Primary structure of a molluscan egg-specific NADase, a second-messenger enzyme*

David L. Glick,[†] Mark R. Hellmich,[†]
Sven Beushausen,[‡] Paul Tempst,[§]
Hagan Bayley,^{||} and Felix Strumwasser^{†||}

[†]Laboratory of Neuroendocrinology
Marine Biological Laboratory
Woods Hole, Massachusetts 02543

[§]Department of Genetics
Harvard University School of Medicine
Boston, Massachusetts 02115

^{||}Worcester Foundation For Experimental Biology
Shrewsbury, Massachusetts 01545

An egg-specific NADase has been purified from the ovotestis of the marine mollusk *Aplysia californica*. The enzyme converts NAD to cyclic ADP-ribose (cADPR), which is a potent mobilizer of Ca^{2+} . It is likely that the NADase serves to raise Ca^{2+} levels in the ova at appropriate times. A 1.2-kb cDNA clone containing the complete coding sequence of the native NADase protein was isolated from an unamplified ovotestis cDNA library and represents the first cloning of an NADase that generates cADPR. In vitro translation studies indicate that the protein initially has a signal sequence that may help to target it to discrete vesicles of the ova in which it is found. There are 12 cysteines in the open reading frame, two of these being in the signal sequence. No part of the sequence has significant similarity to other proteins or known nucleotide binding site consensus sequences. Northern blot analysis of poly(A)⁺ selected ovotestis RNA has identified an NADase mRNA of 1.85 kb. In situ hybridization analysis of cryostat sections from ovotestis has shown that the NADase mRNA is restricted to the immature ova, although the NADase protein is present in both immature and mature eggs.

* Nucleotide sequence has been submitted to GenBank. The accession number is M37644.

‡ Current address: Laboratory of Neurobiology, NINDS, NIH, Bethesda, Maryland 20892.

|| To whom correspondence should be addressed.

Introduction

NAD glycohydrolases (NADases), which cleave NAD into nicotinamide and ADP-ribose, are a diverse class of enzymes found in species from microbes to mammals. They vary in size (20–120 kDa) and in cellular location (membrane bound to aqueous soluble) (Kaplan, 1960; Pekala and Anderson, 1982). Many eukaryotic NADases are membrane-associated ectoenzymes (Muller *et al.*, 1983). Despite their wide distribution, the physiological function of NADases is unknown, and their great diversity suggests that the simple cleavage of NAD is not their only function.

The preceding paper (Hellmich and Strumwasser, 1991) describes the purification and characterization of a 29-kDa NADase from the ovotestis of *Aplysia californica*. It was found that the NADase used NAD as a substrate to produce nicotinamide and cyclic ADP-ribose (cADPR). The latter compound is a second messenger that promotes Ca^{2+} ion release from intracellular compartments (Rusinko and Lee, 1989). The *Aplysia* NADase was found only within oocytes where it was localized, in abundance, to 5- to 10- μm granules or vesicles. Two-dimensional (2-D) electrophoresis revealed several forms of the NADase differing by isoelectric point. Although a definitive function for the enzyme in the physiology of the ova remains to be established, it might play a role in post-fertilization events utilizing calcium.

We decided to clone and sequence the cDNA coding for this interesting NADase because it generates cADPR, a putative second messenger for Ca^{2+} mobilization. This paper describes the characterization of the primary structure of the NADase transcript and protein and our use of in situ hybridization to localize the transcript.

Results

Peptide sequencing and protease resistance of the native NADase

To obtain internal amino acid sequence for designing oligonucleotide probes, fragments of the NADase were generated by proteolytic diges-

