Recent studies have revealed new roles for NAD and its derivatives in transcriptional regulation. The evolutionarily conserved Sir2 protein family requires NAD for its deacetylase activity and regulates a variety of biological processes, such as stress response, differentiation, metabolism, and aging. Despite its absolute requirement for NAD, the regulation of Sir2 function by NAD biosynthesis pathways is poorly understood in mammals. In this study, we determined the kinetics of the NAD biosynthesis mediated by nicotinamide phosphoribosyltransferase (Nampt) and nicotinamide/nicotinic acid mononucleotide adenylyltransferase (Nmnat), and we examined its effects on the transcriptional regulatory function of the mouse Sir2 ortholog, Sir2α, in mouse fibroblasts. We found that Nampt was the rate-limiting component in this mammalian NAD biosynthesis pathway. Increased dosage of Nampt, but not Nmnat, increased the total cellular NAD level and enhanced the transcriptional regulatory activity of the catalytic domain of Sir2α recruited onto a reporter gene in mouse fibroblasts. Gene expression profiling with oligonucleotide microarrays also demonstrated a significant correlation between the expression profiles of Nampt- and Sir2α-overexpressing cells. These findings suggest that NAD biosynthesis mediated by Nampt regulates the function of Sir2α and thereby plays an important role in controlling various biological events in mammals.

Sir2 (silent information regulator 2) proteins are evolutionarily conserved family of NAD-dependent protein deacetylases (1–3). They have been shown to regulate longevity in yeast (4–6) and Caenorhabditis elegans (7). In mammals, it has been shown recently that the mammalian Sir2 ortholog, SIRT1/Sir2α, plays important roles in a variety of biological processes, such as stress and cytokine responses (8–12), differentiation (13, 14), and metabolism (14), by deacetylating transcriptional regulators. Sir2 proteins possess the unique ability to couple the breakdown of NAD and protein deacetylation to the formation of nicotinamide and O-acetyl-ADP-ribose (15, 16). This unique requirement for NAD implies that Sir2 proteins function as energy sensors (17, 18) or redox sensors (13) that link energy metabolism to transcriptional regulation.

Since NAD is essential for the Sir2 deacetylase reaction, the regulation of NAD biosynthesis has attracted new attention. In yeast, a strong connection has been established between NAD biosynthesis and Sir2 (Fig. 1A), which mediates transcriptional silencing at telomeres, silent mating-type loci, and ribosomal DNA repeats (17, 19). It has been demonstrated that increased dosage of NPT1, which encodes nicotinamide phosphoribosyltransferase, enhances Sir2-dependent transcriptional silencing and extends the life span of yeast mother cells (20). Consistent with this finding, deletion of NPT1 causes a loss of Sir2-dependent silencing and abrogates the life span extension by caloric restriction (21, 22). Additional copies of other genes, PNC1, NMA1, and NMA2, which encode nicotinamide and nicotinic acid mononucleotide adenylyltransferase 1 and 2, respectively, also increase telomeric and rDNA silencing (20). Most notably, PNC1 mediates the life span extending effect of caloric restriction, and additional copies of PNC1 increase the replicative life span of yeast mother cells dramatically (23, 24). It has also been shown that the cellular [NAD]/[NADH] ratio is critical to regulate Sir2 activity in calorie-restricted yeast (25).

Even though the [NAD]/[NADH] ratio also modulates Sir2 function in skeletal muscle differentiation in mammals (13), it is not known whether NAD biosynthesis regulates Sir2 activity in these organisms. In fact, NAD biosynthesis in vertebrates is markedly different from that of yeast and invertebrates (Fig. 1). Vertebrates lack any obvious homolog of the yeast nicotinamidase (Pnc1) (26), and the recycling of nicotinamide into NAD is more direct (Fig. 1B). Nicotinamide, rather than nicotinic acid, is the major substrate for NAD biosynthesis in mammals (27). Instead of being deamidated, nicotinamide is converted into NMN by nicotinamide phosphoribosyltransferase (Nampt). 1 NMN is then directly synthesized into NAD by nicotinamide/nicotinic acid mononucleotide adenylyltransferase (Nmnat) (28, 29).

Since Nampt and Nmnat are sufficient to synthesize NAD from nicotinamide in mammals (Fig. 1B), we hypothesized that this NAD biosynthesis pathway regulates mammalian Sir2 activity. We also suspected that Nampt might be the main regulatory component in this pathway, since its presence pro-

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1 The abbreviations used are: Nampt, nicotinamide phosphoribosyltransferase; Nmnat, nicotinic acid mononucleotide adenylyltransferase; GFP, green fluorescent protein; PRPP, phosphoribosyl pyrophosphate; HPLC, high pressure liquid chromatography; RT, reverse transcription; PBef, pre-B-cell colony-enhancing factor.

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vides new dynamics of NAD biosynthesis for vertebrates compared with yeast and invertebrates (Fig. 1B). Nmnt, originally identified in Haemophilus ducreyi (30), was found to have significant homology to the mammalian pre-B-cell colony-enhancing factor (PBEF), a presumptive cytokine capable of stimulating the maturation of B-cell precursors (31). More recently, it has been reported that the mouse PBEF protein immunoprecipitated from liver extracts has the Nmnt enzymatic activity (32). However, the kinetic characteristics of the NAD biosynthesis pathway mediated by Nmnt and Nmnat have not yet been determined.

