA from yeast cells as detected either by pCp [5′-^{32}P] labeling or by oligo(dT) Northern blots compared with mock-treated samples (Fig. 1 *B* and *C*). The enrichment of poly(A)$^+$ mRNA in the ADPRC-selected population suggested that yeast mRNAs contain 5′ NAD$^+$ modifications.

To determine the RNAs enriched in the ADPRC-treated samples, we performed NAD capture sequencing (NAD-CaptureSeq) (7) on ADPRC-treated samples from cells grown in rich or synthetic media compared with mock-treated control samples. Sequencing reactions were performed in triplicate, and were highly reproducible (Fig. S2).

We identified specific mRNAs enriched over the background control from cultures grown in either rich yeast extract peptone dextrose (YEPD) media or synthetic media (Fig. 2 *A* and *B*).

Significance

The knowledge of the number and functional significance of chemical modifications within mRNA has increased dramatically over the past few years. We identify an mRNA modification in the eukaryote *Saccharomyces cerevisiae*, nicotinamide-adenine dinucleotide (NAD$^+$)–linked mRNA, which was previously reported in bacteria. This finding is significant for two reasons. First, NAD$^+$ is located at the 5′ terminus of mRNAs, where the 7-methylguanosine cap is known to modulate many important mRNA functions. Second, we present data that suggest NAD$^+$ is added as the initiating nucleotide during transcription, which suggests an additional layer of transcriptional control. The presence of an alternative cap structure on mRNA 5′ ends suggests a possible unanticipated level of regulation due to this modification.

Author contributions: R.W.W., T.M., L.S.M., B.S.R., D.M., and R.P. designed research; R.W.W., T.M., L.S.M., B.S.R., and D.M. performed research; and R.W.W., T.M., and R.P. wrote the paper.

Reviewers: J.L.-A., University of California, San Diego; and D.T., University of Edinburgh.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo/ (accession nos. GSE90636 and GSM2409726–GSM2409737).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1619369114/-/DCSupplemental.

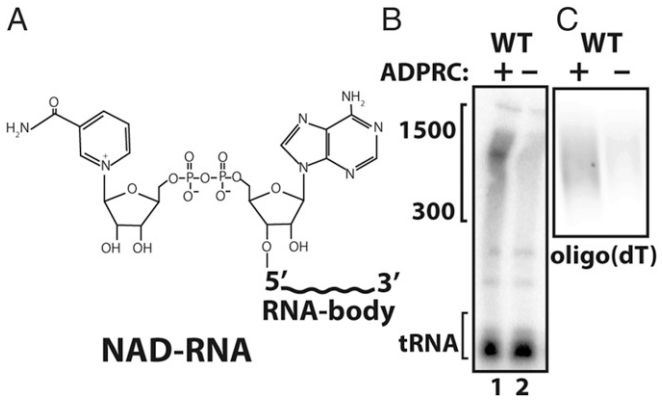

Fig. 1. ADPRC treatment enriches for a biochemical fraction that contains NAD-RNA. (*A*) Structure of NAD-capped RNA. (*B*) PAGE gel of pCp [5′-^{32}P]-labeled RNA of total yeast RNA that was incubated after selection either with or without treatment with ADPRC. (*C*) Similar to *B*, total yeast RNA was either treated with ADPRC or mock-treated, run on a Northern blot, and probed for polyA$^+$ RNA using a poly(dT) DNA probe.

More NAD$^+$-modified mRNAs were detected in yeast grown in synthetic media compared with rich YEPD media (Fig. 2*C*), implying either the biogenesis or metabolism of NAD-RNAs is modulated by growth conditions. NAD-RNAs are not simply the most highly abundant RNAs in the cell, because there is no correlation between overall abundance (as assessed by RNA-Seq reads under the same growth conditions) and the enrichments in the NAD-RNA population (Fig. 2*D*). These results argue that subsets of yeast mRNAs can have a 5′ NAD$^+$ modification.

