THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 35, Issue of August 29, pp. 33056–33059, 2003
Printed in U.S.A.

The Reported Human NADsyn2 Is Ammonia-dependent NAD Synthetase from a Pseudomonad*

Received for publication, March 5, 2003, and in revised form, May 29, 2003
Published, JBC Papers in Press, May 29, 2003, DOI 10.1074/jbc.M302276200

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Nicotinamide-adenine dinucleotide (NAD^+) synthetases catalyze the last step in NAD^+ metabolism in the *de novo*, import, and salvage pathways that originate from tryptophan (or aspartic acid), nicotinic acid, and nicotinamide, respectively, and converge on nicotinic acid mononucleotide. NAD^+ synthetase converts nicotinic acid adenine dinucleotide to NAD^+ via an adenylylated intermediate. All of the known eukaryotic NAD^+ synthetases are glutamine-dependent, hydrolyzing glutamine to glutamic acid to provide the attacking ammonia. In the prokaryotic world, some NAD^+ synthetases are glutamine-dependent, whereas others can only use ammonia. Earlier, we noted a perfect correlation between presence of a domain related to nitrilase and glutamine dependence and then proved in the accompanying paper (Bieganowski, P., Pace, H. C., and Brenner, C. (2003) *J. Biol. Chem.* 278, 33049–33055) that the nitrilase-related domain is an essential, obligate intramolecular, thiol-dependent glutamine amidotransferase in the yeast NAD^+ synthetase, Qns1. Independently, human NAD^+ synthetase was cloned and shown to depend on Cys-175 for glutamine-dependent but not ammonia-dependent NAD^+ synthetase activity. Additionally, it was claimed that a 275 amino acid open reading frame putatively amplified from human glioma cell line LN229 encodes a human ammonia-dependent NAD^+ synthetase and this was speculated largely to mediate NAD^+ synthesis in human muscle tissues. Here we establish that the so-called NADsyn2 is simply ammonia-dependent NAD^+ synthetase from *Pseudomonas*, which is encoded on an operon with nicotinic acid phosphoribosyltransferase and, in some *Pseudomonads*, with nicotinamidase.

NAD^+ is essential as a co-enzyme for oxidation and reduction reactions and as a substrate for NAD^+-consuming enzymes such as the Sir2-related lysine deacetylases, the poly(ADP-ribose) polymerases, and the cyclic ADP-ribose synthetases (1, 2). Because the reaction catalyzed (amidation of nicotinic acid adenine dinucleotide) is the final common step in NAD^+ biosynthesis from the *de novo*, import, and salvage pathways, NAD^+ synthetase is an essential enzyme in yeast (3). Eukaryotic NAD^+ synthetase was first characterized 45 years ago in extracts from yeast and human cells and shown to use glutamine as the ammonia source and to work in an ATP-Mg^{2+}-dependent manner through an adenylylated nicotinic acid adenine dinucleotide intermediate (4, 5). Surprisingly, *Escherichia coli* NAD^+ synthetase (6) and *Bacillis subtilis* NAD^+ synthetase (7) cannot use glutamine while *Mycobacterium tuberculosis* NAD^+ synthetase can use glutamine (8). Although the N terminus of *M. tuberculosis* NAD^+ synthetase was observed to be extended with respect to the ammonia-dependent NAD^+ synthetases by ~300 amino acids, no similarity was observed to any known glutamine amidotransferase domain (8, 9). Two years ago, we classified the nitrilase superfamily into 13 branches, most of which consist of amidases of various specificities, and observed that presence of a nitrilase-related domain correlates with glutamine dependence in prokaryotic NAD^+ synthetases and is found in all of the eukaryotic NAD^+ synthetases (10). Thus, we made the explicit prediction that the nitrilase-related domain accounts for glutamine dependence by serving as the glutamine amidotransferase domain for the associated NAD^+ synthetase (10, 11).

