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Orf186 Represents a New Member of the Nudix Hydrolases, Active on Adenosine(5′)triphospho(5′)adenosine, ADP-ribose, and NADH*

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***orf186*, a new member of the Nudix hydrolase family of genes, has been cloned and expressed, and the protein has been purified and identified as an enzyme highly specific for compounds of ADP. Its three major substrates are adenosine(5′)triphospho(5′)adenosine, ADP-ribose, and NADH, all implicated in a variety of cellular regulatory processes, supporting the notion that the function of the Nudix hydrolases is to monitor the concentrations of reactive nucleoside diphosphate derivatives and to help modulate their accumulation during cellular metabolism.**

The Nudix hydrolases are a family of widely distributed enzymes (1) characterized by a structural motif, the Nudix box, having the highly conserved consensus sequence

G*XXXXX***E***XXXXXXXX***REU***X***EE***X***GU**

SEQUENCE 1

where U represents one of the bulky hydrophobic amino acids, usually I, L, or V. Based on predictive (2) and structural (3) studies, the Nudix box designates a unique loop-helix-loop motif, involved in the binding of the substrate (4) and in catalysis (5, 6). Searches of the protein data banks have uncovered over 100 putative proteins containing the Nudix structural motif, within all three kingdoms, the prokaryota, archaea, and eukaryota, in organisms ranging in complexity from viruses to humans. To date, about 20 of these proteins have been identified as enzymes, and their substrates include nucleoside triphosphates, co-enzymes, sugar nucleotides, and dinucleoside polyphosphates. Although seemingly diverse, these compounds share a common structural feature. They are all **nu**cleoside **di**phosphates linked to some other moiety, ***x***; hence the acronym, Nudix (1). In addition to the structural similarity of these substrates, they are all either potentially toxic or deleterious compounds, cell signaling molecules, regulators, or metabolic intermediates whose concentrations require modulation during the vicissitudes of the cellular environment.

The subject of this paper, Orf186, was identified in a BLAST search (7) as a putative 186-amino acid protein, locus YRFE, in *Escherichia coli* (GenBank accession no. P45799). Herein, we describe the cloning and expression of the *orf186* gene, and the purification and initial characterization of the resulting protein. Orf186 is a novel enzyme, contains the Nudix box, and hydrolyzes the sensitive metabolic intermediates, adenosine-(5′)triphospho(5′)adenosine (Ap_3A),[1] ADP-ribose, and NADH, all derivatives of the nucleoside diphosphate, ADP. Thus, Orf186 is a new member of the Nudix hydrolase family.

EXPERIMENTAL PROCEDURES

Materials

Primers were obtained from Integrated DNA Technologies (Coralville, IA). Biochemicals and enzymes were purchased from Sigma unless otherwise noted. *E. coli* strain MG1655 was kindly provided by Dr. Frederick R. Blattner (University of Wisconsin). Other common organisms and vectors were from laboratory stocks.

Methods

Cloning—The *orf186* gene was amplified from *E. coli* strain MG1655 chromosomal DNA using the polymerase chain reaction. The oligonucleotides d(GCGCGCGGTACCGATGAGCAAATCATTACAAAAACC) and d(GCGCGCGGATCCTTACACTCGCCCCTGCCC) were used as primers to amplify the gene and incorporate a *Kpn*I restriction site at the start of the gene and a *Bam*HI restriction site at the end of the gene. The amplified gene was purified by agarose gel electrophoresis, digested with *Kpn*I and *Bam*HI, and ligated into the *Kpn*I and *Bam*HI restriction sites of plasmid pTRC99A. The resulting plasmid pTRCorf186 was used to transform *E. coli* strain HB101.

The *orf186* gene was subcloned from pTRCorf186 using a modification (8) of the megaprimer method (9) for site-directed mutagenesis. The oligonucleotides d(GGCCGCTGGCGCACATGATGGATTTG) and d(GCCAGGCAAATTCTG) were used to create a megaprimer containing a silent mutation of T to C at position 462 of *orf186*, thereby eliminating the *Nde*I site in the *orf186* gene but retaining the histidine encoded for at this position. The purified megaprimer and the oligonucleotide d(GCGCGCGCATATGAGCAAATCATTACAAAAACC) were used as primers to amplify the *orf186* gene, incorporating the mutation described above, as well as an *Nde*I site at the start of the gene, and a *Bam*HI site at its end. The amplified gene was digested with *Nde*I and *Bam*HI restriction enzymes and ligated into the *Nde*I and *Bam*HI restriction sites of plasmid pET11b to place the *orf186* gene under control of a T7 *lac* promoter for expression. The resulting plasmid, pETorf186(mut462) was used to transform *E. coli* strain DH5α for storage and *E. coli* strain BL21(DE3) for expression.