We observed specific classes of mRNAs as being preferentially modified with NAD$^+$. The mRNAs encoding components of the translation machinery were preferentially modified (Fig. 3*A*). In addition, using Gene Ontology term analysis, we observed an enrichment of mRNAs involved in mitochondrial function (Fig. 3*A*). This result is notable because we also observed enrichment in the NAD-RNA population of the mitochondrial encoded mRNAs, F0-ATP synthase subunit c (*ATP9*) and cytochrome c oxidase subunit 2 (*COX2*), as well as the mitochondrial 15S and 21S rRNAs (Fig. 3 *B–D*). Consistent with NAD$^+$ being at the 5′ end of the identified RNAs, we observed a 5′ end bias in the NAD-RNA-Seq reads for the mitochondrial 21S rRNA.

We verified that specific mRNAs [enhancer of Gal4 DNA binding (*EGD2*), glycerol-3-phosphate phosphatase (*GPP1*), and ribosomal protein of the large subunit 16b (*RPL16b*)] contained NAD-RNA species by showing an enrichment in Northern blots of ADPRC-treated and -purified RNA compared with control lanes, whereas abundant 7S RNA was not enriched (Fig. 3*D*).

We used the ADPRC enrichment method to estimate the percentage of specific mRNAs that are NAD$^+$-modified by calculating