GATTAGAAATGTCTCCCGTCGCCATTATTGCTTGTGTGTCTGGCGGTGACGCTGACA	60
d l e m s p v a i i a c v c l a v t l t	20
AGTATCTCGCCAGCGAGGCCATCGTCCCCACTCGCGAGTTGGAGAATGTGTTCTTGGC	120
s i s p s e a I V P T R E L E N V F L G	40
CGCTGTAAGGATTACGAGATCACCCGATACCTGGATATTCTTCCCAGAGTGAGATCGGAC	180
R C K D Y E I T R Y L D I L P R V R S D	60
TGCTCGGCATTGTGGAAGGACTTTTTCAAAGCCTTCTCCTTCAAAAATCCCTGTGATCTC	240
C S A L W K D F F K A F S F K N P C D L	80
GACCTTGAAGTTACAAAGATTTTTCACCTAGTGCAGCAACAGCAGCTGCCAAAGAACAAA	300
D L G S Y K D F F T S A Q Q Q L P K N K	100
GTAATGTTCTGGTCTGGAGTCTATGACGAGGCTCACGATTATGCCAACACCGGCAGGAAG	360
V M F W S G V Y D E A H D Y A N T G R K	120
TACATCACTTTGGAAGATACGCTTCCGGGTTACATGTGAACTCTCTGGTTTGGTGCGGC	420
Y I T L E D T L P G Y M L N S L V W C G	140
CAACGCGCTAATCCAGGATTCAACGAAAAGGTGTGCCCGGACTTCAAAACGTGTCCAGTG	480
Q R A N P G F N E K V C P D F K T C P V	160
CAAGCTAGAGAGAGCTTCTGGGGAATGGCGTCAAGTTCTTATGCTCACAGTGCAGAGGGA	540
Q A R E S F W G M A S S S Y A H S A E G	180
GAAGTGACCTACATGGTTCGACGGTTCGAATCCCAAGGTACCAGCTTACAGGCCAGACAGT	600
E V T Y M V D G S N P K V P A Y R P D S	200
TTTTTTGGCAAGTACGAACTGCCAATCTGACTAACAAGGTGACAAGGGTAAAGGTCATC	660
F F G K Y E L P N L T N K V T R V K V I	220
GTCCTGCATCGGCTCGGGGAAAAGATCATAGAGAAGTGCGGTGCAGGATCTTTGCTGGAC	720
V L H R L G E K I I E K C G A G S L L D	240
CTTGAAAGCTTGTCAAAGCCAAGCATTTTGCATTTCGATTGCGTGGAAAATCCAAGGGCT	780
L E K L V K A K H F A F D C V E N P R A	260
GTTCTCTTCTGCTGTGTCTCCGACAACCCCAATGCCAGAGAGTGTGCACTCGCTAAACGT	840
V L F L L C S D N P N A R E C R L A K R	280
TTCTACAGGATCGCTAAATAATCCAGCACCGCTCGTCAACTATTTAAGAGGCGTTTCGT	900
F Y R I A stop	285
TAGAGGTTTATAAATTAATTCTTTTTTTTTGAGAACGTGTCGACGGTGGCAAAATGGGGT	960
CGTTTTTTTAGTCATTAGTGTGGAAAATATATTGGGGATAAAAATGGGGTGGGGCTGGG	1020
GCATTGCTGTTGACGATATAAAGTAAAGAGGGAGGGGCGAGCTCTTCACTTCAGTGCACA	1080
CAGCAGTTGTTATGAATTGAGGGCTGAACGTTCTCGAGTACATTTCAATATTAACCTCTT	1140
GATGTAGAGAGGGTACGCTCTTTACCAATTTCAAATTATTTAAAGGTGTG	1191

Figure 1. Complete nucleotide sequence of the *Aplysia* NADase cDNA. The amino acids are shown just below the nucleotide sequence. Underlined peptides represent sequence obtained from automatic Edman degradation of the NADase *N*-terminus or internal peptides after protease digestion. The amino acids starting at the *N*-terminus of the native protein are capitalized. The internal peptides that were sequenced after digestion of the purified protein with endoproteinase lys-c are KC3:DFFK, KC4:AFSFK, KC8:DFFTSAQQLPK, KC10:VMFWSGVYDEAHDYANT, KC7:VPAYRPDSFFGK, KC11:VPAYRPDSFFGKYELPNLTN, and KC5:VIVLHRLGEK.

tion. The native NADase proved to be very refractory to digestion. Internal peptides could be obtained only when the NADase was first denatured in 8 M guanidine hydrochloride and then diluted to 2 M for digestion with subtilisin or endoproteinase lys-C. The subtilisin digestion resulted in ~40 peptides, whereas the endoproteinase lys-C produced a simpler mixture of ~20. The products of the endoproteinase lys-C digest were further purified with high-pressure liquid chromatography (HPLC) for sequencing. The *N*-terminal and internal peptides that were sequenced are underlined in Figure 1.

Isolation of NADase cDNA clones

When 500 000 phage plaques of the ovotestis cDNA library were screened with the degenerate oligonucleotide DG-1, three independent clones were isolated. The three isolated cDNA clones, K, S3, and O3, had inserts of 800, 1000, and 1200 bp, respectively. A parallel screen with the DG-2 oligonucleotide did not yield any clones.