In this study, we characterized the biochemical natures of mouse Nmnt and Nmnat by developing an enzyme-coupled fluorometric assay and reconstituting mammalian NAD biosynthesis in vitro with purified recombinant proteins. We also examined the effect of these enzymes on the function of the mouse Sir2 ortholog, Sir2a, in mouse fibroblasts. Increasing the dosage of Nmnt, but not Nmnat, increased the total cellular level of NAD and enhanced the transcriptional regulatory activity of Sir2a. Finally, we identified common gene expression changes in mouse fibroblasts overexpressing Sir2a and Nmnt. Taken together, our results establish Nmnt as the rate-limiting component of the mammalian NAD biosynthesis pathway from nicotinamide and shed new light on the connection between NAD biosynthesis and the regulation of Sir2 activity in mammals.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. All NIH3T3 cell lines used in this study were established by selecting in the presence of 650–700 µg/ml of G418 (Invitrogen).

**Plasmids**—The coding regions of mouse Nmnt and Nmnat cDNAs were amplified from a mouse liver cDNA library (Clontech) by PCR with PfuTurbo polymerase (Stratagene, CA) and the following forward and reverse primers containing EcoRI sites: Nmnt forward, TTGAAATT-CAGCCCCATTTTCCCTTGCT; Nmnt reverse, TTGAAATCCACAATA-ACACCCGGCCACATG; and Nmnat forward, TTGAAATCCGAGGAGTACAAAGCCGTGTT; Nmnat reverse, TTGAAATCCGCCCCTTGCTGTCAGAGT. The resulting 1584- and 972-bp fragments of Nmnt and Nmnat cDNAs, respectively, were digested with EcoRI and cloned into the pBluescript SK- vector. Nmnt and Nmnat cDNA fragments were then subcloned into the mammalian expression vector pCXN2 (33) (a gift from Dr. Jun-ichi Miyazaki, Osaka University, Japan). To create N-terminal His-tagged recombinant proteins of these two enzymes, Nampt and Nmnat cDNA fragments were re-amplified by PCR to create EcoRI and NdeI sites at the 5’ ends of each cDNA, respectively. The PCR products were cloned into the pET-28a (+) vectors (EMD Biosciences). To create expression vectors for Nmnt and Nmnat proteins fused to GFP at their C termini, the Nmnt and Nmnat cDNA fragments were cloned between EcoRI and BamHI sites of the pEGFP-N1 vector (Clontech) after modifying their stop codons. All Nmnt and Nmnat cDNA inserts were sequenced, and those sequences were deposited in the GenBank™ database as accession numbers AGH679720 and AY679721, respectively.

To make effector plasmids of mouse Sir2a for reporter gene transcription assays, the DNA fragments corresponding to amino acids 220–500 of the wild-type and mutant Sir2a (8, 9) were amplified by PCR with PfuTurbo DNA polymerase (Stratagene) and primers that created EcoRI sites at both ends of each fragment. They were cloned into the EcoRI site of the pM mammalian expression vector (Clontech) to produce the N-terminal fusion to the GAL4 DNA binding domain. To make the expression vector for the C-terminally GFP-fused Sir2a protein, the Sir2a minigene that carries the 2.2-kbp Sir2a cDNA fragment, whose stop codon was modified, and a 1.3-kbp genomic fragment of the Sir2a intron region was inserted between EcoRI and BamHI sites of the pEGFP-N1 vector after removing its cytomeglovirus enhancer/promoter. The mouse Sir2a expression vector, pBabe-Sir2a, was a gift from Dr. Homayoun Vaziri at the University of Toronto. All necessary plasmids were prepared by using the QIAGEN plasmid midi kit (Qiagen).

**Phylogenetic Analysis of Nampt Protein Sequences**—Amino acid sequences of Nampt proteins in different species were compared by using ClustalX software. A phylogenetic tree was created by using ClustalX and NJPLOT.

**Production of Recombinant Nampt and Nmnat Proteins—**BL21(DE3)pLysS cells were transformed with each of His-tagged Nmnt and Nmnat plasmids. Transformed BL21(DE3)pLysS cells were grown overnight at 37 °C in 25 ml of Terrific broth containing 75 µg/ml kanamycin and 37 µg/ml chloramphenicol. Cells were spun down, resuspended in 500 ml of the same media, and grown at 37 °C to an A595 of 0.6. His-tagged recombinant proteins were then induced by 1.5 mM isopropyl-β-thiogalactopyranoside (Sigma). After inducing for 5 h at 37 °C, cells were spun down, resuspended in 20 ml of Tris-HCl (pH 8.0), 300 mM NaCl, 0.1% Triton X-100) with protease inhibitors (Roche Applied Science) and lysozyme. The lysate was then produced with a French press and cleared at 10,000 × g for 30 min. The His-tagged Nmnt and Nmnat recombinant proteins were purified with nickel-nitriotropic acid resin (Qiagen) by washing with lysozyme buffer and wash buffer (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10% glycerol, 0.1% Triton X-100, 40 mM imidazole) and eluting with 150 mM imidazole-containing buffer.