Purification of the Enzyme—E. coli strain BL21(DE3) containing pETorf186(mut462) was grown at 37 °C in three stages. A single colony was inoculated into 10 ml of LB medium containing 100 μg/ml ampicillin and grown to an A_{600} of 0.4. The cells were collected by centrifugation, washed with 0.9% NaCl, and added to 100 ml of fresh LB medium plus ampicillin. These cells were grown to an A_{600} of 0.6, collected and washed as above, and added to 2 liters of fresh medium plus ampicillin. When growth reached an A_{600} of 0.6, the culture was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown for an additional 2 h.

The induced cells were harvested, washed by suspension in a buffered saline solution, resuspended in 2 volumes of buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol), sonicated, centrifuged, and the precipitate was discarded. The protein concentration of the super-

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[1] The abbreviations used are: Ap_3A, adenosine(5′)triphospho(5′)adenosine; Ap_2A, adenosine(5′)diphospho(5′)adenosine; Ap_4A, adenosine(5′)tetraphospho(5′)adenosine.

natant was adjusted to 10 mg/ml with buffer A (Fraction I). A typical preparation yielded approximately 200 mg of protein from 3 g of cells.

To Fraction I was added 0.1 volume of 10% streptomycin sulfate, and after 15 min on ice, the precipitate was discarded. Approximately 60% of the enzymatic activity remained in the supernatant. The concentration of streptomycin was raised to 2% with 25% streptomycin sulfate, and this solution was stored on ice overnight. The precipitate was collected and dissolved in 4 ml of buffer A containing 10% ammonium sulfate (Fraction II). Fraction II was loaded onto a 2.5 × 60-cm column of Sephadex G100 and eluted with buffer A containing 50 mM NaCl. The fractions containing the bulk of the enzyme were combined (Fraction III), and concentrated by precipitation in 45% ammonium sulfate (Fraction IV). The purified enzyme was stable for months when stored at −80 °C.

Enzyme Assay—In the standard assay, the hydrolysis of Ap_3A to AMP and ADP was measured by converting the products to P_i and adenosine with calf intestinal phosphatase. The reaction mixture contained (in 50 μl): 2 mM Ap_3A, 50 mM Tris, pH 9, 0.5 mM $MgCl_2$, 1% glycerol, 0.1 mg/ml bovine serum albumin, 0.2 unit of calf intestinal phosphatase, and 0–1 milliunit of enzyme. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 250 μl of 4 mM EDTA. The P_i produced was measured by the colorimetric method of Ames and Dubin (10). A unit of Orf186 cleaves 1 μmol of substrate/min.

The identity of the products and the stoichiometry of the reaction were analyzed by paper electrophoresis (11) and high performance liquid chromatography in scaled-up standard reaction mixtures, from which calf intestinal phosphatase was omitted.

RESULTS

Subcloning, Expression, and Purification

Elimination of the *Nde*I restriction site in the wild type *orf186* gene, by making a silent mutation at position 462, enabled us to place *orf186* between the *Nde*I and *Bam*HI sites of pET11b, directly down stream from a ribosome binding site and under control of a T7 *lac* promoter for optimal expression. The sequence of the cloned gene agrees with that reported for *orf186* (accession no. P45799), except for the engineered T → C transition at position 462 (data not shown).

The pETorf186 plasmid was unstable in *E. coli* strain BL21(DE3) when the culture was grown to high cell density, presumably due to a selective disadvantage upon depletion of ampicillin. Therefore, the cultures were grown stepwise, centrifuged, and suspended in fresh medium containing ampicillin, as described under "Experimental Procedures." With this procedure, virtually 100% of the cells retained the plasmid, as determined by plating aliquots on selective agar.