Both strands of clone O3 were sequenced and determined to be 1191 bp in length. The cDNA contained a 282-amino acid open reading frame beginning with a start codon ATG at nucleotides 10–12 and ending at a stop codon at nucleotides

Figure 2. Northern blot hybridizations of ovotestis RNA with cloned NADase cDNA. Twenty micrograms of poly(A)⁺ RNA from the ovotestis was electrophoresed and transferred to a nylon filter as described in Methods. The sizes of individual RNA species in an RNA ladder (BRL) are shown, and were used to estimate the size of the hybridizing transcript as 1.85 kb. No hybridization was seen when poly(A)⁻ RNA was used (not shown).

856–858 (Figure 1). The *N*-terminus of the purified protein (I), as determined by amino acid sequencing, was found beginning at nucleotides 82–84, suggesting that a signal sequence preceded the native form of the protein. Each of the peptides, sequenced from the endopeptidase Lys-C digestion, could be identified within the translated nucleotide sequence in Figure 1,

thus verifying the identity of the cDNA. The entire open reading frame with the flanking 5' and 3' sequence is shown in Figure 1.

A potential explanation for the lack of positive plaques obtained with DG-2 was revealed by examination of the relevant nucleotide sequence at bp 124–143. The “correct” oligonu-

Figure 3. In vitro translation of RNA transcribed from the NADase clone. NADase RNA, transcribed with T7 polymerase from the plasmid PKSG14, was translated in a reticulocyte lysate in the presence of [³⁵S]methionine and the translation products were separated on a 12% polyacrylamide gel. The positions of the [¹⁴C] molecular weight markers are indicated. Lane A is the product of the in vitro translation mix when no exogenous RNA was added. Lane B represents the in vitro translation of the NADase RNA.

Figure 4. Sequencing of the in vitro translated NADase by automatic Edman degradation. NADase RNA was translated in a wheat germ system in the presence of [^3H]leucine. Proteins in the translation mix were precipitated in 90% acetone and subjected directly to Edman degradation. The radioactivity released at each cycle is plotted, with the first peak normalized to 1000. Peaks appeared at the same cycles (12 and 16) when translation product purified by SDS-polyacrylamide gel electrophoresis was used.

cleotide from the degenerate DG-2 mixture was TGTAAGGATTACGAGATAC. It is possible that the nucleotides TIAC (from the 3' end) or TTAC (from the middle of the oligonucleotide) could have looped back to base pair with nucleotides GTAA at the 5' end and interfered with hybridization.

Northern hybridization revealed that the mature mRNA transcript from the NADase gene was ~1850 bp in length (Figure 2). The translated protein thus consists of a mature peptide of 258 amino acids, which is preceded by a signal sequence of ≥ 24 amino acid residues.

In vitro translation

Although the native NADase had an apparent molecular mass of 29 kDa, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration (Hellmich and Strumwasser, 1991), the in vitro translation of RNA, transcribed from the NADase cDNA clone, produced a product with an apparent molecular mass of 31 kDa (Figure 3). In vitro translation done in the presence of [^{35}S]methionine and then submitted to automatic Edman degradation demonstrated that ~15% of the radioactivity was released at the first cycle, with no significant release in the next

18 cycles. The translation product synthesized in the presence of [^3H]leucine and then submitted to Edman degradation gave peaks of radioactivity at cycles 12 and 16 (Figure 4). The sequence (Figure 1) shows two leucines at positions 12 and 16 from the initial methionine. These results confirm that the in vitro translated protein is initiated at the methionine, 24 amino acids in front of the mature protein's *N*-terminus, which is an isoleucine. These data suggest that the protein is translated in vivo with a signal sequence that is later cleaved during translocation through membranes.

Sequence analysis

The translated protein beginning with isoleucine at amino acid 28 and terminating with alanine at amino acid 285 was calculated to have a molecular mass of 29.5 kDa. This agrees closely with the apparent molecular mass of the mature protein of 29 kDa, as determined by SDS-PAGE and gel filtration. These results also suggest that the native protein is not heavily glycosylated, despite a potential glycosylation site at AA 209–211 (NLT) (Figure 1) (Bause and Hettkamp, 1979). There are two potential serine phosphorylation sites where basic residues are amino terminal to a serine (Glass and Krebs, 1980; Taylor *et al.*, 1990) at AA 54–60 (LPRVRS) and at AA 196–201 (YRPDSF). A hydrophobicity plot (Kyte and Doolittle, 1982) shows the protein to be generally hydrophilic in nature except for the hydrophobic signal sequence.