**Enzyme-coupled Fluorometric Assays—**Enzymatic activities of recombinant Nampt and Nmnat proteins were measured by an enzyme-coupled fluorometric assay. To establish this assay system, optimal reaction conditions for Nmnt were initially examined by varying ATP and Mg2+ concentrations and pH of the reaction buffer. The resultant reaction buffer for Nmnt contained 50 mM HEPEs (pH 7.4), 0.02% bovine serum albumin, 12 mM MgCl2, 2 mM ATP, and 30 µg/ml alcohol dehydrogenase to convert NAD to NADH. To determine the kinetic parameters for Nmnt, 30 ng of purified His-tagged Nmnt and varying concentrations of NNN were added to 1 ml of the reaction buffer. The reactions were run at 37 °C and quenched at six time points by the addition of 250 µl of 0.5 M EDTA. The production of NADH was measured by excitation at 340 nm and emission at 460 nm in a fluorometer. For kinetic characterization of Nmnt, 500 ng of His-tagged Nmnt and varying concentrations of nicotinamide were reacted at 37 °C in 100 µl of a buffer containing 50 mM Tris-HCl (pH 7.5), 0.02% bovine serum albumin, 12 mM MgCl2, 2.5 mM ATP, 10 µg/ml His-tagged Nmnt, 0.4 mM phosphorous pyrophosphate (PRPP), 1.5% ethanol, and 30 µg/ml alcohol dehydrogenase. NADH production was measured continuously in a fluorometer.

**HPLC Detection of Nampt Reaction Products—**HPLC was performed with Waters 515 pumps and a 2487 detector (Waters) with a Supelco LC-18-T column (15 × 4.6 cm). The Nampt reaction was conducted at 37 °C for 15 min in 500 µl of reaction buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 50 mM nicotinamide, 0.2 mM PRPP) with 50 µg of His-tagged Nampt protein. The reaction was terminated by adding 125 µl of 1 M HClO4. Protein was then precipitated at 18,000 × g, and 500 µl of the supernatant was neutralized with 40 µl of 3 M K2CO3. 100 µl of the neutralized reaction mixture was added to a buffer (A (50 mM K2PO4/KH2PO4, pH 7.0) and loaded into the HPLC system. The products from Nampt reaction were monitored by absorbance at 261 nm.

**Antibody Generation—**Polyclonal rabbit antisera were produced against the purified full-length His-tagged Nampt and Nmnt recombinant proteins (Covance, PA). Polyclonal rabbit anti-mouse Sir2a antisera was also raised against an N-terminal fragment (amino acids 1–131) of mouse Sir2a. Specific antibodies were affinity-purified from these antisera with HitTrap affinity columns (Amersham Biosciences) conjugated with each protein.

**Western Blotting—**Whole cell extracts were prepared with Laemmli’s sample buffer. Proteins were separated in SDS-PAGE with 4–15% gradient or 12% gels and transferred onto Immobilon-P transfer membranes (Millipore, MA). Uniform transfer was confirmed by Ponceau S staining. Membranes were blocked in TBS-T buffer (0.1% Tween 20 (TBS-T buffer) and 5% dry milk (w/v) for 1 h at room temperature and washed three times in TBS-T. Membranes were blotted overnight at 4 °C with primary antibodies diluted at an appropriate dilution ratio in TBS-T with 5% dry milk and then with a secondary donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature. After washing, membranes were developed with the ECL detection system (Amersham Biosciences).

**NAD Measurement—**NAD was measured by HPLC, as described previously (34, 35). Briefly, 5 × 105 cells were plated in 6-cm dishes and harvested 48 h later in 800 µl of ice-cold phosphate-buffered saline. Cells were then spun down and lysed with 300 µl of 1× HClO4 on ice for 10 min. Lysates were cleared by centrifuging at 4 °C at 18,000 × g for 5 min. Cleared lysates (240 µl) were neutralized by adding 50 µl of 3 M
K$_2$CO$_3$ and incubating on ice for 10 min. After centrifuging for 10 min, 100 µl of the supernatant were mixed with 400 µl of Buffer A and loaded onto the column. The HPLC was run at a flow rate of 1 ml/min with 100% Buffer A from 0 to 5 min, a linear gradient to 95% Buffer A and 5% Buffer B from 5 to 6 min, 95% Buffer A and 5% Buffer B from 6–11 min, a linear gradient to 85% Buffer A and 15% Buffer B from 11 to 13 min, 85% Buffer A and 15% Buffer B from 13 to 23 min, and a linear gradient to 100% Buffer A from 23 to 24 min. NAD was eluted as a sharp peak at 22 min. The amount of NAD was quantitated based on the peak area compared with a standard curve.

Luciferase Assay—1.2 × 10$^6$ NIH3T3 cells were plated in 6-cm dishes. 24 h after plating, cells were transfected for 3 h with 380 ng of pUAS$_{tk}$-luc as a reporter, 1.5 µg of pM or pM-GAL4DBD-mCORE as an effector, and 38 ng of pRlSV40 (Promega, WI) as a normalization control by using Superfect (Qiagen). Transfectants were harvested 48 h after transfection, and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, WI) and a SIRIUS luminometer (Berthold Detection Systems, Germany) according to the manufacturers’ protocols. Background luminescence was subtracted and was always less than 1% of measured values. The expression levels of luciferase were normalized based on Renilla luciferase activity. 5 µM nicotinamide or 5 mM nicotinic acid was added 24 h after transfection. To examine the effect of Nmnat on the transcriptional repressive activity of GAL4DBD-mCORE, NIH3T3 cells were co-transfected with the indicated amounts of Nampt and Nmnat expression vectors along with reporter, effector, and normalization control plasmids.