Expression of the gene results in the appearance of a major band on a denaturing gel (Fig. 1), corresponding to a 21.5-kDa protein not detectable in the strain containing pET11b without the insert. When the crude extract is brought to a concentration of 1% streptomycin sulfate to remove nucleic acids, approximately 40% of the enzyme precipitates and 60% remains in solution. Increasing the concentration of streptomycin in the supernatant to 2% results in a quantitative precipitation of the remainder of the enzyme, while leaving many of the other proteins behind. Subsequent fractionation by gel filtration and concentration by ammonium sulfate precipitation led to a preparation substantially free of contaminating proteins (Fig. 1). Orf186 migrated on the SDS gel as expected for the 21-kDa polypeptide predicted from its amino acid composition. However, when chromatographed on a pre-calibrated gel filtration column, it appeared as a symmetrical peak in a region expected for a 43-kDa protein. Thus, it is quite possible that the enzyme exists as a homodimer in its native state; however, at present, no further work has been done to investigate this issue.

Properties of the Enzyme

Requirements of the Reaction—The enzyme has optimal activity at a distinctly alkaline pH. In Tris buffer, the maximum rate of hydrolysis for Ap_3A is at pH 9.0, with the rate falling to <50% at pH 7.5. In glycine buffer, the maximum is at pH 10 with a 50% reduction in rate at pH 8. This alkaline pH optimum is typical of four other Nudix hydrolases we have characterized, namely MutT dGTPase, pH 9 (12); NADH pyrophosphatase, pH 8.5 (13); GDP-mannose hydrolase, pH 9.3 (14); and Orf17 dATPase, pH 8.6 (15). These relatively high pH optima may implicate the guanido group of arginine in the binding of the pyrophosphate-containing substrates, and in the mechanism of catalysis, as has been implied for several other enzymatic reactions involving phosphate esters (16). We have noted that arginine is one of the four absolutely conserved amino acids in all of the proteins containing the Nudix box (1), and we have observed that the Nudix hydrolases are inactivated by carbonyl reagents targeting arginine.[2]

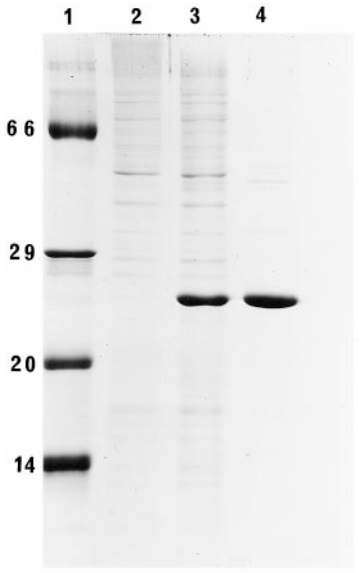

FIG. 1. **Expression and purification of the Orf186 protein.** A polyacrylamide gel (15%) containing 1% SDS, stained with Coomassie Blue, contained the following: *lane 1*, reference proteins including serum albumin (66 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa); *lanes 2* and *3*, 1 μl of crude extracts from cells transformed with pET11b (no insert) or pETorf186, respectively; *lane 4*, enzyme Fraction IV, containing approximately the same number of enzyme units as present in *lane 3*.

The enzyme has an absolute requirement for a divalent cation, achieving optimal activity with 0.5 mM Mg^{2+}, but Zn^{2+} and Mn^{2+} can partially substitute, sustaining rates of hydrolysis of 34% and 17%, respectively, on Ap_3A. No activity was detected in the presence of Co^{2+} or Ca^{2+}. These metal ion effects clearly distinguish the Orf186 enzyme from another Ap_3A hydrolase purified from *E. coli* (17) that cannot substitute Mg^{2+} with Mn^{2+} or Zn^{2+}. Furthermore, it does not hydrolyze NAD^+ as does Orf186 (see below), and it has a specific activity (units per mg) about 300-fold lower than Orf186.

Specificity—Table I lists a number of biochemicals tested as potential substrates for Orf186. Several features are noteworthy. In contrast to MutT, the first member of the Nudix family studied (18), and Orf17 (15), both of which hydrolyze ribo- and deoxyribonucleoside triphosphates, Orf186 has no detectable activity on these compounds. Instead, it seems to be specific for nucleoside pyrophosphates in which one of the moieties is ADP. Thus, Ap_2A, Ap_3A, ADP-ribose, and NADH are all good substrates, as well as Ap_3G and $Ap_3$7-methyl-G. However, when the remaining adenine is replaced by guanine, as in Gp_3G, the activity falls dramatically. Activity on the sugar nucleotides reinforces this observation. Both ADP-glucose and ADP-man-

[2] C. A. Dunn and M. J. Bessman, unpublished observations.

TABLE I
Substrate specificity of Orf186

All substrates were present at a concentration of 2 mM and were assayed using the colorimetric procedure described under "Experimental Procedures" except for the (deoxy)nucleoside triphosphates, which were assayed as described in Ref. 15.

