Identification and characterization of YLR328W, the *Saccharomyces cerevisiae* structural gene encoding NMN adenylyltransferase. Expression and characterization of the recombinant enzyme

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Abstract The enzyme nicotinamide mononucleotide (NMN) adenylyltransferase (EC 2.7.7.1) catalyzes the transfer of the adenylyl moiety of ATP to NMN to form NAD. A new purification procedure for NMN adenylyltransferase from *Saccharomyces cerevisiae* provided sufficient amounts of enzyme for tryptic fragmentation. Through data-base search a full matching was found between the sequence of tryptic fragments and the sequence of a hypothetical protein encoded by the *S. cerevisiae* YLR328W open reading frame (GenBank accession number U20618). The YLR328W gene was isolated, cloned into a T7-based vector and successfully expressed in *Escherichia coli* BL21 cells, yielding a high level of NMN adenylyltransferase activity. The purification of recombinant protein, by a two-step chromatographic procedure, resulted in a single polypeptide of 48 kDa under SDS-PAGE, in agreement with the molecular mass of the hypothetical protein encoded by YLR328W ORF. The N-terminal sequence of the purified recombinant NMN adenylyltransferase exactly corresponds to the predicted sequence. Molecular and kinetic properties of recombinant NMN adenylyltransferase are reported and compared with those already known for the enzyme obtained from different sources.

Key words: Nicotinamide mononucleotide adenylyltransferase; NAD biosynthesis; Cloning; Yeast

1. Introduction

Besides its fundamental function as a coenzyme in redox reactions, NAD may also serve as a precursor for molecules involved in regulatory processes. Indeed, the ADP-ribose moiety of NAD may be transferred to proteins, thus severely influencing their functional state [1]. More recently, it was discovered that NAD glycohydrolases are able to synthesize cyclic ADP-ribose, a compound which exerts Ca^{2+}-mobilizing activity [2]. Therefore, in recent years the enzymes involved in NAD cleavage have gathered increasing interest, while no comparable effort appears to have been devoted to the study of the biosynthetic routes leading to NAD formation. In both de novo and salvage synthesis of nicotinamide nucleotides, a central role is played by the nuclear enzyme nicotinamide mononucleotide (NMN) adenylyltransferase, which catalyzes the formation of NAD (or deamido-NAD) from the mononucleotide and ATP, with the concomitant release of pyrophosphate. For the first time observed and partially kinetically characterized in yeast autolysate by Kornberg [3], this enzymatic activity appears to be of substantial importance, as shown by its correlation with crucial cellular events, like mitosis and DNA synthesis [4–6]. NMN adenylyltransferase, which in prokaryotes is critical for cell survival, is very low in tumor cells, thereby representing a potential target for chemotherapy [5,7,8]. More recently, the involvement of NMN adenylyltransferase in the cellular metabolism of potent antitumor agents, like tiazofurin, selenazofurin and benzamide riboside, has also been demonstrated [9–11].

The first homogeneous enzymatic preparation of NMN adenylyltransferase has been obtained from the yeast *Saccharomyces cerevisiae* [12]; later NMN adenylyltransferase has been purified to homogeneity also from human placenta, bull testis, and thermophilic bacteria [13–15].

The level of eukaryotic NMN adenylyltransferase is low in most tissues and the purified enzyme is very unstable. A recombinant enzyme source is therefore needed for the study of its structure and function.

In our laboratory we have identified, cloned and expressed the gene for NMN adenylyltransferase from the thermophilic archaeon *Methanococcus jannaschii*, which to date is the only known gene for this enzyme [15]. In addition we have recently demonstrated that *Synechocystis* sp. slr0787 protein is a novel bifunctional enzyme endowed with both nicotinamide mononucleotide adenylyltransferase and 'Nudix' hydrolase activities [16].

Determination of the sequence of NMN adenylyltransferase tryptic fragments allowed us to identify the YLR328 open reading frame (ORF) from the *S. cerevisiae* genome sequence as the NMN adenylyltransferase gene. In this paper, we report on the first identification, cloning and expression of a eukaryotic NMN adenylyltransferase gene, the major molecular and kinetic properties of the recombinant protein are also described. A preliminary version of this paper has been published in abstract form as an adjunct to a meeting [17].