The fact that there are 10 cysteine residues in such a small protein (2 additional cysteines are found in the signal sequence), together with the signal sequence, is consistent with a secreted protein. The numerous potential disulphide bonds might help to maintain a tight globular structure, which could explain the resistance to proteases seen with the NADase.

Several computer searches of the NADase against other protein sequences in the various protein databases did not reveal any similarities except for one found by Dr. Russell F. Doolittle (University of California, San Diego). Amino acid residues 138(C)–185(G) of rabbit muscle phosphorylase b kinase and residues 220(I)–267(S) of the *Aplysia* NADase could be aligned. Within this alignment of 48 residues, 13 were identical (*) and 4 were conservative (#) replacements:

CALHKLNI	VHRDL	KPENI	LLDDDMNI	KL	TDFGF	SCQLDP	GEKLRE	VCG	kinase
***#	#		***#	#	*	*	*	*	
IVLHRLGEK	I	EKCGAGS	LLDLK	LVKAKH	FAFDC	VENPRA	VLFL	LLCS	NADase

The underlined residues in the kinase are highly conserved between all eukaryotic protein kinases. A more specific search against proteins that utilize NAD as a coenzyme provided no additional major similarities.

Localization of the NADase transcript in Aplysia

The in situ hybridization showed that the NADase mRNA transcript was specifically localized to the eggs of the ovotestis when the antisense cRNA probe was used (Figure 5). No other cell type found in the ovotestis, such as sperm or follicle cells, showed hybridization. No hybridization occurred with the sense cRNA probe. The smaller, immature eggs with few granules or vesicles had large amounts of NADase transcript, whereas the larger, mature eggs, full of vesicles, contained low levels of the transcript.

Discussion

***Aplysia* NADase cDNA**

This report describes the isolation and characterization of a cDNA clone that encodes an *Aplysia* egg-specific NADase and represents the first cloning and sequencing of an NADase that generates cADPR, a putative second messenger for Ca^{2+} mobilization. While the cDNA of 1191 bp contained the complete open reading frame, Northern blot analysis demonstrated the presence of a single, much larger transcript of ~1850 bp. Large, untranslated regions in *Aplysia* mRNA transcripts have been seen for the catalytic subunit of the *Aplysia* cAMP-dependent protein kinase, where a 6500-bp mRNA

transcript contains an open reading frame of only 1056 bp (Beushausen *et al.*, 1988).

The single transcript suggests that the different isoforms of the NADase seen on 2-D gels (Hellmich and Strumwasser, 1991) are due to posttranslational modifications rather than to multiple transcripts. If these isoforms are not due to purification artifacts (e.g., proteolytic removal of a few amino acids during purification), then one possibility is that the isoforms represent different amounts of phosphorylation occurring at the two potential serine phosphorylation sites, as deduced from the sequence.

Protein similarity searches

Computer searches of protein databases revealed no major similarities with any other protein, even when the search was narrowed to those proteins that have binding sites for nucleotides. Although the NADase and rabbit muscle phosphorylase b kinase had a short stretch of similarity, they are unlikely to be functionally related. The NADase had only one identical amino acid and one conservatively replaced residue out of the seven residues that are conserved between many kinases in this region (see underlined residues in Results) (Taylor *et al.*, 1990). As additional NADases are sequenced, the significance of the identities in this region of the two rather different enzymes might become clearer.

The generally accepted adenine dinucleotide and nucleotide binding site consensus sequences, which are GXGXXG for many dehydrogenases (Wierenga *et al.*, 1986) and GXXXXGK(T/G)XXXXXX(I/V) for many ATPases (Walker *et al.*, 1982), are absent in the NADase.

Figure 5. In situ hybridization of *Aplysia* ovotestis tissue sections with anti-sense NADase RNA probes. The hybridization of the NADase ^{35}S -labeled anti-sense RNA probe can be seen as exposed silver grains over the immature oocytes (thin arrows). The mature eggs (thick arrows) and other cell types such as spermatocytes (below star) showed no reactivity. Scale = 100 μm .

This is surprising, but might indicate that among the NADases there could be alternative motifs for binding nucleotides. Alternatively, the fact that the *Aplysia* NADase generates a cyclic form of ADP-ribose might indicate that the nature of the binding site for NAD is not the nucleotide portion of the substrate, but rather the nicotinamide moiety.