Substrate[a]	Specific activity[b]	Relative activity
	units mg^{-1}	%
Ap_3A	29.0	(100)
Ap_2A	19.0	66
Ap_4A	0.3	1
Ap_5A	<0.1	<1
Ap_3G	25.2	87
Ap_3(7-methyl)G	23.0	75
Gp_3G	0.8	3
ADP-ribose	27.0	93
ADP-glucose	3.0	10
ADP-mannose	2.0	7
NADH	21.0	72
NAD^+	2.5	9
Deamino-NADH	0.1	1
FAD	11.0	38
GDP-glucose, GDP-mannose, UDP-glucose, UDP-mannose, (deoxy)nucleoside triphosphates	<0.1	<1

[a] Ap_3A, adenosine (5′)triphospho(5′)adenosine. Other dinucleoside polyphosphates are abbreviated in an analogous manner.
[b] A unit of enzyme hydrolyzes 1 μmol of substrate/min.

nose, although not indigenous to *E. coli*, are hydrolyzed at a significant rate, whereas the corresponding GDP- and UDP-sugars, more characteristic metabolites, are not. Additionally, when adenine is replaced by hypoxanthine as in deamino-NADH, the presence of the 6-amino instead of a keto group reduces the activity 100 fold.

In the Ap_nA series, activity falls off sharply when $n > 3$. This could be due to steric constraints at the active site of the enzyme, or to electrostatic effects related to the additional phosphates, since ATP, itself, is not a significant substrate. Table II compares kinetic constants for some of the favored substrates. Their turnover numbers (k_{cat}) are all in the same range. This is true, also, for the K_m values of the first four compounds. However, the K_m for NAD^+ is approximately 100-fold higher than the rest, leading to a "catalytic efficiency" (k_{cat}/K_m) 50–80-fold lower than the other substrates. This may have important physiological consequences for the cell (see "Discussion").

Products of the Reaction—The course of hydrolysis of Ap_3A was measured in a standard reaction mixture (minus alkaline phosphatase) scaled up 20-fold. At intervals, aliquots were removed and analyzed by high performance liquid chromatography. Fig. 2 shows the disappearance of Ap_3A and the commensurate appearance of AMP and ADP. For every molecule of Ap_3A hydrolyzed, one molecule each of AMP and ADP was formed, *e.g.* at 7.5 min, 37 nmol of Ap_3A were lost, and 35 and 34 nmol of AMP and ADP were formed, respectively. At 30 min, the values (in order) were 69, 67, and 67. No P_i was detected throughout the course of the reaction. Therefore, the reaction may be written as shown below.

$$Ap_3A + H_2O \rightarrow AMP + ADP$$

REACTION 1

The products formed from NADH, ADP-ribose, Ap_2A, Ap_3G, and adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) were also examined. In each case, AMP was always formed as one of the products, and the remainder of the molecule was liberated intact. Two of the substrates are of special interest.

$$Ap_3G + H_2O \rightarrow AMP + GDP$$

REACTION 2

Neither GMP nor ADP is produced, indicating that the substrate is oriented asymmetrically at the catalytic site. Nucleophilic attack by water could occur on the α or β phosphorus (with respect to the adenosine moiety) leading to the observed products. It is noteworthy that $H_2{}^{18}O$ studies have shown that two other Nudix hydrolases, the MutT dGTPase and the Orf17 dATPase, attack the less favorable (more shielded) β phosphorus, forming dGMP (or dAMP) and PP_i (15, 19). It would be of interest to do similar studies with the Orf186 diadenosine triphosphatase to test the generality of this mechanism. Another substrate worth noting is Ap_4A. Although it is hydrolyzed at only 1% of the rate of Ap_3A, our analysis of the products (data not shown) indicate that it is cleaved according to the following reaction.

$$Ap_4A + H_2O \rightarrow AMP + ATP$$

REACTION 3

This is the first example of an "asymmetric" Ap_4A hydrolase in prokaryotes. Previous reports had relegated this activity to eukaryotes, since all other enzymes prepared from eubacteria or archaea hydrolyze Ap_4A "symmetrically," producing 2 mol of ADP (for review, see Ref. 20).