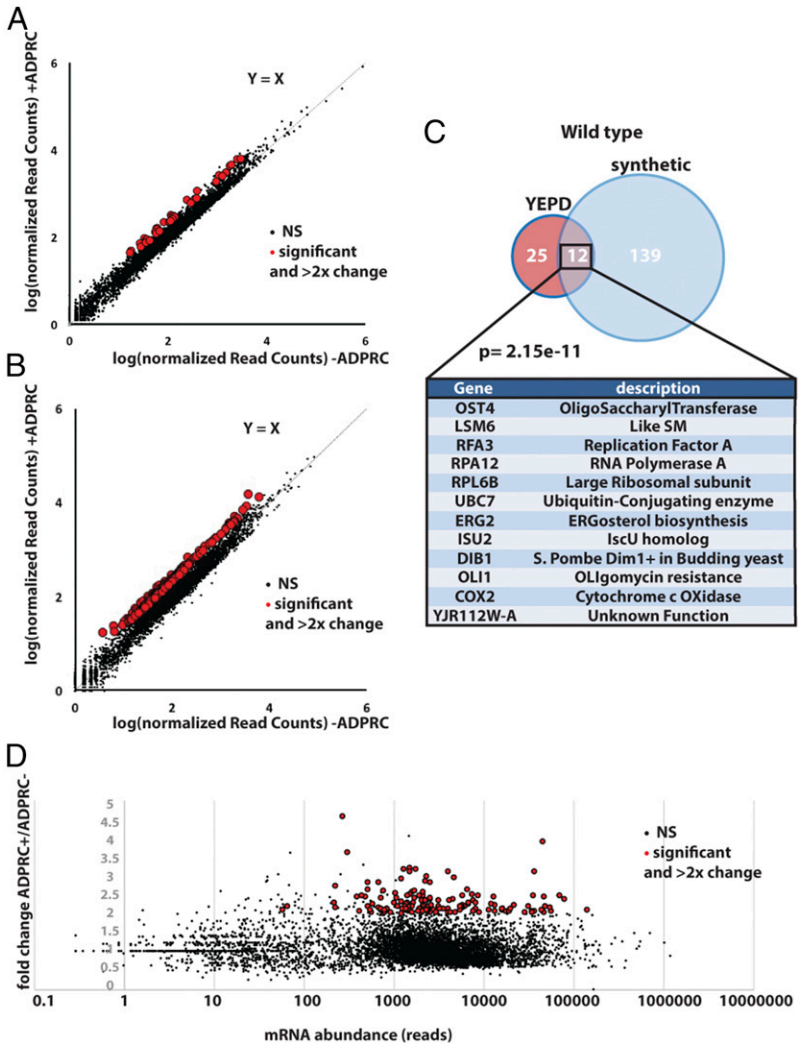

Fig. 2. NAD-Capture-Seq identifies NAD-RNA in yeast. Abundance of ADPRC-treated RNAs vs. mock-treated samples in rich media (*A*) and synthetic defined media (*B*). Red triangles indicate mRNAs enriched at least twofold and statistically enriched with *P* values <0.05 using standard parameters in DESeq software. Black dots indicate non-enriched RNAs. (*C, Top*) Overlap of NAD-RNAs in a WT yeast strain grown in YEPD and synthetic media (*P* = 2.15e-11). (*C, Bottom*) List of NAD-RNAs found in both growth conditions in WT yeast. (*D*) NAD-RNAs (ADPRC$^+$/ADPRC$^-$) (red triangles, statistically enriched and greater than twofold change) plotted vs. total mRNA abundance.

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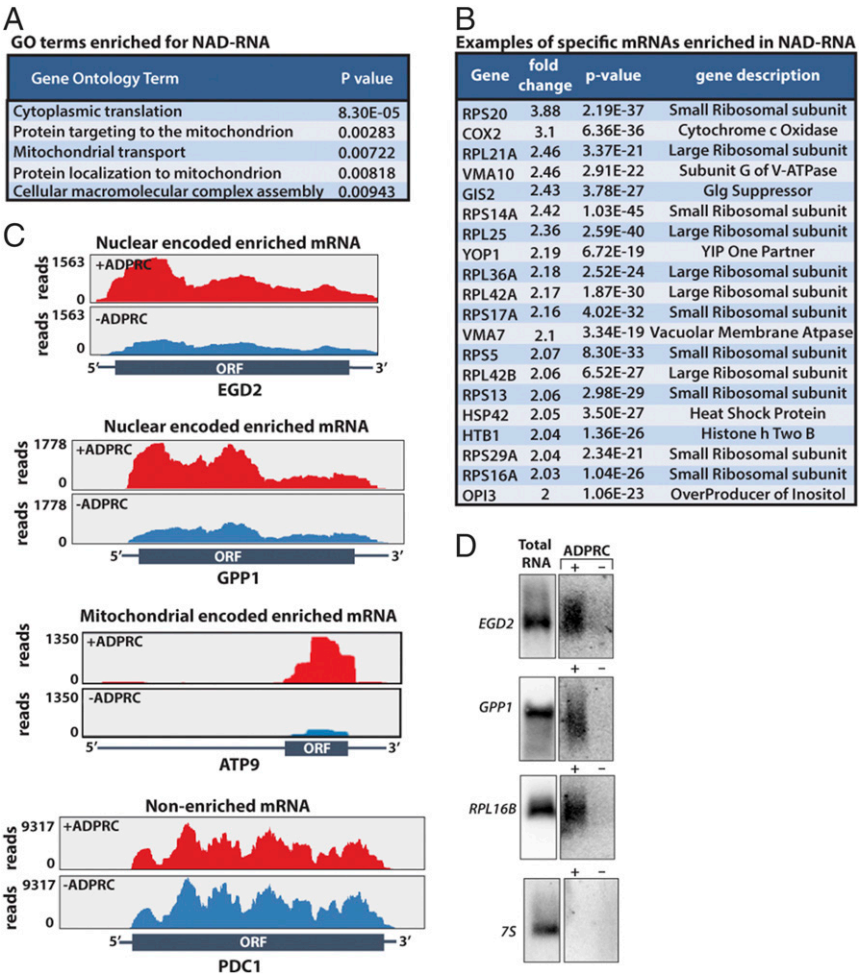


Fig. 3. NAD-mRNAs are encoded in both the nuclear and mitochondrial genomes. (*A*) Gene Ontology (GO) term analysis of yeast NAD-RNAs. (*B*) Twenty most highly enriched NAD-RNAs in WT yeast grown in synthetic media. (*C*) Gene tracks depicting RNA-Seq reads of two enriched nuclear encoded mRNAs (*EGD2* and *GPP1*), a mitochondrial encoded mRNA (*ATP9*), and a nonenriched mRNA (*PDC1*). (*D*) Northern blots of mRNAs identified by NAD-capture as well as 7S rRNA control.