Cloning of the yeast Gln-dependent *N*AD^+ *s*ynthetase, Qns1, allowed us to show that Cys-175 is required for glutaminase activity and glutamine-dependent NAD synthetase activity *in vitro* (3). *In vivo* experiments established that neither the active-site mutants in the NAD^+ synthetase domain nor in the glutaminase domain allow physiological function despite the residual ammonia-dependent activity of the latter mutants (3). Moreover, genetic experiments showed that the ammonia released from the glutaminase active site follows an obligate intramolecular path to the pyridine-nucleotide substrate bound at the other active site (3). Without reference to structures (12, 13) and classification of enzymes in the nitrilase superfamily including glutamine-dependent NAD^+ synthetase (10, 11), Tsuchiya and co-workers (14) purified human Qns1, which they term NADsyn1, from COS7 cells and report that glutamine-dependent but not ammonia-dependent NAD^+ synthetase activity depends on Cys-175. Surprisingly, Tsuchiya and co-workers (14) claim that a second glutamine-independent NAD^+ synthetase they term human NADsyn2 is expressed in skeletal muscle and heart and that this enzyme may largely mediate NAD^+ synthesis in these tissues. Here we show that NADsyn2 is an ammonia-dependent NAD^+ synthetase of *Pseudomonal* origin, which is encoded on an operon with nicotinic acid phosphoribosyltransferase and, in some *Pseudomonads*, with nicotinamidase.

EXPERIMENTAL PROCEDURES

Nucleic Acid and Protein Sequence Analyses—DNA sequence searches were performed with MEGA-BLAST (15) against all of the non-redundant data available at the National Center for Biotechnology Information and the Sanger Center. Protein sequence homologs were identified using BLASTP without position specific iteration (15). Neighboring genes were located in the ERGO database (16). Phylogenetic analysis of amino acid sequences was performed with PHYLIP (17).

* This work was supported by Research Grant CA77738 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviation used is: NAD^+, nicotinamide-adenine dinucleotide.

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                .MetThrAlaCysAsnGlyGlnProValAlaLysIleSerAspGluAlaGlyLysThrHisCysThrAspProAsnPheValAlaTyrLeu
HSA236685       AATGACCGCCTGTAATGGCCAGCCCGTCGCAAAGATCTCTGATGAAGCGGGCAAGACCCACTGCACCGATCCGAACTTCGTCGCTTATTTG
P.fluorescens AATGACCGCCTGCAATGGCCAGCCCGTCGCGAAGATCTCCGATGAAGCGGGCAAGACCCACTGCACCGACCCGAATTCGTCGCCTATTTG

ArgHisValPheLysValProAlaLeuProSerLysGluSTP~~~MetGlnAlaValGlnArgGluIleAlaGluGlnLeuLysValGlnProProPheLysAsp
CGCCACGTTTTCAAAGTACCTGCCCTACCCAGCAAGGAGTGAATCATGCAAGCCGTACAGCGCGAGATTGCTGAACAGCTCAAGGTCCAACCGCCGTTCAAGGAC
CGTCATGTTTTCAAAGTACCTGCCATCTCTAGCAAGGAGTGAATCATGCAAGCCGTACAGCGTGAGATTGCTGAACAGCTCAAGGTCCAAGCGCCGTTTAACGAC

GlnAlaAlaLeuGluAlaGluValAlaArgArgValThrPheIleGlnAspCysLeuLeuAsnSerGlyLeuLysThrLeuValLeuGlyIleSerGlyGlyVal
CAGGCCGCCCTTGAGGCGGAGGTCGCCCGGCGCGTTACCTTTATCCAGGACTGCCTGCTCAATTCAGGCCTCAAGACGTTGGTGCTGGGCATCAGCGGCGGCGTC
CAGGCCGCCCTTGAGGCCGAGGTTGCCCGCCGCGTGACCTTTATCCAGGATTGCCTGCGCAATTCCGGGCTCAAAACGTTGGTGCTGGGCATCAGTGGTGGCGTC

AspSerLeuThrAlaGlyLeuLeuAlaGlnArgAlaMetGluGluLeuArgAlaAlaLysGlyAspGlyAlaTyrArgPheIleAlaValArgLeuProTyrGlu
GACTCGCTGACCGCCGGCCTGCTGGCCCAGCGTGCGATGGAAGAGTTGCGGGCCGCCAAGGGCGACGGCGCTTACCGCTTTATCGCGGTGCGCCTGCCCTACGAA
GACTCCCTGACTGCCGGCCTGCTCGCCCAACGCGCGATGCAGGAACTGCGCGCCAGCACCGGTGATGAGGCCTACCGTTTTATCGCCGTGCGCCTGCCCTACGAA