DISCUSSION

Fig. 3 shows the results of a recent BLAST search (7) for polypeptide sequences homologous to the Nudix box. The broad biodiversity is self-evident, suggesting that representatives of this family of proteins are of primordial origin and were conserved during evolution. We have instituted a systematic study of the members of the group to delimit the scope of reactions they catalyze, and to ascertain whether they exhibit a functional commonality in cellular metabolism. At present, the genes coding for 11 of the proteins listed in Fig. 3 have been cloned, and the expressed proteins were identified as enzymes. These are shown in Fig. 4 along with their major substrates, which, although quite different in structure and metabolic origin, are all derivatives of nucleoside diphosphates. The first of these enzymes, the MutT nucleoside triphosphatase (12, 18), was found to be defective in the original *mutT1* mutator strain (21), thereby accounting for the >1000-fold increase in spontaneous mutation frequency in organisms defective in this gene (22). Since then, homologous enzymes containing the Nudix box have been found in *Streptococcus pneumoniae* (6), *Proteus vulgaris* (23), human (24), rat (25), and mouse (26). This subset of Nudix hydrolases most likely prevent the specific AT → CG transversions seen in the mutator strains (27) by sanitizing the nucleotide pool of a mutagenic form of dGTP (12), possibly 8-oxo-dGTP (28). The next enzyme in Fig. 4, Orf17 dATPase (15), may play a similar role in hydrolyzing the recently discovered, and potentially mutagenic, 2-hydroxy-dATP (29), or in monitoring the accumulation of dATP, the major negative effector of deoxynucleotide synthesis (30). The following enzyme, Orf257, is an unusual NADH pyrophosphatase, 100 times more active on the reduced form of the coenzyme than on NAD^+ (13). It could play a role in maintaining the pivotal cellular NADH/NAD^+ ratio important in balancing the anabolic *versus* catabolic pathways, as has been suggested previously for hydrolases active on NAD^+ (31). The next enzyme, GDP-mannose hydrolase, could play a role in recycling nucleoside diphosphate sugar intermediates and diverting them for reutilization in different pathways during cell maturation, as originally suggested for other sugar nucleotides (32, 33). Also in Fig. 4, the Ap_4A hydrolase, purified from human placenta (34) contains the Nudix box signature sequence, as does the enzyme from pig (35, 36). Ap_4A is a member of the general class of diadenosine polyphosphates, Ap_nA, where n = 2–6. Since the discovery of

TABLE II
Kinetic parameters for Orf186

The standard assay as described under "Experimental Procedures" was used with concentrations of 0.05–2 mM for all substrates except NAD^+ which was present at concentrations of 0.5–50 mM. K_m *and* V_{max} were determined from a non-linear regression analysis (53), and k_{cat} was calculated from V_{max} assuming one active site per monomer. A unit of enzyme hydrolyzes 1 μmol of substrate/min.

Substrate	V_{max}	k_{cat}	K_m	k_{cat}/K_m
	units mg^{-1}	s^{-1}	*n*M	$10^4 M^{-1} s^{-1}$
Ap_3A	31.5 ± 0.9	11.1 ± 0.3	0.147 ± 0.016	7.6 ± 1.0
Ap_2A	24.3 ± 0.4	8.6 ± 0.1	0.151 ± 0.009	5.7 ± 0.4
ADP-ribose	28.0 ± 1.0	9.9 ± 0.4	0.220 ± 0.027	4.5 ± 0.7
NADH	22.1 ± 0.5	7.8 ± 0.2	0.119 ± 0.011	6.6 ± 0.8
NAD^+	39.3 ± 2.4	13.9 ± 0.9	14.9 ± 2.2	0.090 ± 0.018

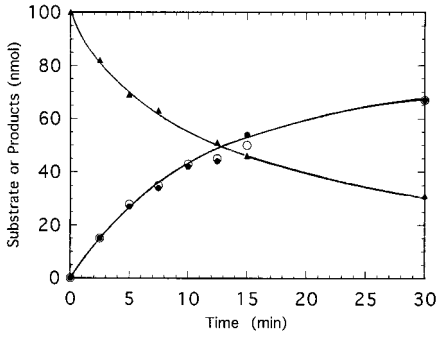

FIG. 2. **Products of the reaction.** The standard enzyme assay as described under "Experimental Procedures" was scaled up 20-fold and monitored by high performance liquid chromatography to quantify the substrate, Ap_3A (▲) and the products, AMP (○) and ADP (●).

these compounds (37), considerable interest has been generated by reports of their involvement in several areas of cell physiology including the responses to heat shock, oxidative stress, and starvation, in cell proliferation and DNA replication and repair, as neurotransmitters, effectors of platelet aggregation, vasotone regulators etc. (for reviews, see Refs. 38 and 39). Although the mechanism(s) of action of these compounds is still in question, the plethora of reports citing their complicity augurs their importance.