NADase signal sequence

The in vitro translation experiments suggested the presence of a signal sequence on the NADase. The 24-amino acid sequence, beginning with methionine, preceding the *N*-terminal residue of the native protein, has many characteristics in common with known signal sequences. It has a hydrophobic core sequence (VAII-ACVCLAV) followed by a more polar set of amino acids (TSISPSE) next to the *N*-terminus of the exported, native protein. In addition, the positions of serine and alanine, which are -3 and -1 from the *N*-terminus isoleucine of the processed protein, are typical of signal sequences and follow the common -3, -1 rule (Briggs and Gierasch, 1986).

The in vitro translation experiments do not definitively determine that translation in vivo begins at the indicated methionine. They do show that translation begins ≥ 24 amino acids in front of the amino terminal end of the native protein. There is some evidence that this methionine is not the true start of translation. Most *N*-terminal residues in eukaryotic signal sequences are charged, although there are exceptions. Because these charged residues are absent in the 24-amino acid sequence seen here, it is possible that the signal sequence is longer than 24 amino acids (up to 36 is known) and extends 5' beyond the available sequence to a methionine not contained within the cloned NADase cDNA.

Localization of the NADase

The in situ hybridization studies presented here and immunochemical studies from the companion paper indicate that the NADase mRNA transcript is produced primarily in immature eggs and that the translated protein is localized to vesicles or granules within ova of all stages. Ultrastructural studies of *Aplysia* ova at the electron microscope (EM) level (Coggeshall, 1970) have demonstrated the presence of only two types of granules or vesicles: small (1- to 2- μ m diam) lipid droplets and larger (5- to 10- μ m diam) membrane-bound vesicles characteristic of proteinaceous yolk granules. The vesicles, containing NADase, correspond in size to

the latter, but immunostaining at the EM level will be needed for positive identification. The signal sequence may play a role in the targeting of the NADase to these vesicles. The fact that the NADase is sequestered in this manner probably protects the ova from NAD depletion and allows the cell to control the release of the NADase.

Function of the egg-specific NADase

The companion paper presented data showing that the *Aplysia* NADase uses NAD as a substrate to produce cADPR and nicotinamide. The cADPR is a potent mobilizer of internal Ca^{2+} (Rusinko and Lee, 1989). This immediately suggests that the oocyte utilizes the NADase to release calcium, possibly for a series of calcium waves, such as those that occur after fertilization in the ova of the sea urchin *Lytechinus* (Poenie *et al.*, 1985). Although the exact function of the NADase is unclear, the cell must regulate it because of its potent activity. The NADase is located in vesicles or granules in the unfertilized egg, and this may prevent it from altering calcium levels. Further work will have to be done on the distribution of the NADase after fertilization, but presumably it is released from the vesicles. After release, the NADase activity would have to be regulated in some other manner than sequestration in vesicles. The guanylate cyclase of the sea urchin (*Arbacia*) spermatozoan is activated by phosphorylation and inactivated by dephosphorylation (Bentley *et al.*, 1986). Because it has two potential phosphorylation sites, the *Aplysia* NADase could be regulated in this manner.

The roles of other eukaryotic NADases in cellular functions have not been determined. Most of the mammalian NADase activity has been localized to macrophages and erythrocytes, where they have been shown to be ectoenzymes (Muller *et al.*, 1983; Amar-Costesec *et al.*, 1985; Kim *et al.*, 1988). These mammalian NADases and others should be reexamined in light of our reports to determine whether they are also capable of producing cADPR.

Methods

Amino acid sequencing

The NAD glycohydrolase from *Aplysia* ovotestis was purified as described by Hellmich and Strumwasser (1991). The *N*-terminal amino acid sequence was obtained by automated Edman degradation using an Applied Biosystems (Foster City, CA) Protein Sequencer. Internal peptides were obtained by denaturing the protein in 5 μ l of 8 M guanidine hydrochloride for 30 min at 50°C, diluting it with 15 μ l 0.2 M NH_4HCO_3 , and digesting it with (1/15 wt/wt enzyme/substrate) *Achromobacter* endoproteinase lys-C (Wako Chemicals, Osaka, Japan) for 5 h or subtilisin for 3 h at 37°C

(Vangrype et al., 1989; Riviere and Tempst, 1990). The peptide mixture was separated by HPLC using an Aquapore (Applied Biosystems, Foster City, CA) RP-300 column, and amino acid sequence of the purified peptides was determined by automatic Edman degradation (Tempst et al., 1990).