2. Materials and methods

2.1. Materials

Phage clone of *S. cerevisiae* DNA segment (lambda PM-2035) (No. 70036), containing the YLR328 gene, was purchased from the Amer-

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Abbreviations: NMN, nicotinamide mononucleotide; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; BisTris-HCl, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane hydrochloride; IPTG, isopropyl-1-thio-β-D-galactopyranoside

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ican Type Culture Collection (Rockville, MD). Oligonucleotide primers were obtained from Life Technologies (Milan, Italy). Restriction enzymes were obtained from Promega Biotec (Madison, WI), and T4 DNA ligase, PCR reagents, and enzymes were obtained from Boehringer Mannheim GmbH. The basic molecular biology procedures for bacterial growth, plasmid DNA purification, and preparation and transformation of competent cells as described by Sambrook et al. [18] were followed.

2.2. Sequencing of S. cerevisiae NMN adenylyltransferase proteolytic fragments

The original enzyme purification procedure [12] has been revised. Briefly, the steps were as follows: crude extract from yeast; pH 5.0 precipitation; heat treatment; hydroxyapatite chromatography; dye-ligand chromatography on Matrex Green A; FPLC on phenyl Superose HR 5/5. The final SDS-PAGE [10% (w/v) gel] was performed by the method of Schagger and von Jagow [19]. After Coomassie brilliant blue staining the band corresponding to NMN adenylyltransferase was excised and subjected in situ to tryptic digestion. The fragments generated were separated by reverse-phase liquid chromatography on a ABI 173 A Capillary LC/Microblotter System and sequenced by using an Applied Biosystems Procise Model 491 sequencer.

2.3. Cloning of the YRL328 gene

The synthetic oligonucleotide primers 5′-ACA GTC GAC ATG GAT CCC ACA AGA GCT CCG-3′ and 5′-CTA AAG CTT TCA TTC TTT GTT TCC AAG AAC-3′ were used in a PCR to amplify the YLR328 ORF and to insert the *Sal*I and *Hin*dIII restriction sites at its 5′ and 3′ ends, respectively. PCR was performed with 8 ng of *S. cerevisiae* DNA segment (lambda PM-2035) as the template, with 20 pmol of each primer in a final volume of 100 μl. Each cycle was set for 1 min of denaturation at 95°C, 1 min of annealing at 42°C, and 1 min of elongation at 72°C, and 30 reaction cycles were carried out in a DNA thermal cycler. The amplified DNA contained a 1.2 kb product visible by ethidium bromide staining, after electrophoresis in a 1% agarose gel. The product was digested with *Sal*I and *Hin*dIII, and cloned into *Sal*I-*Hin*dIII-digested pT7-7 plasmid vector [20] to obtain the construct pT7-7-YLR328. The nucleotide sequence of the insert was confirmed by direct sequencing, to ascertain that no mutations had been introduced during the amplification reaction. The construct was used to transform *Escherichia coli* TOP10 (Invitrogen) for plasmid preparation and *E. coli* BL21 (DE3) for protein expression.

2.4. Purification of the YLR328 ORF-encoded protein

2.4.1. Growth and expression. Single colonies of strain BL21 (DE3) harboring the pT7-7-YLR328 plasmid were inoculated into 50 ml of Luria-Bertani medium (supplemented with ampicillin at 100 μg/ml) and grown at 37°C to an A_{600} of 0.700. A 10 ml portion was used to inoculate 1 liter of fresh Luria-Bertani medium (supplemented with ampicillin at 100 μg/ml), and the mixture was incubated overnight at 120 rpm at 37°C. The culture was then induced with 1 mM IPTG. Incubation was continued for 5 h at 37°C with shaking before the cells were harvested by centrifugation (10 000 × *g* for 10 min at 4°C), and either carried on for purification or stored at −80°C.

2.4.2. Crude extract. All steps were performed at 4°C. The cell pellet was suspended in 50 ml of 50 mM Tris-HCl pH 8.0, containing 1 mM DTT and disrupted by sonication. The lysate was centrifuged at 15 000 × *g* for 30 min (crude extract).