Orf186, described in this paper, is a bona fide member of the Nudix hydrolases, of particular interest, since it recognizes three different substrates characteristic of this family of enzymes. A role played by its NADH hydrolase activity in maintaining the optimal NADH/NAD^+ ratio, referred to above, may be of particular importance during periods of anaerobiosis, since the essentially reversible NAD^+-linked oxidoreduction reactions would shut down due to the accumulation of NADH in the absence of acceptor oxygen.

A second major substrate of Orf186 is Ap_3A, a member of the broadly distributed Ap_nA family implicated in the diverse physiological responses mentioned above. Recently, Barnes *et al.* (40) reported that the human fragile histidine triad protein (FHIT) is an Ap_3A hydrolase. This enzyme is the product of the putative human tumor suppressor gene, *FHIT*, at 3p14.2, found to be aberrant in cell lines derived from cancers of the lung, esophagus, stomach, and colon (41, 42). The connection between neoplasia and a defect in Ap_3A hydrolysis is not readily apparent, but it focuses attention on this member of the Ap_nA family and stimulates interest in the role of Ap_3A in *E. coli* metabolism, which may be more amenable to investigation than human cell lines.

The third significant substrate, ADP-ribose, is a by-product of the ubiquitous NAD^+-linked ADP-ribosylation reactions implicated in a broad range of cellular regulatory processes (for review, see Refs. 43 and 44). Hydrolytic removal of the mono- or poly(ADP-ribosyl) groups from modified proteins produces free ADP-ribose. In addition, the normal cellular turnover of NAD^+ itself, which amounts to approximately 30% and 90% of the total NAD^+ synthesis in *E. coli* and HeLa cells, respectively (45), is in part due to the production of ADP-ribose and nicotinamide by NAD^+-glycohydrolase. A role for Orf186 would be the recycling of the ADP-ribose moiety thus formed. Perhaps a more important function is to sanitize the metabolic pool of the potentially harmful accumulation of ADP-ribose. Because of its free aldehydic group, ADP-ribose can modify proteins in non-enzymatic glycation reactions, derivatizing terminal amino groups, cysteines, and lysines (46, 47). Non-enzymatic glycation of long-lived proteins by reducing sugars increases with age, producing what have been called advanced glycosylation end products, which may be targets for degradation and thereby trigger apoptosis in eukaryotic cells (48–50). Ridding the metabolic pool of ADP-ribose may be especially important for two reasons. First, ADP-ribose is a more active glycating agent, by several orders of magnitude, than the free sugars (51). Second, random or nonspecific ADP-ribosylation could misdirect the cellular recognition systems to, or away from, impostors masquerading as properly tagged proteins. A testimony to the importance of this ADP-ribose pyrophosphatase activity is its widespread distribution. We are presently characterizing an additional highly active ADP-ribose hydrolase from *E. coli* (Orf209), and other ADP-ribose pyrophosphatases from yeast (YSA1), from *Bacillus subtilis* (YQKG), from *Haemophilus influenzae* (YZZG), and from an archaeon, *Methanococcus janaschii* (MJ1149), all of which contain the Nudix box.[3] One of the human genome expressed sequence tags (EST 348875) codes for a Nudix polypeptide highly homologous to the yeast (YSA1) enzyme, and specific ADP-ribose pyrophosphatases have been partially purified from rat liver and Artemia (52) although the sequences of the latter two enzymes have not been reported.

At present, no mutants defective in Orf186 are available to test whether the loss of these enzymatic activities elicits a recognizable phenotype. We are in the process of constructing a null mutant of the yeast YSA1 gene (mentioned above), coding for a highly active ADP-ribose pyrophosphatase, to address the question of phenotype. However, ablating one gene may not seriously compromise the cell, since in our experience, there are several Nudix hydrolases with overlapping specificities that might compensate for the loss of one. This is not altogether unexpected, if the enzymatic process is important for survival. In this respect, it is interesting to note that in a recent search of the partially sequenced genome of *Deinococcus radiodurans*, an organism noted for its extraordinary resistance to ionizing radiation, we have identified 17 distinct polypeptides containing the Nudix box.