Construction of the *Aplysia ovotestis* cDNA library

Total ovotestis RNA was isolated by the use of the hot guanidinium isothiocyanate/phenol method of Feramisco et al. (1982). Poly(A)⁺ RNA was recovered by chromatography over oligo d(T) cellulose (Collaborative Research, Bedford, MA). Complementary cDNA libraries were generated from poly(A)⁺ RNA by random priming using the method of Gubler and Hoffman (1983). *NotI*/*EcoRI* adapters were ligated to the cDNA, which was then size selected by passing over a 2 ml Bio-Rad (Richmond, CA) A50 column. The cDNA was ligated into *EcoRI* cut, non-dephosphorylated lambda gt-10 phage arms (Stratagene, La Jolla, CA) and packaged by the use of Stratagene's Gigapack Gold packaging extract. Three libraries totaling ~10⁷ recombinants were produced.

Screening for a cDNA clone encoding the NADase

Oligonucleotide probe DG-1 was derived from amino acids 108–115, whereas probe DG-2 was derived from amino acids 42–48 (Figure 1). Both oligonucleotides utilized inosine to reduce degeneracy (Ohtsuka et al., 1985):

DG-1:

5' TA(T/C)GA(T/C)GA(A/G)GC(CA/T/G)GA(T/C)TA(T/C)GC 3'

DG-2: 5' TG(T/C)AA(A/G)GA(T/C)TA(T/C)GA(A/G)ATAC 3'

The oligonucleotides DG-1 and DG-2 were labeled at their 5' ends with T4 polynucleotide kinase and [γ -³²P]ATP. The ovotestis cDNA libraries were plated at a density of 40 000 plaques per 150-mm plate. After transfer of the plaques to nitrocellulose filters, the hybridizations were done overnight with DG-1 at 25°C, in 6× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7), 0.2% SDS, 10× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.2% bovine serum albumin) or with DG-2 at 20°C, in 9× SSC, 0.2% SDS, 10× Denhardt's solution. The filters were washed using 6× SSC, 0.5% SDS at 37°C (2 × 15 min each) for DG-1 and 9× SSC, 0.5% SDS at 27°C (2 × 15 min each) for DG-2.

DG-1 hybridizing plaques were rescreened by hybridizing overnight at 41°C in 6× SSC, 20% formamide, 50 mM sodium phosphate, pH 6.8, 1 mM sodium pyrophosphate, 5× Denhardt's, 50 µg/ml salmon sperm DNA and then washed at 41°C using 2× SSC, 0.1% SDS (2 × 20 min each). DG-2 hybridizing plaques were rescreened by hybridizing at 31°C under the same conditions as DG-1 and then washed at 31°C using 6× SSC, 0.1% SDS (3 × 10 min each).

Subcloning and sequencing

The inserts of the positive lambda clones were subcloned into M13mp18 and Bluescript KS[−] (Stratagene) in both orientations for DNA sequencing. A series of overlapping deletions was made in the Bluescript vector, containing the insert, using exonuclease III and S1 nuclease (Promega [Madison, WI] Erase-a-Base kit based on the paper by Henikoff, 1984).

Sequencing of the single-stranded M13 DNA and the double-stranded Bluescript deletions was performed with the use of [α -³⁵S]dATP and Sequenase (Version 2, United

States Biochemical, Cleveland, OH) with the M13 universal and reverse primers.

Northern blots

Poly A⁺ RNA was extracted from the ovotestis using the Fastrack Kit from Invitrogen (San Diego, CA). It was run in a 1% agarose gel containing 20 mM MOPS pH 7.5 and 0.7 M formaldehyde, and transferred to Hybond-N (Amersham, Arlington Heights, IL) by capillary action in 10× SSC. The NADase cDNA insert was agarose gel isolated after *EcoRI* digestion of the Bluescript plasmid in which it had been subcloned. It was labeled by the use of random priming and α -³²P-dCTP (Feinberg and Vogelstein, 1983) and hybridized to the transferred RNA overnight at 42°C in 5× Denhardt's solution, 5× SSC, 50 mM sodium phosphate, pH 6.8, 1 mM sodium pyrophosphate, 20% formamide, 60 µg/ml sonicated calf thymus DNA, and 1% SDS. A low-stringency wash of 2× SSC, 0.1% SDS at 55°C for 20 min was followed by higher stringency washes using 0.2× SSC, 0.1% SDS at 55°C for 15 min and finally 0.1× SSC, 0.1% SDS at 68°C for 25 min. Autoradiography was performed using Kodak (Rochester, NY) X-AR film.