2.4.3. Heat treatment. The crude extract was heated to 60°C for 10 min, and centrifuged at 16 000 × *g* for 30 min. The resulting supernatant is referred to as the 60°C fraction.

2.4.4. Matrex Gel Green A chromatography. The 60°C fraction was applied to a Matrex Gel Green A dye-ligand chromatography column, previously equilibrated with 20 mM Tris-HCl pH 8.0, containing 1 mM DTT. The column was washed with the same buffer, containing 1 M NaCl and then eluted with a linear gradient of 1–3 M NaCl in the equilibration buffer (Green A fraction).

2.4.5. FPLC on phenyl Superose HR 5/5. The Green A fraction was made 3 M in NaCl and injected in 1 ml aliquots into a phenyl Superose HR 5/5 FPLC column, previously equilibrated with 20 mM Tris-HCl pH 8.0, containing 1 mM DTT and 3 M NaCl. The column was washed with the same buffer and eluted with a discontinuous gradient from 3 to 0 M NaCl in 20 mM Tris-HCl pH 8.0, containing 1 mM DTT. Active fractions were pooled, concentrated by ultrafiltration and stored at 4°C (phenyl Superose fraction).

2.5. NMN adenylyltransferase assay and kinetic characterization

Enzyme activity was routinely measured by a continuous spectrophotometric coupled enzyme assay [21]. In an alternative assay procedure, the enzyme activity was calculated by HPLC [21]. The optimal reaction conditions were established by varying both the divalent cation concentration and the pH of the reaction buffer, using 30 mM BisTris-HCl and 30 mM Tris-HCl adjusted to the desired pH (5.0–9.0). One enzyme unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NAD^+ per minute at 37°C.

2.6. Other determinations

Gel electrophoresis of the recombinant *S. cerevisiae* NMN adenylyltransferase was carried out according to the method of Schagger and von Jagow in 10% polyacrylamide gel [19]. Gel filtration of recombinant *S. cerevisiae* NMN adenylyltransferase was performed by FPLC with a Superose 12 HR 10/30 (Pharmacia) column equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl and 1 mM DTT.

N-terminal sequence analysis of recombinant *S. cerevisiae* NMN adenylyltransferase spotted on a polyvinylidene difluoride membrane was performed by automated Edman degradation as above described. Protein concentration was determined by the method of Bradford [22].

3. Results and discussion

3.1. Identification, cloning, and expression of S. cerevisiae NMN adenylyltransferase gene

When examined after SDS-PAGE, the purified enzyme (phenyl Superose fraction) showed a Coomassie-stained major band of approx. 46 000, in good agreement with the molecular mass of *S. cerevisiae* NMN adenylyltransferase. Attempts to determine the NH_2-terminal sequence of the protein failed, presumably because of the presence of a modified amino acid residue at the NH_2-terminus. Therefore, the protein migrating at 46 000 was excised, digested with trypsin, and the resulting peptides were separated and sequenced, as described in Section 2.

A search of the *S. cerevisiae* genome sequence database, with the BLAST [23] network service at the National Center for Biotechnology Information, revealed that two peptides, DLEEVPHGIVR and VLDHFNHEIN, were identical to residues 129–139 and 256–265 encoded by the YLR328w gene, located on chromosome XII. In addition, the molecular mass of 45.9 kDa predicted for the polypeptide encoded by the YLR328w ORF closely corresponds to the subunit molecular mass obtained for the *S. cerevisiae* enzyme. To confirm that the YLR328w ORF indeed encodes NMN adenylyltransferase, the gene was isolated and cloned into a T7-based vector.

E. coli BL21(DE3) cells harboring the recombinant plasmid were grown, induced with IPTG, and extracted as described in Section 2. Cell extracts were assayed for NMN adenylyltransferase activity. Even in the absence of added IPTG, a high level of NMN adenylyltransferase activity could be detected in BL21 cells transformed with the recombinant plasmid, while BL21(DE3) containing the expression vector alone had no detectable NMN adenylyltransferase activity. A two-fold greater NMN adenylyltransferase total activity was obtained 5 h after induction with IPTG. In the crude extract of BL21(DE3) cells transformed with the recombinant plasmid, upon SDS-PAGE, the presence of a polypeptide of the expected size was evidenced, whereas it was absent in crude extract of bacteria lacking the YLR328w coding sequence (data not shown).