the percentage of each mRNA enriched by the ADPRC method, which was ~20% efficient based on control NAD-RNAs generated in vitro (Fig. S1). Based on this approach, we estimate that the amount of the *EGD2*, *GPP1*, and *RPL16b* mRNAs containing NAD^+-modified 5′ ends are 10%, 7.5%, and 5.5%, respectively. However, these numbers can only be used as very rough estimates of the amount of NAD-RNA in cells because these calculations assume that every NAD-linked RNA is as efficiently selected as control RNAs. Thus, we conclude that at steady state in normal growth conditions, the mRNAs with NAD^+ modifications represent a small, but not insignificant, subset of the RNA molecules, on the order of 1–5% of a given mRNA population.

NAD^+ Appears to Be Added to RNAs Cotranscriptionally. In principle, NAD^+ could be added to the 5′ end of mRNAs after cytoplasmic or nuclear decapping, or during the process of transcription. Previous results suggest that in bacterial cells, NAD^+ is added to RNAs in a cotranscriptional process, because RNA polymerases can use NAD^+ instead of ATP at transcription initiation sites that begin with an adenosine residue (9, 19). Two observations suggest that this cotranscriptional mechanism is also true in *S. cerevisiae*. First, for NAD^+ mRNAs with introns, we observed a similar degree of enrichment in the exon and intron reads, whereas the reads from all mRNAs did not show intron enrichment (Fig. 4). This observation argues that NAD^+ is added to mRNA transcripts before splicing.

A second key observation is that there is a strong correlation between the NAD-containing mitochondrial transcripts (*ATP9*, *COX2*, 15S, and 21S) and those mitochondrial RNAs that do not undergo 5′ end processing (20) (Fig. 5 *A* and *B*). This correlation is consistent with NAD^+ being added to 5′ ends cotranscriptionally. Moreover, transcription initiation in mitochondria begins with an adenosine residue at all promoters (20). Surprisingly, we saw no enrichment of the *COX1* mRNA, which is at the 5′ end of a transcription unit in the mitochondrial genome and is not known to undergo 5′ end processing (20). However, closer inspection of the RNA-Seq reads for *COX1* revealed that the reads do not extend to the transcriptional start site (Fig. 5*C*), although they do for the *COX2* and *ATP9* mRNAs, which contain NAD^+ (Fig. 5 *D* and *E*). This finding is consistent with the *COX1* mRNA undergoing a previously unknown 5′ end-processing reaction, thereby removing any NAD^+ linked to the nascent 5′ ends. These observations suggest that NAD^+ is added to the 5′ ends of mRNAs cotranscriptionally in the mitochondria, as well as during nuclear transcription.

Discussion

We present evidence that a fraction of specific mRNAs in *S. cerevisiae* can contain a 5′ NAD^+ moiety. The key observation is