ThrGlnPheAspGluHisAspAlaGlnAlaSerValAspPheIleGluProAspGluArgHisThrValAsnIleGlyProAlaValLysSerLeuAlaAsnGlu
ACCCAGTTCGACGAACACGATGCCCAAGCGTCCGTGGACTTTATCGAACCCGACGAGCGCCACACGGTGAACATCGGCCCGGCGGTGAAATCCCTGGCCAACGAA
ACCCAGTTCGATGAGCTCGACGCCCAGGCCTCGGTGGACTTTATCGAGCCCGACGAGCGTCACACCGTGAACATCGGCCCAGCGGTGAAAGCCCTGGCCAATGAA

ValAlaAlaPheGluGlyLysAlaAlaValSerArgAspPheValLeuGlyAsnThrLysAlaArgMetArgMetValAlaGlnTyrThrIleAlaGlyAlaAla
GTGGCGGCGTTTGAAGGCAAGGCCGCGGTCTCCCGGGACTTCGTGCTGGGCAACACGAAGGCGCGCATGCGCATGGTGGCGCAGTACACCATCGCCGGCGCGGCC
GTGGCGGCGTTCGAAGGCAAGGCGCGGTGTCCCGCGATTTCGTGCTGGGCAACACCAAGGCGCGCATGCGCATGGTGGCGCAGTACACCATCGCGGGCGCGGCG

SerGlyLeuValIleGlyThrAspHisAlaAlaGluAlaValMetGlyPhePheThrLysPheGlyAspGlyAlaCysAspLeuAlaProLeuSerGlyLeuVal
AGTGGTTTGGTGATTGGTACCGACCATGCGGCGGAAGCGGTGATGGGCTTTTTCACCAAGTTCGGTGATGGCGCCTGCGACCTGGCGCCGCTGAGCGGGCTGGTG
GGTGGGCTGGTGATCGGTACCGACCATGCGGCGGAAGCGGTGATGGGTTTTTTCACCAAGTTCGGTGATGGGCCTGCGATTTGGCACCGTTGAGCGGGCTGGTG

LysAsnGlnValArgAlaIleAlaArgHisPheGlyAlaProGluSerLeuValGluLysIleProThrAlaAspLeuGluAspLeuSerProGlyLysProAsp
AAGAACCAGGTGCGCGCGATTGCCCGGCACTTTGGTGCACCGGAATCGTTGGTGGAGAAAATCCCGACGGCCGACCTGGAAGACCTCTCGCCCGGGCAAGCCGGAC
AAGAACCAGGTGCGTGCGATTGCACGGCACTTTGGGGCGCCGGAGTCGCTGGTGGAGAAGGTTCCGACGGCCGACCTGGAAGACTTGTCACCGGGCAAGCCGGAT

GluAlaSerHisGlyValThrTyrAlaGluIleAspAlaPheLeuHisGlyGluProValArgGluGluAlaPheArgIleIleCysAspThrTyrArgLysThr
GAAGCGTCCCATGGCGTGACCTATGCCGAGATCGATGCGTTCCTGCACGGCGAGCCGGTGCGCGAGGAAGCGTTCCGGATCATTTGTGACACCTATCGCAAGACC
GAAGCGTCCCATGGCGTGACCTACGCCGAGATCGATGCCTTTCTGCACGGTGAGCCGGTGCGTGAGGAAGCGTTCAAGATTATCTGCGAGACGTACCGCAAGACT

GluHisLysArgValMetProPheAlaProSTP
GAGCACAAGCGGGTGATGCCGTTTGCACCCTGAGGT-AACTCAGCAGCTCATTGTGGCAAAAGGGCTTGTTGTGGTG
GAGCACAAGCGGGTCATGCCGTTTGCGCCGTGAGGTGAAC-CAG
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FIG. 1. **HSA236685 shows full-length similarity to a genomic fragment from *P. fluorescens* strain SBW25.** HSA236685 was used as the genetic information used to clone human NADsyn2. Here, the 1007-nucleotide sequence of HSA236685 is aligned with a 975-nucleotide fragment from the unfinished *P. fluorescens* SBW25 sequencing project. The translation (*frame 2*) of HSA236685 is provided. Amino acids and nucleotides that differ are in *boldface*. The stop codon of the first predicted polypeptide and the start and stop codons of the second polypeptide are *underlined*.