Orf186 has proven to be an interesting new member of the Nudix hydrolases, a broadly distributed family of enzymes sharing a common structural motif, a related series of substrates and, perhaps, a common function, *i.e.* securing the cellular environment. As a result of the vigorous activity in

[3] C. A. Dunn, S. F. O'Handley, S. Sheikh, D. N. Frick, K. Finney, and M. J. Bessman, unpublished observations.

		NUDIX BOX Sequence	
Protein	**Accession**		**Organism**
Orf186	P45799	LGFSK**G**LIDPG**E**SVYEAAN**REL**K**EE**V**G**FGANDL	E. coli
Orf149	AB001488	WDLPG**G**RVDPG**E**SAEEAAV**REI**L**EE**T**G**YNAALS	B. subtilis
Orf176	U29581	WQFPQ**G**GINPG**E**SAEQAMY**REL**F**EE**V**GL**SRKDV	E. coli
YSA1	Q01976	IEMPA**G**LIDAG**E**DIDTAAL**REL**K**EE**T**G**YSGKII	S. cerevisiae
Ap_4Ase	P50584	WTPPK**G**HVEPG**E**SDLQTAL**RET**Q**EE**A**GI**DAGQL	S. scrofa
Y38A8.1	U55855	VCFPG**G**RMDPG**E**TTTETAL**RET**F**EE**I**GV**NAESV	C. elegans
MTCY349	Z83018	WSLPK**G**KVDPG**E**TAPVGAV**REI**L**EE**T**G**HRANLG	M. tuberculosis
MutT	P44932	LEFPG**G**KVDAG**E**TPEQALK**REL**E**EEI**G**I**VALNA	H. influenzae
Ap_4Ase	P50583	WTPPK**G**HVEPG**E**DDLETAL**RE**TQ**EE**A**GI**EAGQL	H. sapiens
MLCL581	Z96801	WITVG**G**EVRPG**E**RLAAAAA**REL**A**EE**T**GL**RVIPT	M. leprae
InvA	P35640	WQFPQ**G**GIDEG**E**EPLDAAR**REL**Y**EE**T**G**MRSVNL	B. bacilliformis
T26E3.h	Z82053	WYMPA**G**RVEAG**E**TIEEAVV**REV**K**EE**T**G**YSCDVV	C. elegans
YZGD	P46351	WGLPS**G**HVERG**E**SVEEAIV**REI**R**EE**T**GL**QVEVM	B. subtilis
MutT	P08337	LEFPG**G**KIEMG**E**TPEQAVV**REL**Q**EE**V**GI**TPQHF	E. coli
MJ1149	D64443	FALPG**G**FVECG**E**TVEEAVV**REI**K**EE**T**GL**IPKVK	M. jannaschii
sll1054	D90899	LEFPA**G**TVEVG**E**NPAETVK**REL**E**EE**A**G**YRGHTW	C. synechocystis
slr1134	D90905	WSLPG**G**LIDWG**E**TVAITAA**REL**Q**EE**T**GL**RLIKI	C. synechocystis
antiBFGF	P53370	WKFPG**G**LSEPG**E**DIGDTAV**REV**F**EE**T**GV**KSEFR	H. sapiens
InvA	E64101	WQFPQ**G**GINDN**E**SAEQAMY**REL**H**EE**V**GL**QPKDV	H. influenzae
PSU1	P53550	WSFPR**G**KISKD**E**NDIDCCI**REV**K**EE**I**G**FDLTDY	S. cerevisiae
Orf209	P36651	LEMVA**G**MIEEG**E**SVEDVAR**RE**A I**EE**A**GL**IVKRT	E. coli
antiBFGF	U58289	WKFPG**G**LSEPG**E**DIGDTAV**REV**F**EE**T**GV**KSEFR	R. norvegicus
MTCY1A10	Z95387	WFTVG**G**QVRPG**E**RLAQAAA**REL**A**EE**T**GL**RVAPA	M. tuberculosis
F13H10.2	Z68748	FAVVS**D**LKESG**E**SMAECAR**REI**A**EE**V**GI**EVDSI	C. elegans
YGL067W	P53164	YSTIA**G**FMEPS**E**TIEEACI**REI**W**EE**T**GI**SCKNI	S. cerevisiae
Ap_4Ase	U89841	WPQMQ**G**GIDEG**E**DPRNAAI**REL**R**EE**T**GV**TSAEV	L. angustifolius
VD10	P33071	AIYPG**G**ILKRG**E**NVPECLS**REI**K**EE**VN**I**DNSVF	Variola virus
Orf17	P24236	WQSVT**G**SVEEG**E**TAPQAAM**REV**K**EE**VT**I**DVVAE	E. coli
MutT	P36639	WNGFG**G**KVQEG**E**TIEDGAR**REL**Q**EE**S**GL**TVDAL	H. sapiens
MutT	P53368	WNGFG**G**KVQEG**E**TIEDGAK**REL**L**EE**S**GL**SVDTL	M. musculus
MutT	P53369	WNGFG**G**KVQEG**E**TIEDGAK**REL**L**EE**S**GL**RVDTL	R. norvegicus
YQKG	P54570	VEIPA**G**KLEKG**E**EPEYTAL**REL**E**EE**T**G**YTAKKL	B. subtilis
YJAD	P44710	YTTLA**G**FVEVG**E**TFEQAVQ**REV**F**EE**T**GI**SIKNL	H. influenzae
VD10	P04312	AIYPG**G**IPKRG**E**NVPECLS**REI**K**EE**VN**I**DNSFV	Vaccinia virus
Orf257	P32664	HTVLA**G**FVEVG**E**TLEQAVA**REV**M**EE**S**GI**KVKNL	E. coli
MutX	P41354	WIGVG**G**KLERG**E**TPQECAV**REI**L**EE**T**GL**KAKPV	S. pneumoniae
VD9	P21011	LILLG**G**KLDKK**E**SIKDCLR**REL**K**EE**SDERITVK	Vaccinia virus
MutT	P32090	WEFPG**G**KLEDN**E**TPEQALL**REL**Q**EE**I**GI**DVTQC	P.vulgaris
antiBFGF	M27398	GEDI**G**ATAVQG**E**DIGATAV**REV**L**EE**T**GI**HSEFK	X. laevis
Orf1.9	P32056	WFVPG**G**RVQKD**E**TLEAAF**ERL**TMA**E**LG**L**RLPIT	E. coli