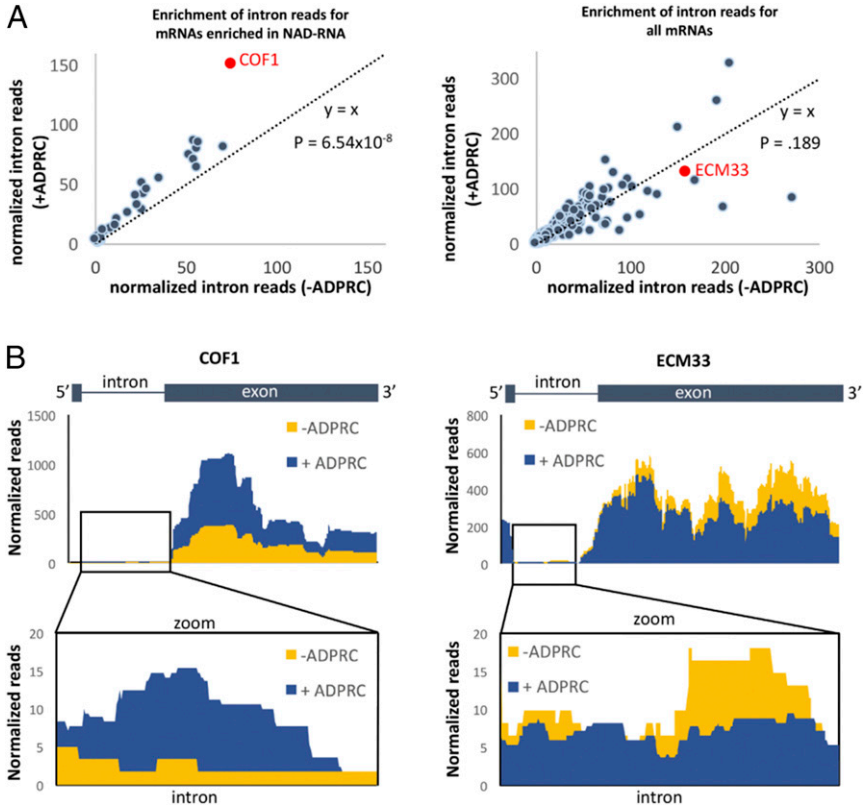


Fig. 4. NAD-mRNAs contain NAD^+ on their cognate pre-mRNAs. (*A*, *Left*) Introns of NAD-capped RNAs are also enriched in NAD-Capture-Seq. (*A*, *Right*) Introns of all mRNAs are not significantly enriched. (*B*, *Left*) Representative gene track of *COF1* mRNA, enriched in NAD-Seq. The intron of *COF1* is also enriched. (*B*, *Right*) Gene track of *ECM33* mRNA, not enriched in NAD-Seq. The intron of *ECM33* is also not enriched.

that a subset of RNAs, including cytoplasmic and mitochondrial encoded transcripts, reacts with ADPRC. In addition, *N*-ribosylnicotinamide, which would be consistent with NAD-capped RNAs, has been detected in mass spectroscopy of digested yeast RNA (21). The presence of NAD-RNA is unlikely to be limited to *S. cerevisiae*, and we anticipate this modification will be found in wide range of eukaryotes.

Several observations suggest that NAD-RNA is produced cotranscriptionally. First, it has been observed in vitro that bacterial RNA polymerases, as well as yeast RNA polymerase II, can use NAD^+ instead of ATP to initiate transcription when the transcript starts with an adenosine residue (9, 19). Second, we observe that NAD-RNA is similarly enriched on the pre-mRNAs and mature mRNAs for mRNAs with introns (Fig. 4 *A* and *B*). Third, we observe that the only mitochondrial transcripts enriched in the NAD-RNA population are those mitochondrial transcripts that do not undergo 5′ end processing (Fig. 5). Because we observe NAD-RNA arising from both nuclear and mitochondrial transcripts, the simplest explanation is that NAD-RNA is produced in eukaryotes by competition between ATP and NAD^+ as the initiating nucleotide, both for the mitochondrial RNA polymerase and RNA polymerase II. Interestingly, because bacterial promoter sequences can affect this competition (9), specific promoters or transcription factors might also alter this competition in eukaryotes. However, it remains possible that additional mechanisms of producing NAD-capped RNA exist wherein NAD^+ is added to transcripts posttranscriptionally.

The existence of NAD-RNA demonstrates that there can be an alternative 5′ cap structure for eukaryotic cells, and therefore that cellular machinery exists for its metabolism. Because RNA polymerases, including yeast RNA polymerase II, can also initiate with other adenosine-containing derivatives, such as dephospho-CoA, FAD^+, and NADH (9, 19), it remains possible that a number of other 5′ caps of eukaryotic RNAs are formed.