Microarray Analysis—RNA samples were purified from Nampt-overexpressing (Nampt1, Sir2α-overexpressing (Sir2α), and neomycin-resistant control (Neo1) NIH3T3 cell lines by using a RNeasy kit (Qiagen). Total RNA was incubated with RNase inhibitor (Promega) according to the manufacturer’s protocol. The quality of RNA was examined by capillary electrophoresis. Eight micrograms of total RNA from each of the NIH3T3 cell lines were converted to cDNA by using the 3DNA Array 350 Expression array detection kit (Genisphere) according to the manufacturer’s protocol. Microarray hybridization was then conducted with Cy3- and Cy5-labeled dendraimers as described (36) with the following modifications. Hybridization was conducted at 42 °C for 18 h in Mouse NIH3T3 cell line hybridization buffer (MWG Biotec), an approximate 4 × 10$^5$ NIH3T3 cells were used for each pairwise comparison. A ScanArray Express HT scanner and accompanying software (PerkinElmer Life Sciences) was used to scan the slides and analyze the raw data, including normalization according to the Lowess method (37). Spots used for statistical analysis satisfied the following criteria on at least three of the six repeats of comparisons: 1) signal intensity $>5$; 2) signal to noise ratio $>2$ in both channels. Spot-specific dye bias was corrected by subtracting a correction factor from the log$_2$ of theLowess-normalized median of ratios in the Cy5 and Cy3 channels. It has been reported that combining dye swapping and filtering out spots with signal intensities near background enables highly reproducible detection of gene expression changes with ratios as low as 1.2-fold (38), which we confirmed in our preliminary microarray experiments. Therefore, genes determined to be changed were considered of at least 1.2-fold differences between experimental and control cell lines with 95% confidence intervals that did not overlap the fold change of 1.

Quantitative Real Time RT-PCR—Total RNA samples were purified as described above. For each sample, cDNA was synthesized from 10 µg of total RNA using an Omniscript kit (Qiagen) with random hexamer primers and RNase inhibitor (Promega) according to the manufacturer’s protocol. The real time quantitative RT-PCR was carried out in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with a SYBR Green PCR Master Mix kit (Applied Biosystems) and gene-specific primers. Primer sequences are available upon request.

RESULTS

Biochemical Characterization of Two Critical Enzymes in the Mouse NAD Biosynthesis Pathway Starting from Nicotinamide—In mammals, NAD biosynthesis from nicotinamide is catalyzed by two enzymes, Nampt and Nmnat (Fig. 1B). To examine the connection between this NAD biosynthesis pathway and Sir2 activity, full-length cDNAs of the mouse Nampt and Nmnat genes were isolated from a mouse liver cDNA library by PCR. We isolated the mouse Nampt cdNA, based on a homology search in the mouse EST data base to the amino acid sequence of H. ducreyi Nampt (30). We also isolated the mouse ortholog to the human NNMAT-1 gene, which we refer to as Nmnat in this paper. The mouse Nmnat gene was previously cloned as a fusion gene from the slow Wallerian degeneration mutant mouse (39).

We first reconstituted the NAD biosynthesis pathway in vitro with His-tagged recombinant enzymes and developed an enzyme-coupled fluorometric assay (Fig. 2A). In this enzyme-coupled reaction, NAD is converted to NADH by alcohol dehydrogenase, and the fluorescence of NADH is detected in a fluorometer. Bacterially produced, His-tagged recombinant mouse Nampt and Nmnat proteins showed molecular masses of ∼59 and 35 kDa, respectively, which are consistent with those predicted from their amino acid sequences (Fig. 2B). The in vitro reconstituted NAD biosynthesis reaction generated NAD from nicotinamide, phosphoribosyl pyrophosphate (PRPP), and ATP (Fig. 2C). No NAD was produced in the absence of nicotinamide or PRPP, the substrates of Nampt (Fig. 2C). We further confirmed by HPLC that the mouse Nampt produced NNM from nicotinamide and PRPP (Fig. 2D). Nampt failed to catalyze the synthesis of nicotinic acid mononucleotide from nicotinic acid and PRPP (see supplemental Fig. 1), confirming the substrate specificity of this enzyme. In isolated reactions, we also confirmed that Nmnat catalyzed the synthesis of NAD from NMN and ATP (data not shown).

By using this enzyme-coupled fluorometric assay, we determined the kinetic parameters of purified recombinant mouse Nampt and Nmnat for nicotinamide and NMN, respectively (Table I). The Lineweaver-Burk plots for these two enzymes are shown in Fig. 3. Compared with reported kinetic parameters of other enzymes in the NAD biosynthesis pathways (40), Nampt shows very high affinity for its substrate ($K_m$ = 0.92 µM). The $K_m$ and $V_{max}$ values of mouse Nmnat are consistent with the values reported previously for human NNMAT-1 (28). The catalytic efficiency of Nampt is ∼46-fold less than that of Nmnat, suggesting that the reaction of Nampt is the rate-limiting step in the synthesis of NAD from nicotinamide.

Nampt Regulates the Cellular Level of NAD in Mouse Fibroblasts—If the reaction of Nampt is indeed the rate-limiting step in the mammalian NAD biosynthesis pathway starting from nicotinamide, increasing the dosage of Nampt should increase total NAD levels in mammalian cells. To test this hypothesis, we overexpressed the mouse Nampt gene in mouse NIH3T3 fibroblasts. In the original and neomycin-resistant control NIH3T3 cells, we detected low amounts of the 56-kDa Nampt protein with an affinity-purified rabbit polyclonal antibody raised against the recombinant full-length protein (Fig. 4A). Two Nampt-overexpressing NIH3T3 cell lines, Nampt1 and -2, showed 23- to 15-fold higher amounts of the protein, respectively, compared with control neomycin-resistant cell lines, Neo1 and -2 (Fig. 4A). The amounts of Nmnat did not change in these cell lines. We also overexpressed the mouse Nmnat and Sir2α genes in NIH3T3 cells (Fig. 4A). Nmnat (32 kDa) and Sir2α (apparent molecular mass of 110 kDa) were detected.
with affinity-purified rabbit polyclonal antibodies against these proteins. Overexpression levels of Nmnat and Sir2 are ~14- and 4-fold, respectively. The amount of Nampt did not change in these cell lines. By using GFP fusion expression vectors, we also determined that overexpressed Nmnat and Sir2 proteins were localized exclusively in the nucleus, whereas overexpressed Nampt protein was mainly localized in cytoplasm, as described previously (8, 29, 41) (see supplemental Fig. 2).