RESULTS AND DISCUSSION

DNA Sequences from a Human Chromosome 22 CpG Library Do Not Consist Entirely of Chromosome 22 cDNAs—In their attempt to clone human NAD synthetase(s), Tsuchiya and co-workers (14) interrogated the public nucleic acid repositories with the sequence of *B. subtilis* NAD^+ synthetase (14). One of two sequences they identified as encoding an NAD^+ synthetase homolog (GenBank™ accession number HSA236685) had been deposited as a 1007-nucleotide sequence from a CpG island library prepared from flow-sorted human chromosome 22 (18). Ninety-nine clones from the chromosome 22 library had been sequenced. The authors reported that the majority represented known genes or sequences found among human expressed sequences (18). Additional clones represented chromosome 22 genomic DNA, human repetitive DNA, or Epstein-Barr viral DNA. These investigators were careful to point out that three clones from a similarly prepared chromosome 18 library matched *E. coli* sequences (18). Four clones including HSA236685 provided no match to any sequence, and no warrantee was expressed about the "humanity" or expression of this deposition (18).

Conceptual translation of a 275 amino acid open reading frame from HSA236685 produces a polypeptide that is homologous to *B. subtilis* NAD^+ synthetase. Tsuchiya and co-workers (14) designed primers to amplify this sequence by reverse transcriptase-PCR from human glioma cell line LN229 and report cloning of a "human cDNA" that encodes a polypeptide with 271 of 275 identities to that encoded by HSA236685. The sequence was deposited in GenBank™ with accession number AB091317. Despite noting that there is no evidence for expression of this molecule from the extensive expressed sequence tag databases and with no Southern data or location of the gene in a human or other animal genomic assembly, the clone was termed "human NADsyn2 cDNA" and claimed to encode the first strictly ammonia-dependent NAD^+ synthetase in eukaryotes (14). In fact, what was deposited has no feature diagnostic of a cDNA, such as an untranslated leader or sequences 3′ of the stop codon terminating in poly(A). Surprised by the claim of a human ammonia-dependent NAD synthetase without a genomic clone for such a sequence from any eukaryote, we performed numerous searches through the publicly accessible databases. Because Tsuchiya and co-workers (14) did not identify a genomic DNA fragment encoding their "human NADsyn2," we examined the 1007-nucleotide sequence read of HSA236685 and found an 88% identical match (Fig. 1) over a common length of 975 nucleotides (864 of 975 nucleotides; p value = 6.2×10^{-170}) with the partially assembled genome of *Pseudomonas fluorescens* SBW25 (www.sanger.ac.uk/Projects/P_fluorescens/). Thus, independent of coding potential, AB091317 appears to be of *Pseudomonal* origin.

HSA236685 Represents a Fragment of an Operon Containing pncB and nadE and Possibly pncA Orthologs—Tsuchiya and co-workers never published genomic or transcript sequences 5′ to the initiator of "human NADsyn2." However, they did state that the NADsyn2 sequence contains an in-frame stop codon upstream of the initiator methionine in the same location as HSA236685 and encodes a polypeptide with 98.5% identity to that encoded by HSA236685 (14). As Tsuchiya and co-workers referred to the HSA236685 clone as a fragment of human genomic DNA, we examined the coding potential of the 136

nucleotides of HSA236685 preceding the NAD^+ synthetase initiator codon. Immediately 5′ of the NAD^+ synthetase initiator codon, we discovered an uninterrupted run of 43 amino acids followed by a TGA stop codon and one ATC codon. A BLASTP search (15) of nonredundant peptide data revealed that the 43 amino acid segment is the C terminus of nicotinic acid phosphoribosyltransferase (EC 2.4.2.11), the first enzyme in the Preiss-Handler pathway of NAD^+ biosynthesis from nicotinic acid (NAD^+ synthetase (EC 6.3.5.1) is the last) (4, 5). We then searched the ERGO database of completely and partially assembled genomes (16) to examine whether nicotinic acid phosphoribosyltransferase genes (bacterial orthologs usually termed *pncB*) have been found clustered with NAD^+ synthetase genes (bacterial orthologs usually termed *nadE*). Indeed, as shown in Fig. 2, we found that *pncB* orthologs are the immediate upstream cistron with respect to *nadE* orthologs in *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus gasseri*, *Staphylococcus aureus*, *Staphlococcus epidermidis, Streptococcus equi*, *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. Moreover, we found that a *pncA* ortholog encoding nicotinamidase (EC 3.5.1.19), which salvages nicotinamide to nicotinic acid for its subsequent use in the Preiss-Handler pathway, is located first in a three cistron operon in *Pseudomonas aeruginosa* (19). At the time of consulting the ERGO database, 513 complete and partial genomes were included, including 93 eukaryotic genomes (16). No eukaryotic NAD^+ synthetase homolog was found without an N-terminal nitrilase-related domain, and no NAD^+ synthetase gene was found in a eukaryote in an operon with genes for nicotinic acid phosphoribosyltransferase or nicotinamidase. Thus, the genomic organization of Tsuchiya's NADsyn2 is bacterial and highly typical of a *Pseudomomad*.