Table 1
Purification of recombinant *S. cerevisiae* NMN adenylyltransferase[a]

Fraction	Total protein (mg)	Total activity (U)	Sp. act. (U/mg)	% Yield	Purification factor
Extract	121	154	1.27	100	–
60°C fraction	100	178	1.78	116	1.4
Green A	2.35	126	53.6	82	42
Phenyl Superose	0.86	63.8	74.2	41	58

[a]The purification procedure and the enzyme assay were performed as described in Section 2.

3.2. Purification and properties of recombinant S. cerevisiae NMN adenylyltransferase

Recombinant NMN adenylyltransferase was purified 58-fold to homogeneity in two chromatographic steps, as described in Section 2 (Table 1). Dye-ligand chromatography on Matrex Gel Green A was successful in removing most of the contaminating proteins, the recombinant NMN adenylyltransferase being by far the most abundant component in the column eluate (Fig. 1). The final FPLC on phenyl Superose HR 5/5 was able to yield an enzymatic preparation, with a specific activity of 74.2 U/mg. By means of this purification procedure a yield of approx. 0.9 mg of pure recombinant NMN adenylyltransferase per liter of culture was obtained. SDS-PAGE analysis indicated that the purified recombinant protein had a molecular mass of 48 kDa, which corresponds with the molecular mass predicted for the amino acid sequence (Fig. 1). The native molecular mass of the active recombinant enzyme was determined to be 195 kDa by gel filtration, suggesting that it is an oligomer composed of four identical subunits, as previously observed for the wild type enzyme [12].

Identification of the 48 kDa protein as that encoded by the cloned YLR328 gene was performed by N-terminal amino acid sequencing of pure recombinant protein. Sequencing of the first 22 amino acid residues yielded an exact match with the predicted sequence.

The recombinant NMN adenylyltransferase has an absolute requirement for divalent cations, achieving optimal activity with 5 mM Ni^{2+}; various heavy metal ions markedly depress the enzyme activity, the most effective being Hg^{2+}, completely abolishing NMN adenylyltransferase activity when present in the assay mixture at a concentration of 0.05 mM, similar to what previously reported for human NMN adenylyltransferase [14]. In contrast to pig liver NMN adenylyltransferase, which utilizes various purine nucleoside triphosphates [24], the recombinant NMN adenylyltransferase seems to be specific for adenylic nucleoside triphosphates, no activity being detected in the presence of ITP and GTP. The optimum pH is identical to the wild type enzyme [12].

3.3. Structural analysis of recombinant S. cerevisiae NMN adenylyltransferase

The hydrophobicity profile, performed according to Kyte and Doolittle [25] (Fig. 2), suggests the existence of several hydrophobic fragments likely corresponding to transmembrane regions. Another distinctive feature is the existence, at the NH_2-terminal region, of a hydrophilic segment, which involves a cluster of histidines (amino acids 61–67), which should represent a potential metal binding site.

It is interesting that the consensus sequences both for dinucleotide binding proteins and for mononucleotide binding proteins, as proposed by Moller and Amons [26], are not present in the recombinant protein sequence. Using the MOTIFS program, we identified in recombinant *S. cerevisiae* NMN adenylyltransferase the consensus motif p..[de].y.[ekq].g[filvy], which represents a feature of many ad-

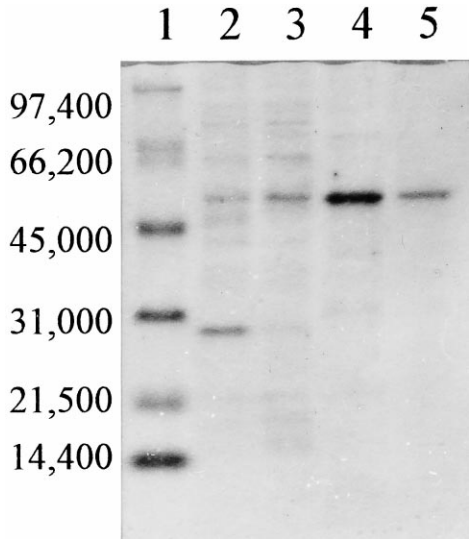

Fig. 1. Expression and purification of recombinant *S. cerevisiae* NMN adenylyltransferase. A polyacrylamide gel (10%) containing 0.1% SDS was stained with Coomassie blue. Lane 1 contains reference proteins; lanes 2, 3, and 4 contain 10 μg of crude extract, heat treatment fraction, and Green A fraction, respectively. Lane 5 contains 5 μg of the purified recombinant NMN adenylyltransferase.