We then measured total cellular levels of NAD in the NIH3T3 cell lines overexpressing the untagged enzymes. As expected, total NAD levels increased 47 and 35% in Nampt1 and -2 cell lines, respectively, compared with those in control cell lines (Fig. 4B). In contrast, the total NAD levels did not change in cells overexpressing Nmnat or Sir2 (Fig. 4B). Addition of 5 mM nicotinamide to the medium, which otherwise contains only 33 μM nicotinamide, did not increase NAD (Fig. 4B). Consistent with the biochemical characteristics of these

**Fig. 1.** The NAD biosynthesis pathways from nicotinamide in yeast and mammals. A, NAD biosynthesis from nicotinamide in *S. cerevisiae* is depicted (26, 61). Pnc1, Npt1, Nma1, Nma2, and Qns1 are nicotinamidase, nicotinic acid phosphoribosyltransferase, nicotinic acid mononucleotide adenylyltransferase 1 and 2, and NAD synthetase, respectively. This pathway is also conserved in *C. elegans, Drosophila,* and other invertebrates (26). B, NAD biosynthesis from nicotinamide and nicotinic acid in mammals is shown. These pathways are also conserved throughout vertebrates. Nicotinamide is the main precursor for NAD biosynthesis in mammals (27). Npt, Nampt, and Nmnat are nicotinic acid phosphoribosyltransferase, nicotinamide phosphoribosyltransferase, and nicotinamide/nicotinic acid mononucleotide adenylyltransferase, respectively. Among the enzymes that break down NAD into nicotinamide, only Sir2 is a topic of this paper. NaMN, nicotinic acid mononucleotide NMN, nicotinamide mononucleotide.
The NAD biosynthesis pathway from nicotinamide is reconstituted in vitro with recombinant Nampt and Nmnat proteins. A, the scheme of the NAD biosynthesis reactions in the enzyme-coupled fluorometric assay is shown. The in vitro synthesized NAD was converted to NADH by alcohol dehydrogenase (ADH), and the fluorescence of the resulting NADH was measured by a fluorometer. PPi, inorganic pyrophosphate. B, His-tagged recombinant proteins of mouse Nampt and Nmnat were produced in Escherichia coli and purified to homogeneity. One microgram of each protein was electrophoresed and stained in SDS-polyacrylamide gels. C, a time course of the NADH production was measured in the enzyme-coupled fluorometric assay using purified Nampt and Nmnat recombinant proteins. Filled squares indicate the reaction with nicotinamide, PRPP, and ATP. Open squares and triangles indicate reactions without nicotinamide and PRPP, respectively. D, the products of mouse Nampt reaction were analyzed by HPLC. Chromatograms at 0- and 15-min time points are shown. Elution times for each chemical were confirmed by running standards in the same HPLC conditions.
enzymes, these results suggest that Nampt is the rate-limiting component of the NAD biosynthesis pathway starting from nicotinamide in mouse fibroblasts.

The Increased Dosage of Nampt Enhances the Transcriptional Repressive Activity of the Mammalian Sir2 Catalytic Core Domain Recruited onto a Reporter Gene—Since mammalian Sir2 requires NAD for its enzymatic activity, we postulated that increasing the dosage of Nampt would enhance Sir2 activity through the increase in cellular NAD. To monitor the transcriptional regulatory activity of the mouse Sir2 ortholog, Sir2α, we developed a reporter gene transcription assay using a GAL4 DNA binding domain (GAL4DBD) fusion system that has been used extensively to evaluate the in vivo functions of mammalian histone deacetylases (42, 43). This assay system used the GAL4DBD fused to the Sir2α catalytic core domain (GAL4DBD-mCORE) and a luciferase reporter that has a thymidine kinase minimal promoter and four GAL4-binding sites.

![Fig. 3. The Lineweaver-Burk plots of mouse Nampt (A) and Nmnat (B). Each data point and their S.D. were determined by three independent assays. The $K_m$, $V_{max}$, and $k_{cat}$ values for each enzyme calculated from these plots are shown in Table I.](image)

![Fig. 4. Nampt is the rate-limiting component in the mammalian NAD biosynthesis pathway initiated from nicotinamide.](image)