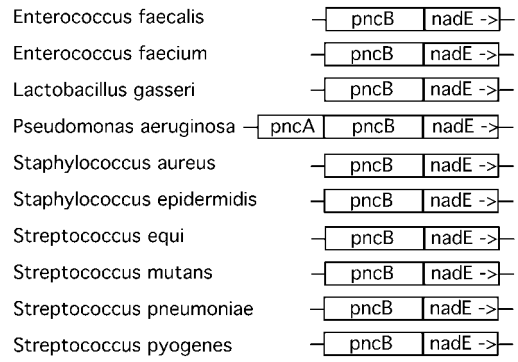

FIG. 2. ***pncB* and *nadE* homologs, which are in an apparent operon in HSA236685, occur in the same organization exclusively in related bacterial genomes.** Genome organization of *pncB* (nicotinic acid phosphoribosyltransferase), and *nadE* (ammonia-dependent NAD synthetase) cistrons in *E. faecalis*, *E. faecium*, *L. gasseri*, *P. aeruginosa*, *S. aureus*, *S. epidermidis, S. equi*, *S. mutans*, *S. pneumoniae*, and *S. pyogenes*. As with the sequence of HSA236685 and the fragment from *P. fluorescens* SBW25, *pncB* and *nadE* sequences are arranged as consecutive cistrons in these genomes. In *P. aeruginosa*, *pncA* (nicotinamidase) is in the operon as well.

NADsyn2 and Its Associated pncB Ortholog Are Pseudomonal Enzymes—Whereas the ERGO search confirmed that single domain NAD^+ synthetases have not been found in eukaryotes, we thought it important to confirm the *Pseudomonal* origin of the HSA236685 genes on the basis of amino acid sequence conservation, independent of the genomic data presented above. BLASTing the 43 amino acid C terminus of the HSA236685 *pncB*, we obtained hits against predicted polypeptides annotated either as "hypothetical protein" or as "nicotinate phosphoribosyltransferase." In descending order of significance, the genomes from which these sequences were derived were *P. fluorescens*, *Pseudomonas syringae*, *Pseudomonas putida*, and *P. aeruginosa* followed by three related γ-proteobacteria and three enterobacteria. Taking the BLASTP results to diminished E-values, a few β-proteobacteria were identified but nothing resembling a eukaryotic sequence was identified. It should be noted that the power of this BLASTP search was limited by the 43 amino acid probe, such that eukaryotic *pncB* homologs were not identified. The shortness of the query sequence served as a high stringency test for the origin of the *pncB* ortholog from HSA236685.

Tsuchiya and co-workers (14) provided the predicted amino acid sequence of "human NADsyn2." We submitted the sequence to a BLASTP search and discovered that its top four hits were from precisely the same *Pseudomonads* that matched the *pncB* cistron upstream of the *nadE* cistron on HSA236685. Although the NADsyn2 sequence is not a perfect match with *P. fluorescens*, it is demonstrably more similar to *nadE* of *P. fluorescens* than to *P. syringae*, other *Pseudomonads*, or other organisms within the huge eubacterial domain. The PHYLIP program (17) was used to display the phylogenetic inferences that emerge from multiple sequence alignment of NADsyn2, the *nadE* homolog obtained by conceptual translation of HSA236685, and eight *nadE* homologs from our BLASTP search. As shown in Fig. 3, an unrooted phylogenetic tree clearly locates HSA236685 and NADsyn2 as deriving from two strains of the same unidentified species of *Pseudomonas*. In this analysis, genus *Pseudomonas* was clearly separated from the other γ-proteobacteria. As other more distantly related *nadE* homologs were added to these alignments, it was