In vitro translation

The Bluescript plasmid containing the cDNA insert (PKSG14) was linearized with *EcoRV* and transcribed in vitro with T7 RNA polymerase in the presence of m⁷G(5')ppp(5')G to produce sense RNA. The RNA was translated in a nuclease-treated reticulocyte lysate at a final salt concentration of 60 mM potassium acetate and 0.2 mM magnesium acetate in the presence of [³⁵S]methionine (3.5 µCi in 12.5 µl) and/or [³H]leucine (50 µCi in 25 µl) for 1 h at 30°C. Alternatively, in vitro translation was performed in a wheat germ extract (Promega) containing 80 mM potassium acetate for 1 h at 25°C.

The translation product was acetone precipitated and used either directly for protein sequence analysis or it was first purified by SDS-PAGE and then sequenced.

In situ hybridization

The in situ hybridization procedure was adapted from that of Angerer et al. (1987). *Aplysia* ovotestis was fixed in 4% paraformaldehyde, 100 mM sodium phosphate, pH 7.5; infiltrated with 25% sucrose; quickly frozen; and cut on a cryostat to provide 5-µm sections.

[³⁵S]-labeled sense (negative control) or antisense cRNA was transcribed with T7 or T3 RNA polymerase, respectively, from linearized plasmid DNA, containing the NADase cDNA insert, and added to the slide in 0.1 ml of hybridization solution such that the final concentration of the probe was 3 × 10³ cpm/µl. After 16 h of hybridization at 50°C, appropriate washes were performed and the sections were rinsed in 0.5 M NaCl and treated with RNase (25 µg/ml RNase A and 50 U/ml RNase T1) in 0.5 M NaCl for 15 min at 37°C. Final washes were 0.5× SSC, 20 mM 2-mercaptoethanol and then 0.1× SSC, 20 mM 2-mercaptoethanol for 15 min at 37°C. The slides were dehydrated in an ethanol series, dried under vacuum for 1 h, then coated with Kodak NTB-2 nuclear track emulsion and exposed.

Database searches

Comparisons between the amino acid sequence of the NADase and other known proteins were done using the Fastdb program from Intelligenetics Ig Suite. In addition, Dr. Russell Doolittle (University of California, San Diego) performed protein database searches utilizing his program Qscan.

Acknowledgments

This research was supported by grants from NIH (NS-21046 to F.S., NS-26760 to H.B.) and the Rockefeller Foundation (to F.S.). We thank Drs. R. Doolittle for protein database searches, J. Brosius and M. Miller for advice on screening cDNA libraries with oligonucleotides, A. Kuzirian for assistance with histology, N. Cornell for helpful discussions about NADases, D. Prasher, and H. Chikarmane for careful review of the manuscript.

Received: November 14, 1990.

Revised and accepted: January 18, 1991.