In this assay, the GAL4DBD-mCORE significantly repressed transcription compared with the activity of the GAL4DBD control (Fig. 5A). When the H355A mutation, which destroys more than 90% of the NAD-dependent deacetylase activity of Sir2α (9), was introduced to the core domain, this repression was abolished (Fig. 5A), demonstrating that the NAD-dependent deacetylase activity is required for this repressive activity. The repressive activity of GAL4DBD-mCORE was also abolished by the addition of 5 mM nicotinamide, a chemical inhibitor for Sir2, but not by 5 mM nicotinic acid (Fig. 5A), which further confirms that this system measures Sir2 activity. By using this reporter assay, we examined the effect of the increased Nampt dosage on the transcriptional repressive activity of GAL4DBD-

| Enzyme | Substrate | $K_m$ | $V_{max}$ | $k_{cat}$ | Catalytic efficiency ($k_{cat}/K_m$) |
|--------|-----------|-------|-----------|----------|-------------------------------------|
| Nampt  | Nicotinamide | 0.92  | 0.021     | 0.020    | 2.17 × 10^4                        |
| Nmnat  | NMN       | 20.1  | 34.1      | 20.0     | 9.95 × 10^5                        |
mCORE. Transient co-transfection of the Nampt gene enhanced the repressive activity of GAL4DBD-mCORE in a dose-dependent manner (Fig. 5B). In contrast, transient co-transfection of the Nmnat gene did not enhance the activity of GAL4DBD-mCORE (Fig. 5B), consistent with the result that overexpression of Nmnat did not increase NAD (Fig. 4B). Co-transfection of both Nampt and Nmnat genes also failed to increase the repressive activity of GAL4DBD-mCORE beyond the effect of the Nampt gene alone (data not shown). We then measured the repressive activity of GAL4DBD-mCORE in the stable Nampt-overexpressing NIH3T3 cell lines, Nampt1 and -2. In these cell lines, the repressive activity of GAL4DBD-mCORE was significantly enhanced (Fig. 5C). Additionally, a strong correlation was observed between the total cellular NAD levels and the repressive activities of GAL4DBD-mCORE in control and Nampt-overexpressing NIH3T3 cell lines (Fig. 5D). Taken together, these results support the hypothesis that the increased dosage of Nampt enhances the transcriptional regulatory activity of Sir2a through the increase of total cellular NAD levels in mammalian cells.

Increasing the Dosage of Nampt and Sir2a Overexpressing Cells Induces Common Gene Expression Changes in Mouse Fibroblasts—To examine further the effect of the NAD biosynthesis mediated by Nampt on mammalian Sir2 function, we compared gene expression profiles between Nampt- and Sir2a-overexpressing NIH3T3 cells (Nampt1 and Sir2a, see Fig. 4) by oligonucleotide microarrays. Combining dye swaps and strict filtering criteria allows us to reproducibly detect gene expression changes with ratios as low as 1.2-fold (38) (see “Experimental Procedures”). The gene expression profiles of Nampt- and Sir2a-overexpressing cell lines were independently determined in comparison to a neomycin-resistant control, Neo1, as illustrated in Fig. 6A. Experiments were repeated with biologically duplicated samples. As shown in Fig. 6B, the gene expression profiles of Nampt- and Sir2a-overexpressing cells are significantly correlated (R = 0.5617, p ≤ 6.359 × 10−13), suggesting that increased dosage of Nampt enhances the transcriptional regulatory function of endogenous Sir2a in mouse fibroblasts. From a total of 9746 unique genes reliably detected in all three cell lines, 171 and 982 genes showed ≥1.2-fold expression changes with 95% confidence in Nampt- and Sir2a-overexpressing cell lines, respectively (Fig. 6C). 44 genes overlapped between these two groups (p < 2 × 10−9), and 36 of these genes showed the same directions of expression changes, listed in Table II. The magnitude of the observed expression changes was higher in Sir2a-overexpressing cells (supplemental Table I) than in Nampt-overexpressing cells (supplemental Table II), consistent with the modest increase in total NAD levels and the
Nampt and Sir2 overexpression induces common gene expression changes in mouse fibroblasts. A, scheme of microarray experiments. Four microarray hybridizations with dye swaps were conducted for each pair-wise comparison using biologically duplicated samples. B, the gene expression profiles of Nampt- and Sir2α-overexpressing cells are significantly correlated. All genes changed with 95% confidence in both Nampt- and Sir2α-overexpressing cell lines are plotted. Statistical analysis of the correlation was determined by the Spearman non-parametric test. C, a Venn diagram for genes exhibiting ≥1.2-fold expression changes with 95% confidence in Nampt- and Sir2α-overexpressing cell lines. Statistical significance was determined by the hypergeometric distribution test. D and E, measurements of relative transcript levels of selected genes in Nampt- and Sir2α-overexpressing cell lines. The transcript levels were measured relative to the glyceraldehyde-3-phosphate dehydrogenase gene and normalized to the Neo1 control. Averages and S.D. were calculated from three independent RNA samples for each gene. Sir2α, Nampt, six down-regulated and two up-regulated genes were examined. Ptn, pleiotrophin; Pttx3, pentaxin-related gene 3; Cxcl1, chemokine (CXC motif) ligand 1; Ccl7, chemokine (CC motif) ligand 7; Gadd45, growth arrest and DNA damage-inducible 45; Atf6, activating transcription factor 6; Ang4, angiotensin-like 4; Odz4, odd Oz/10-m homolog 4. Fabp4 (fatty acid-binding protein 4), which did not meet the criterion of 95% confidence, was also examined since it is a known target for Sir2 (58).