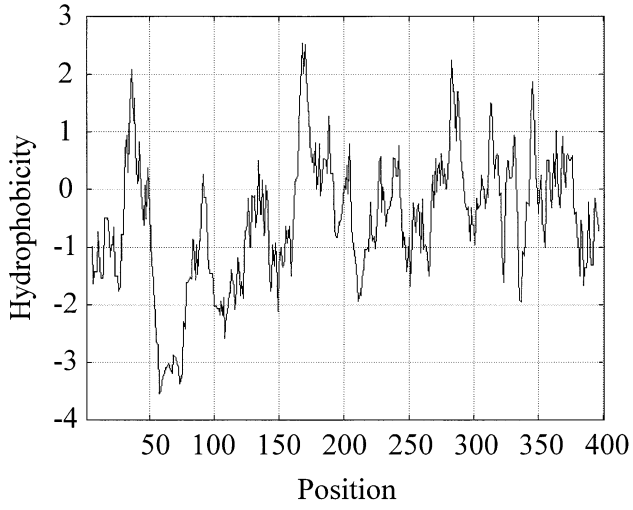

Fig. 2. Hydrophobicity profile of recombinant *S. cerevisiae* NMN adenylyltransferase. The protein was analyzed using the method of Kyte and Doolittle [25]. The hydropathicity value of each amino acid residue is plotted against its position in the polypeptide (*x* axis), starting with the amino-terminus.

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F26H9.4         ------------------------------------------------------------
W06B3.1         ------------------------------------------------------------
YGR010W         MDPTKAPDFKPPQPNEELQPPPDPTHTIPKSGPIVPYVLADYNSSIDAPFNLDIYKTLSS 60
YLR328W         MDPTRAPDFKPPSADEELIPPPDPESKIPKSIPIIPYVLADANSSIDAPFNIKRKKKHPK 60

F26H9.4         ------------------------------------------------------------
W06B3.1         ------------------------------------------------------------
YGR010W         RKKNANSSNR------MDHIPLNTSDFQPLSRDVSSEEESEGQSNGIDATLQDVTMTGNL 114
YLR328W         HHHHHHHSRKEGNDKKHQHIPLNQDDFQPLSAEVSSEDDDADFRSKERYGSDSTTESETR 120

F26H9.4         ----------------------------------------MKRVALLAVGSFNPPTIA 18
W06B3.1         ---------------------------------------MGTEKVVILAVGSFNPPTNG 20
YGR010W         GVLKSQIADLEEVPHTIVRQARTIEDYEFPVHRLTKKLQDPEKLPLIIVACGSFSPITYL 174
YLR328W         GVQKYQIADLEEVPHGIVRQARTLEDYEFPSHRLSKKLLDPNKLPLVIVACGSFSPITYL 180
                                                       : ::* ***.* *

F26H9.4         HLRMLEVARSHL-ETINTQVVEGIMSPVADSYNNKPTLIKSNFRIQMVRAATK-SSDWIR 76
W06B3.1         HLCMMEDAKYSL-EKSGKIVLEGIMSPVSDGYAKK-SLISAKHRLAQTEAATY-DSDWIH 77
YGR010W         HLRMFEMALDDINEQTRFEVVGGYFSPVSDNYQKR-GLAPAYHRVRMCELACERTSSWLM 233
YLR328W         HLRMFEMALDAISEQTRFEVIGGYYSPVSDNYQKQ-GLAPSYHRVRMCELACERTSSWLM 239
                ** *:* *   : *     *: *  ***:*.* ::  *  : .*:   . *    *.*:

F26H9.4         ADDWECTRTTWTRTIDVLRHHRELVQEKFG---------SDVGMMLVVGGDVVDSFTRIL 127
W06B3.1         ASGWECAQSEWTATVNVLKHHQQ--DNKLG---------SDVNVLLLFGGDVIESFDKFY 126
YGR010W         VDAWESLQSSYTRTAKVLDHFNHEINIKRGGIMTVDGEKMGVKIMLLAGGDLIESMGE-- 291
YLR328W         VDAWESLQPSYTRTAKVLDHFNHEINIKRGGVATVTGEKIGVKIMLLAGGDLIESMGE-- 297
                .. **. :. :* * .** *...  : * *          .* ::*: ***:::*: .