The biological role of NAD-RNA remains to be established. One possibility is that the production of NAD-RNA solely represents an aberrant transcription initiation event due to the fundamental ability of NAD to compete with ATP at initiation. More interestingly, it seems likely that biology has taken advantage of the ability to produce NAD-RNA to modulate mRNA function in some contexts. This possibility is suggested by the importance of the 5′ cap structure in controlling mRNA splicing, export, translation, and stability in eukaryotic cells (11). Moreover, it has recently become clear that multiple cap-independent modes of translation initiation exist in eukaryotes (22), suggesting the possibility that NAD^+ capped mRNAs could be recognized and enter translation by a novel mechanism. Such roles would synergize with the expanding diversity of mRNA modifications to create an even more diverse epitranscriptome than previously realized, with the potential for diverse effects on mRNA function.

Materials and Methods

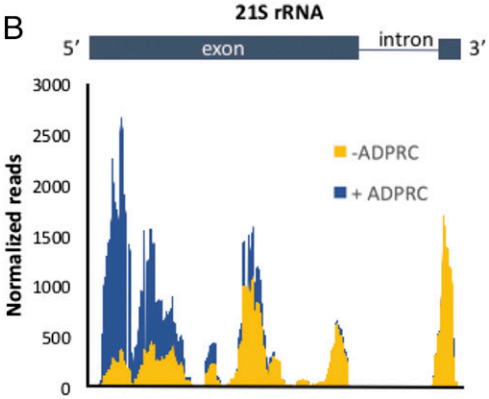
Yeast Growth Conditions. Yeast strains were grown in either rich (YEPD) or minimal (synthetic defined) media according to standard protocols. All strains were harvested for experiments during exponential or midlog (OD_{600} = 0.4–0.5) growth.

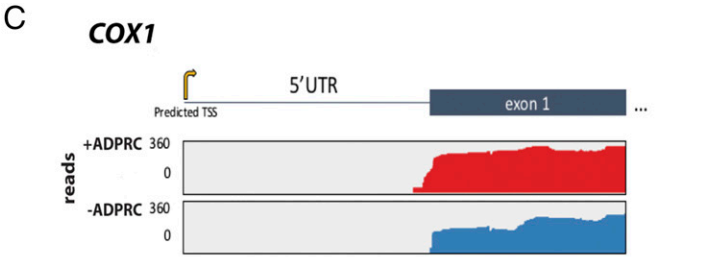
NAD-RNA Capture.

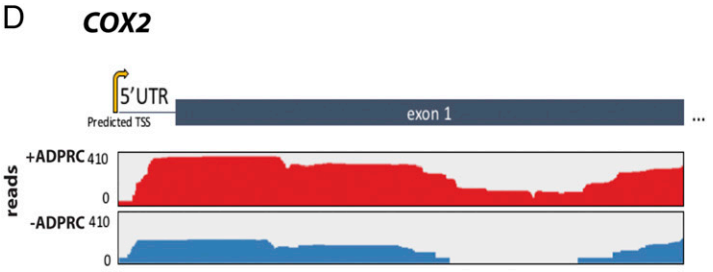
From in vitro-transcribed RNAs. Following gel purification, in vitro-transcribed RNAs were manipulated basically as described (7). Equal radioactive counts of NAD-RNA or A-RNA were incubated with 0.4 μM ADPRC, 3 μL 4-pentyn-1-ol, 50 mM Hepes, and 5 mM $MgCl_2$ (pH 7) in a total of 60 μL at 37 °C for 60 min. RNA was then purified using acid-phenol and chloroform, and was precipitated with ethanol. This template was then used in a reaction containing

A

Mito RNA	Enriched in NAD-RNA?	5′ end processed?	Fold change
COX2	yes	no	3.01
21S rRNA	yes	no	2.10
ATP9	yes	no	2.06
15S rRNA	yes	no	1.47
COX1	no	yes	0.561
COX3	no	yes	0.469
ATP6	no	yes	0.437
SCE1P	no	yes	0.395
CYTB	no	yes	0.365
VAR1	no	yes	0.248
ATP8	no	yes	NA