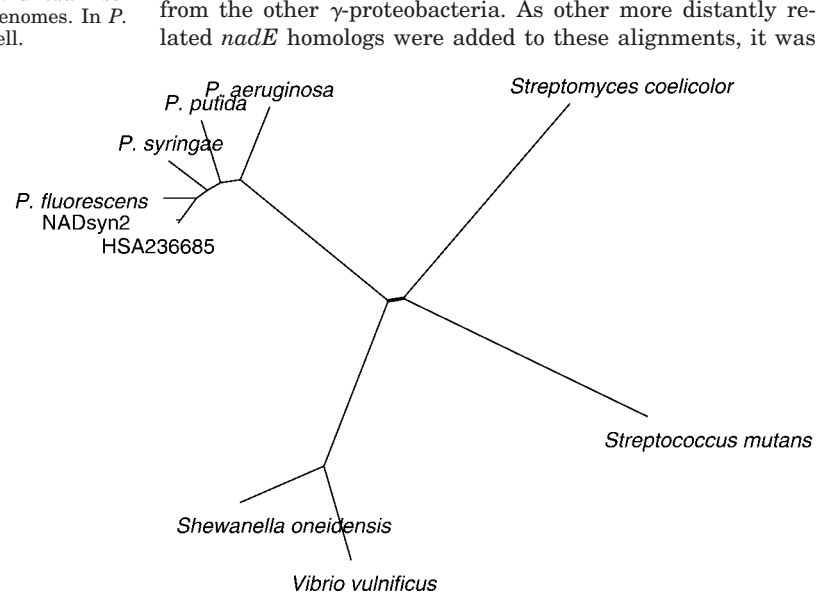

FIG. 3. **Phylogenetic analysis shows that HSA236685 and NADsyn2 *nadE* homologs are *Pseudomonal*.** The top eight unique *nadE*-homologous sequences pulled out by BLASTP were aligned and submitted to phylogenetic analysis with PHYLIP. A nonrooted tree identified HSA236685 and NADsyn2 as encoded by two related strains of genus *Pseudomonas* in a species closer to *fluorescens* than to other *Pseudomonads*.

possible to construct phylogenies that included more of the bacterial domain and, eventually, archaea and eukaryotes. Not surprisingly, vertebrate sequences were tightly grouped extremely far apart from the γ-proteobacteria (data not shown).

CONCLUSION

Tsuchiya and co-workers (14) amplified "human NADsyn2" from human cell lines using primers designed to amplify an ammonia-dependent NAD^+ synthetase gene they spotted in GenBank™ deposited sequence HSA236685 (14). No data were presented, suggesting that the NADsyn2 clone required reverse transcriptase for amplification (14). The NADsyn2 clone is absent from all of the human expression databases (14) and genomic assemblies, whereas DNA sequence homologs with *p* values as low as 6.2×10^{-170} are found in *Pseudomonal* and other bacterial genomic assemblies (this work). *Pseudomonas* species are well known laboratory contaminants capable of growing on disinfectants such as benzalkonium chloride as their sole carbon source (20). Northern blotting of murine tissues with a NADsyn2 probe showed extremely weak hybridization that was interpreted as specific signals in heart, skeletal muscle, and other tissues, whereas robust "expression" was seen in four human cell lines (14). Our interpretations are cross-hybridization and *Pseudomonal* contamination, respectively. Clone HSA236685, which had been isolated by enrichment of human chromosome 22 CpG islands, was reported to be a rare clone with no match to human nucleic acids. Three clones from a similarly constructed chromosome 18 library were reported to match *E. coli* sequences (18). Here we reported that HSA236685 is full-length homologous to a fragment of the unassembled genome of *P. fluorescens*. Moreover, HSA236685 is a fragment of an operon that is found in *E. faecalis*, *E. faecium*, *L. gasseri*, *S. aureus*, *S. epidermidis, S. equi*, *S. mutans*, *S. pneumoniae*, and *S. pyogenes* consisting of *pncB* followed by *nadE* cistrons. In *P. aeruginosa*, the operon consists of *pncA*, *pncB*, and *nadE*, such that nicotinamidase, nicotinic acid phosphoribosyltransferase, and NAD synthetase are coordinately expressed. In contrast, no such chromosomal organization has been found in any animal. Both the *pncB* fragment from HSA236685 and the reported sequence of NADsyn2 are more similar to homologous enzymes in four species of *Pseudomonas* than they are to enzymes from other γ-proteobacteria or any other form of life. Finally, the biochemical properties and the domain structure of NADsyn2 (14) are consistent with those of bacterial ammonia-dependent NAD synthetase (6) and inconsistent with those of any eukaryotic NAD synthetase (3–5,10). The simplest conclusion is that NADsyn2 is a typical *Pseudomonal* ammonia-dependent NAD synthetase.

Acknowledgment—We thank Andrei Osterman for helpful discussion and guidance with the ERGO database.

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