F26H9.4         PDGSNLWNSSDIRTIITEFGLIVLSREGSNPLNTIQSMPAISEFCDRIIQVKDEVCPSGV 187
W06B3.1         ADGTPVWDREDVEEIIS-AGIVVRSRPGSDPEQTLKKLN-LNENSDKVHFIKNAISSNSI 184
YGR010W         ---PHVWADSDLHHILGNYGCLIVERTGSDVRSFLLSHDIMYEHRRNILIIKQLIY-NDI 347
YLR328W         ---PNVWADADLHHILGNYGCLIVERTGSDVRSFLLSHDIMYEHRRNILIIKQLIY-NDI 353
                   . :*   *:. *:   * :: .* **:  . : .   : *   .:  :*: :  ..:

F26H9.4         SSTRLRAAIMNKKSIKYSTPDEVINFIRENNLYQKI------------ 223
W06B3.1         SSTSLRAALKEHRSIKYTTPDSVIKYIKDHRLYE-------------- 218
YGR010W         SSTKVRLFIRRGMSVQYLLPNSVIRYIQEYNLYINQSEPVKQVLDSKE 395
YLR328W         SSTKVRLFIRRAMSVQYLLPNSVIRYIQEHRLYVDQTEPVKQVLGNKE 401
                *** :*  : .  *::*  *:.**.:*:: .**
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Fig. 3. Alignment of the amino acid sequence of recombinant *S. cerevisiae* NMN adenylyltransferase (YLR328W), YGRO10W ORF-encoded protein, F26H9.4 ORF-encoded protein, WO6B3.1 ORF-encoded protein, using the ClustalW program. Asterisks indicate identical amino acids, double and single dots indicate a conserved amino acid substitution, strong and weak, respectively.

enylate kinases family members. The significance of this segment, starting at amino acid 206, is at present unknown.

Comparison of the amino acid sequences of the three NMN adenylyltransferases to date known shows that *S. cerevisiae* NMN adenylyltransferase is quite different from the others. The *M. jannaschii* NMN adenylyltransferase displays 41% identity and 60% similarity with the first 65 residues at the N-terminus of the *Synechocystis* sp. NMN adenylyltransferase [15], while no significant similarity was found with respect to *S. cerevisiae* NMN adenylyltransferase. When we searched the SwissProt data base we found an ORF, designated YGR010w, which encodes a putative protein with 395 amino acids, showing 72% amino acid identity to the *S. cerevisiae* NMN adenylyltransferase (Fig. 3). The ORF YGR010w is located on chromosome VII and might represent a duplication of NMN adenylyltransferase gene, even though, at present, no additional experimental data substantiate this hypothesis.

A Yeast-Worm ClustalW alignment revealed also that *S. cerevisiae* NMN adenylyltransferase displays a high degree of amino acid sequence homology with two hypothetical proteins of *Caenorhabditis elegans*, designated F26H9.4 and WO6B3.1. The similarity was 52 and 50%, respectively, thus suggesting that the two unknown proteins probably possess NMN adenylyltransferase activity (Fig. 3). Therefore, from an evolutionary perspective it appears that the NMN adenylyltransferase gene into the diverging branches of the eukaryotic subtree has retained common features, while it has arrived at separate solutions with respect to its prokaryotic counterpart.

Although the enzyme NMN adenylyltransferase has been studied for a long time, its regulation and mechanism are still poorly understood. Cloning of NMN adenylyltransferase gene from a eukaryotic organism and availability of large amounts of recombinant enzyme will be instrumental both for studying its expression in different cells and organs and for undertaking more detailed structural studies of the enzymatic protein.

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