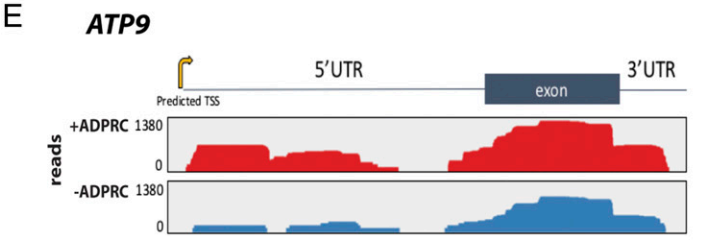


Fig. 5. Non-5′ end-processed mitochondrially encoded RNAs selectively contain NAD^+. (*A*) Table of mitochondrial RNAs showing their enrichment in RNA-Seq and 5′ end-processing properties. (*B*) Representative gene track and read density along the 21S rRNA. Read density shows a 5′ bias. (*C*) Gene track of RNA-Seq reads across the COX1 transcript. (*D* and *E*) Gene tracks showing the 5′ ends of the *COX2* and *ATP9* mitochondrial NAD-capped transcripts. Ellipses indicate that the gene continues.

29 μM biotin-PEG_3-azide (Sigma–Aldrich), 1.25 mM fresh $CuSO_4$, 0.625 mM Tris(3-hydroxypropyltriazolylmethyl)amine (THTPA), 2.5 mM sodium ascorbate, 50 mM Hepes, and 5 mM $MgCl_2$ (pH 7). This mixture was incubated at 25 °C for 60 min and then added to 1 mg of MyOne Streptavidin C1 beads (Thermo Fisher). Streptavidin beads were prewashed per the manufacturer's protocol, and blocked with BSA as described (7) at 100 μg/mL. This RNA/streptavidin bead mixture was incubated for 15 min at 25 °C. This mixture was then washed according to the manufacturer's protocol, incubated with formamide loading dye, heated for 5 min at 100 °C, and separated via magnetic stand, and the supernatant was run on 15% sequencing denaturing PAGE gels.

From total RNA. NAD-RNAs were purified essentially as described (7). Total RNA was extracted from yeast strains using previously described methods (23). RNA was treated with TURBO-DNase (8) (Invitrogen; 0.04 U/μL at 25 °C for 30 min) and then treated with Proteinase K (0.06 U/μL at 25 °C for 30 min). RNA (100 μg) was then incubated with 0.85 μM ADPRC (or left out as a control), 10 μL of 4-pentyn-1-ol, 50 mM Hepes, and 5 mM $MgCl_2$ (pH 7) at 37 °C for 30 min. RNA was extracted with acid phenol and then chloroform, and it was then precipitated with ethanol. This RNA was then incubated with 250 μM biotin-PEG_3-azide, 1 mM fresh $CuSO_4$, 0.5 mM THTPA, 2 mM sodium ascorbate, 50 mM Hepes, and 5 mM $MgCl_2$ (pH 7) at 25 °C for 30 min. RNA was again extracted with acid phenol and chloroform, and it was then precipitated with ethanol. This RNA was incubated with 1 mg of preblocked MyOne Streptavidin C1 beads as described for in vitro-transcribed RNAs.

In Vitro Transcription. Complementary oligonucleotides, one carrying the T7 class II promoter (Φ2.5) sequence (40 μM each), were annealed in 10 mM Tris·HCl, 50 mM NaCl, and 1 mM EDTA (pH 8) at 80 °C for 5 min, and then allowed to cool to room temperature over the course of 30 min. This mixture (4 μM) was subsequently used in each in vitro transcription reaction. In vitro reactions were carried out basically as described (8). Reactions contained 1× RNA pol buffer (New England Biolabs); 1 mM CTP, GTP, and UTP; 1 mM NAD^+ or 1 mM ATP; 0.01% Triton X-100; 5 mM DTT; and 5 U/μL T7 RNA polymerase (New England Biolabs) at 37 °C for 2 h. Unincorporated nucleotides were removed using G-25 columns (GE Life Sciences). RNAs were treated with TURBO-DNase at 0.4 U/μL at 37 °C for 30 min, extracted using acid phenol and chloroform, and then precipitated with ethanol. Where indicated, reactions also contained α-^{32}P-GTP (3,000 Ci/mmol, 10 mCi/mL).