**TABLE II**

| GenBank accession no. | Name | Symbol | Nampt-fold change | Nampt S.D. | Sir2-fold change | Sir2 S.D. |
|-----------------------|------|--------|-------------------|------------|-----------------|----------|
| D90225                | Pleiotrophin | Ptn   | -1.92             | 0.18       | -3.43           | 0.17     |
| NM_008987             | Ptnx3, pentaxin-related gene | Ptx3 | -1.34             | 0.15       | -3.93           | 0.76     |
| NM_007913             | Early growth response 1 | Egr1 | -2.08             | 0.75       | -2.71           | 0.80     |
| NM_008176             | Chemokine (CXC motif) ligand 1 | Cxcl1 | -1.27             | 0.96       | -2.96           | 0.06     |
| AK010675              | Serum amyloid A 3 | Saa3  | -1.24             | 0.05       | -2.09           | 0.43     |
| AK007378              | RIKEN cDNA 1810008K03 gene | 1810008K03Rik | -1.28          | 0.13       | -2.04           | 0.30     |
| K02782                | Complement component 3 | C3   | -1.21             | 0.07       | -2.02           | 0.29     |
| NM_009117             | Serum amyloid A 1 | Saa1 | -1.22             | 0.05       | -1.79           | 0.32     |
| L04694                | Chemokine (CC motif) ligand 7 | Ccl7 | -1.22             | 0.44       | -1.77           | 0.09     |
| NM_011415             | Snail homolog 2 (Drosophila) | Snai2 | -1.13             | 0.17       | -1.59           | 0.37     |
| NM_007896             | Growth arrest and DNA damage-inducible 45α | Gadd45a | -1.23          | 0.12       | -1.65           | 0.17     |
| AF128835              | Polyadenylate binding protein-interacting protein 1 | Paip1 | -1.28           | 0.10       | -1.47           | 0.18     |
| AF357407              | Stromal interaction molecule 2 | Stim2 | -1.33           | 0.12       | -1.39           | 0.13     |
| AK020727              | RIKEN cDNA A330102H22 gene | A330102H22Rik | -1.40          | 0.24       | -1.26           | 0.09     |
| AF357404              | Unknown                                             |       | -1.32           | 0.06       | -1.27           | 0.12     |
| NM_005821             | Inhibitor of DNA binding 3 | Idib3 | -1.24           | 0.08       | -1.34           | 0.12     |
| AK020070              | Activating transcription factor 6 | Atf6 | -1.20           | 0.02       | -1.34           | 0.04     |
| AK013649              | RIKEN cDNA 2900045N06 gene | 2900045N06Rik | -1.23         | 0.09       | -1.27           | 0.12     |
| AK005117              | Adult male cerebellum cDNA |          | 1.22             | 0.10       | 1.24            | 0.23     |
| AK016238              | Unknown                                             |       | 1.25             | 0.08       | 1.22            | 0.21     |
| NM_026473             | RIKEN cDNA 2310057H16 gene | 2310057H16Rik | 2.11           | 0.04       | 1.31            | 0.07     |
| Z12572                | Unknown                                             |       | 1.24             | 0.13       | 1.34            | 0.35     |
| AK019844              | Adult male testis cDNA |       | 1.20             | 0.06       | 1.39            | 0.41     |
| AK013967              | RIKEN cDNA 4933434L15 gene | 4933434L15Rik | 1.25           | 0.09       | 1.35            | 0.38     |
| AK015276              | Adult male testis cDNA |       | 1.38             | 0.10       | 1.23            | 0.24     |
| AK007471              | Insulin-induced gene 1 |       | 1.23             | 0.12       | 1.41            | 0.37     |
| AK000394              | Protein phosphatase 1F (PP2C domain containing) | Insig1 |             |           |                 |          |
| AK021280              | γ-Aminobutyric acid (GABA-A) receptor, subunit α2 | Gabra2 | 1.24           | 0.08       | 1.43            | 0.48     |
| NM_005806             | Fibroblast growth factor 2 | Fgf2 | 1.29           | 0.15       | 1.38            | 0.18     |
| AF131212              | Solute carrier family 29 (nucleoside transporters), member 1 | Slc29a1 | 1.20           | 0.09       | 1.50            | 0.07     |
| NM_025670             | RIKEN cDNA 5730403B10 gene | 5730403B10Rik | 1.34           | 0.17       | 1.42            | 0.33     |
| NM_013793             | Killer cell lectin-like receptor, subfamily A, member 1 | Kdra1 | 1.31           | 0.07       | 1.49            | 0.40     |
| NM_020838              | Angiotensin-like 4 | Angptl4 | 1.25           | 0.12       | 1.72            | 0.36     |
| AK017143              | 3 days neonate thymus cDNA |       | 1.21           | 0.14       | 1.85            | 0.40     |
| NM_028133              | EGL nine homolog 3 (C. elegans) | Egln3 | 1.28           | 0.10       | 1.85            | 0.60     |
| D87034                | Odd Oz/10-m homolog 4 (Drosophila) | Odz4 | 1.20           | 0.09       | 2.00            | 0.57     |
transcriptional repressive activities of GAL4DBD-mCORE in Nampt-overexpressing cells (see “Discussion”). The accuracy of microarray measurements was confirmed for representative genes with quantitative real time RT-PCR (Fig. 6, D and E). Consistent with the results from NAD measurements and reporter gene transcription assays, these results demonstrate that NAD biosynthesis regulated by Nampt controls Sir2α activity in mammalian cells.