References

- Amar-Costesec, A., Prado-Figueroa, M. Beaufay, H., Nagelkerke, J.F., and Van Berkel, T.J.C. (1985). Analytical study of microsomes and isolated subcellular membranes from rat liver. IX. Nicotinamide adenine dinucleotide glycohydrolase: a plasma membrane enzyme prominently found in Kupffer cells. *J. Cell Biol.* 100, 189–197.
- Angerer, L.M., Cox, K.H., and Angerer, R.C. (1987). Demonstration of tissue-specific gene expression by in situ hybridization. In: *Guide to Molecular Cloning Techniques*, ed. S.L. Berger and A.R. Kimmel, New York: Academic Press, 649–661.
- Bause, E., and Hettkamp, H. (1979). Primary structural requirements for N-glycosylation of peptides in rat liver. *FEBS Lett.* 108, 341–344.
- Bentley, J.K., Tubb, D.J., and Garbers, D.L. (1986). Receptor-mediated activation of spermatozoan guanylate cyclase. *J. Biol. Chem.* 261, 14859–14862.
- Beushausen, S., Bergold, P., Stürner, S., Elste, A., Roytenberg, V., Schwartz, J.H., and Bayley, H. (1988). Two catalytic subunits of cAMP-dependent protein kinase generated by alternative RNA splicing are expressed in *Aplysia* neurons. *Neuron* 1, 853–864.
- Briggs, M.S., and Gierasch, L.M. (1986). Molecular mechanisms of protein secretion: the role of the signal sequence. In: *Advances in Protein Chemistry*, vol. 38, ed. C.B. Anfinsen, T.J. Edsall, and F.M. Richards, New York: Academic Press, 109–180.
- Coggeshall, R.E. (1970). A cytological analysis of the bag cell control of egg laying in *Aplysia*. *J. Morphol.* 132, 461–486.
- Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132, 6–13.
- Feramisco, F.R., Smart, J.E., Burridge, K., Helfman, D.M., and Thomas, G.P. (1982). Co-existence of vinculin and a vinculin-like protein of higher molecular weight in smooth muscle. *J. Biol. Chem.* 257, 11024–11031.
- Glass, D.B., and Krebs, E.G. (1980). Protein phosphorylation catalyzed by cyclic AMP-dependent and cyclic GMP-dependent protein kinases. In: *Annu. Rev. Pharmacol. Toxicol.* 20, ed. R. George, R. Okun, and A.K. Cho, Palo Alto: Annual Reviews, 363–388.
- Gubler, U., and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* 25, 263–269.
- Hellmich, M.R., and Strumwasser, F. (1991). Purification and characterization of a molluscan egg-specific NADase, a second-messenger enzyme. *Cell Regul.* 2, 193–202.
- Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* 28, 351–360.
- Kaplan, N.O. (1960). The pyridine coenzymes. In: *The Enzymes*, vol. 3, ed. P.D. Boyer, H. Lardy, and K. Myrback, New York: Academic Press, 105–169.
- Kim, U-H., Rockwood, S.F., and Daynes, R.A. (1988). Membrane-associated NAD glycohydrolase from rabbit erythrocytes is solubilized by phosphatidylinositol-specific phospholipase C. *Biochim. Biophys. Acta* 965, 76–81.
- Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157, 105–132.
- Muller, H.M., Muller, C.D., and Schuber, F. (1983). NAD glycohydrolase, an ectoenzyme of calf spleen cells. *Biochem. J.* 212, 459–464.
- Ohtsuka, E., Matsuki, S., Ikehara, M., Takahashi, Y., and Matsubara, K. (1985). An alternate approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. *J. Biol. Chem.* 260, 2605–2608.
- Pekala, P.H., and Anderson, B.M. (1982). Non oxidation-reduction reactions of pyridine nucleotides. In: *The Pyridine Nucleotide Coenzymes*, ed. J. Everse, B.M. Anderson, and K.S. You, New York: Academic Press, 326–377.
- Poenie, M., Alderton, J., Tsien, R.Y., and Steinhardt, R.A. (1985). Changes of free calcium levels with stages of the cell division cycle. *Nature* 315, 147–149.
- Riviere, L., and Tempst, P. (1990). Enzymatic endoproteolysis in the presence of detergents and chaotropic agents. *J. Protein Chem.* 9, 295–296.
- Rusinko, N., and Lee, H.C. (1989). Widespread occurrence in animal tissues of an enzyme catalyzing the conversion of NAD into a cyclic metabolite with intracellular Ca^{++} -mobilizing activity. *J. Biol. Chem.* 260, 11725–11731.
- Taylor, S.S., Buechler, J.A., and Yonemoto, W. (1990). cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. In: *Annu. Rev. Biochem.*, vol. 59, ed. C.C. Richardson, J.N. Abelson, P.D. Boyer, and A. Meister, Palo Alto: Annual Reviews, 971–1006.
- Tempst, P., Link, A., Riviere, L., Fleming, M., and Elicone, C. (1990). Internal sequence analysis of proteins separated on polyacrylamide gels at the sub microgram level: improved methods, applications, and gene cloning strategies. *Electrophoresis* 11, 537–553.
- Vangrype, W., Ampe, C., Kerssters-Hilderson, H., and Tempst, P. (1989). Single active-site histidine in D-xylose isomerase from *Streptomyces violaceoruber*. *Biochem. J.* 263, 195–199.
- Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982). Distantly related sequences in the α - and β -subunits of ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* 1, 945–951.
- Wierenga, R.K., Terpstra, P., and Hol, G.J.W. (1986). Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$ -fold in proteins using an amino acid sequence fingerprint. *J. Mol. Biol.* 187, 101–107.