Radiolabeling of RNAs Using pCp [5′-^{32}P]. RNA samples (on streptavidin beads) were incubated in 1× T4 RNA Ligase Buffer (New England Biolabs), 1 mM ATP, 3 μL of DMSO, 1–4 μL of pCp [5′-^{32}P] (3,000 Ci/mmol, 10 mCi/mL), and 1 μL of T4 RNA ligase (New England Biolabs) in a final volume of 30 μL at 4 °C for ∼18 h. These reactions were washed three times in wash buffer [5 mM Tris, 0.5 mM EDTA, 1 M NaCl (pH 7.5)]. Samples were eluted from streptavidin beads by incubation with 95% formamide loading dye at 95 °C for 5 min.

Quantification of Individual NAD-RNAs. All measurements are averages based on multiple biological replicates. First, the efficiency of the NAD-capture protocol was calculated using radioactive counts of in vitro-transcribed NAD-RNA measured using a Typhoon FLA 9500 instrument and quantified by ImageJ (NIH) (24). We calculated the efficiency of the NAD-capture protocol by determining the percentage of input RNA pulled down in the NAD-capture method by first determining the specific selection of NAD-RNAs by subtracting the background in the ADPRC(–) lane from the ADPRC(+) lane; then, this amount was divided by the input counts to get a percentage of specific selection. Similarly, using quantification of Northern blots (Fig. 3*D*), we assessed levels of individual NAD-RNAs by the formula ADPRC(+) – ADPRC(–)/total individual mRNA, followed by correction for differences

in loading in each lane, and the estimated efficiency of the NAD-capture protocol.

Sequencing.

Library construction. Strand-specific RNA-Seq libraries were prepared with modification (25) using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). NAD-RNA was purified (as discussed above) from total RNA samples, bound to streptavidin beads, and then washed three times with wash buffer for subsequent library preparation. First-strand and second-strand cDNA synthesis was carried out per the NEBNext Ultra Directional RNA Library Prep Kit protocol, whereas the RNA was bound to streptavidin beads. The cDNA was then eluted from streptavidin beads with 60 μL of nuclease-free dH_2O. Library preparation quality was assessed by Qubit (Thermo Fisher). Double-stranded cDNA libraries were purified using Agencourt AMPure XP beads, and subsequent end preparation of libraries was done according to the manufacturer's protocol.

Sequencing data analysis. All reads were subject to quality control using FastQC (26). Adaptors were trimmed using the program Trimmomatic (27). Reads were aligned to a yeast reference genome using Bowtie (28). Alignments were visualized using Integrative Genomics Viewer (IGV) (29). Alignments were counted for each gene using HTSeq count (30). Differential expression was determined using DESeq (31). A hit was only considered to be significant if the fold change between +ADPRC and −ADPRC samples was >2 and if the adjusted *P* value from DESeq was <0.05. All figures containing RNA-Seq reads mapped to individual genes were generated through IGV or through Excel (by manually inputting read numbers for each nucleotide position from IGV) (29). Three-dimensional replicate plots and r^2 values were generated using MATLAB (MathWorks). All other graphs were generated using Excel (Microsoft Corporation).

The statistical analysis for intron-containing genes was calculated using a binomial test, where genes were bifurcated into two classes (enriched or not enriched) based on whether they lay above or below the line $y = x$, respectively. The probability of any given gene being up-regulated was assumed to be 0.5.

ACKNOWLEDGMENTS. We thank Dr. Olke Uhlenbeck (University of Colorado Boulder) for advice, Anne Webb for aesthetics, and the R.P. and Dowell laboratories for assistance. We thank Dr. Jamie Kershner at the BioFrontiers Next Generation Sequencing Facility (University of Colorado Boulder) and Dr. Katrina Diener (Genomics and Microarray Core at the University of Colorado Denver). We thank Amber Sorenson, Aaron Odell, and Dr. Robin Dowell (University of Colorado Boulder) for assistance with sequence analysis. This work was supported by the Howard Hughes Medical Institute and NIH Grant GM045443.

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