**DISCUSSION**

In this study, we have provided four lines of evidence that Nampt is the rate-limiting component in the NAD biosynthesis pathway starting from nicotinamide and regulates the transcriptional function of Sir2α in mammalian cells. First, our biochemical analyses with the enzyme-coupled fluorometric assays showed that Nampt has a high affinity for nicotinamide ($K_m = 0.92 \mu M$), whereas the catalytic efficiency of Nampt is ~46-fold lower than that of Nmnat (Table I). Second, overexpression of Nampt significantly increased total cellular NAD in mouse fibroblasts, whereas increased dosage of Nmnat and addition of 5 mM nicotinamide were unable to increase NAD (Fig. 4B). Third, overexpression of Nampt, but not Nmnat, enhanced the transcriptional repressive activity of the GAL4DBD-fused Sir2α core domain (GAL4DBD-mCORE) recruited onto a reporter gene (Fig. 5B), consistent with their effects on total cellular NAD levels. Furthermore, there was a strong correlation between the total cellular NAD levels and the transcriptional activities of GAL4DBD-mCORE in control and stable Nampt-overexpressing NIH3T3 cell lines (Fig. 5D). Fourth, gene expression profiling with oligonucleotide microarrays showed a significant correlation between expression changes in Nampt- and Sir2α-overexpressing cell lines (Fig. 6B). This correlation was confirmed by measuring transcript levels of representative genes with quantitative real time RT-PCR (Fig. 6, D and E). Taken together, these results establish for the first time that Nampt plays an important role in regulating NAD biosynthesis and consequently Sir2α activity in mammalian cells.

Although Sir2 proteins are highly conserved among bacteria, archaea, and eukarya (1, 44), the NAD biosynthesis enzymes that metabolize nicotinamide have a peculiar phylogenetic distribution. The gene encoding Haemophilus Nampt, nadV, is carried on a plasmid called pNAD1 (30), which has recently been found to contain homologs of the genes originated from a single-stranded bacteriophage (45). Most surprisingly, the entire pyridine nucleotide salvage cycle containing genes homologous to Nampt, Nmnat, and Sir2 has been found in the T4-like, broad host range vibriophage KVP40 (46). Based on these findings, the functional connection between NAD biosynthesis mediated by Nampt and Sir2 seems ancient and fundamental. Considering this connection, it is intriguing that Nampt is present only in vertebrates and a subset of bacterial species (26) (see supplemental Fig. 3). Organisms that do not have Nampt, such as Saccharomyces cerevisiae, C. elegans, and Droso phila, unanimously carry a gene encoding nicotinamidase (26, 47), which converts nicotinamide to nicotinic acid, the main precursor for NAD biosynthesis in those organisms (Fig. 1A). In yeast, PNC1 encodes nicotinamidase and enhances the function of Sir2 in response to a variety of stresses (23, 24). Since no obvious homologs of Pnc1 have been found in vertebrates (26), the presence of Nampt, which allows a more direct pathway for NAD biosynthesis from nicotinamide (Fig. 1B), clearly distinguishes the vertebrate NAD biosynthesis and its relationship with Sir2.

Compared with yeast and invertebrates, mammals also seem to have a different intracellular environment for NAD biosynthesis. The low $K_m$ value of Nampt is consistent with concentration of nicotinamide in mammals, which have been reported at 0.4–0.5 $\mu M$ in human serum (48) and 0.34 $\mu M$ in fasted human plasma (49). Although it has been suggested that nicotinamide plays a critical role as an endogenous inhibitor of Sir2 in yeast (23, 24), the intracellular concentration of nicotinamide in mammalian cells is likely below the $IC_{50}$ values reported for Sir2 family members, which are 40–50 $\mu M$ for human SIRT1 (50, 51) and 130 $\mu M$ for yeast HST2 (52). Instead, nicotinamide could promote Sir2 activity in mammals by acting as a substrate for NAD biosynthesis mediated by Nampt. It has long been known that nicotinamide administration to mammals causes an increase in NAD levels in tissues such as liver and kidney (53, 54). Further investigation will be required to test thoroughly whether administration of nicotinamide activates Sir2 in mammals.

Since Nampt constitutes such an important step in NAD biosynthesis from nicotinamide, the dosage and/or the enzymatic activity of Nampt may be regulated in response to environmental stimuli. It has been reported that the expression of the Nampt gene (referred to as PBEF) is up-regulated in mouse spleen cells stimulated by an anti-CD3ε antibody (32), in human macrophages stimulated by bacterial pathogens (55), and in neutrophils and monocytes in response to inflammatory stimuli (56). Nampt protein levels are also increased under these conditions, which should in turn elevate intracellular NAD levels and consequently activate Sir2. If this is the case, some of the biological consequences ascribed to the function of PBEF/Nampt, such as inhibition of neutrophil apoptosis (56), might be mediated by Sir2, which is also known to have anti-apoptotic effects (8–11). We also suspect that either a cofactor or protein modification might be required to fully activate Nampt activity, since the magnitude of the increase in total NAD levels and gene expression changes were relatively moderate in the Nampt-overexpressing cell lines despite strong overexpression of the protein. We are currently investigating this possibility since we observed a transient shift of the molecular mass of Nampt in response to certain stimuli (our preliminary observations).

Sir2 proteins have been demonstrated to play critical roles in regulating aging and longevity in lower eukaryotes, such as yeast and C. elegans (3). Sir2 proteins are also required for the life span-extending effects of caloric restriction (57–59). NAD biosynthesis plays an important role in regulating Sir2 activity and thereby controls aging, at least in yeast (20, 23, 24). If Sir2 can promote longevity in mammals, the findings presented here imply that NAD biosynthesis would play a significant role in enhancing this effect. Indeed, it has been suggested recently that increasing NAD biosynthesis enhances Sir2 activity in neurons and may increase the resistance to neurodegenerative diseases (60). It would be of great interest to investigate the molecular mechanism of NAD biosynthesis regulation and its impact on aging and longevity in mammals.

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