FIG. 3. **Members of the Nudix family.** Polypeptides identified in a Blast search (7) against the 23 amino acids spanning the Orf186 signature sequence are shown.

Enzyme	Sequence	Substrates
MutT		
E.coli	**G**KIEMG**E**TPEQAVV**REL**Q**EE**V**GI**	dGTP, 8-oxo-dGTP
S.pneumoniae	**G**KLERG**E**TPQECAV**REI**L**EE**T**GL**	" "
P.vulgaris	**G**KLEDN**E**TPEQALL**REL**Q**EE**I**GI**	" "
Human	**G**KVQEG**E**TIEDGAR**REL**Q**EE**S**GL**	" "
Rat	**G**KVQEG**E**TIEDGAK**REL**L**EE**S**GL**	" "
Mouse	**G**KVQEG**E**TIEDGAK**REL**L**EE**S**GL**	" "
Orf17	**G**SVEEG**E**TAPQAAM**REV**K**EE**VT**I**	dATP
Orf257	**G**FVEVG**E**TLEQAVA**REV**M**EE**S**GI**	NADH
Orf1.9	**G**RVQKD**E**TLEAAF**ERL**TMA**E**LG**L**	GDP-mannose
Ap_4Aase	**G**HVEPG**E**DDLETAL**RE**TQ**EE**A**GI**	Ap_4A
Orf186	**G**LIDPG**E**SVYEAAN**REL**K**EE**V**G**F	Ap_3A, ADP-ribose, NADH

FIG. 4. **Enzymes of the Nudix hydrolase family.** Listed are those proteins containing the Nudix box characterized as enzymes, along with their preferred substrates.

cloning and sequencing of the various genomes, new candidates containing the Nudix box motif are being uncovered at a rapid pace. At present, over 100 have been reported in various prokaryotes, 5 in yeast, and in terms of expressed sequence tags, 2 in rice, 3 in *Caenorhabditis elegans*, 2 in Arabidopsis, 4 in mouse, and 7 in human. In our limited experience, the Nudix hydrolase activities so far identified in the prokaryotes and archaea have their counterparts in yeast and and higher eukaryotes. If this pattern continues, the use of prokaryotes to discover and characterize new members of this family should simplify the task of identifying their activities, and perhaps their physiological functions in the more complex organisms.

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REFERENCES

1. Bessman, M. J., Frick, D. N., and O'Handley, S. F. (1996) *J. Biol. Chem.* **271,** 25059–25062
2. Koonin, E. V. (1993) *Nucleic Acids Res.* **21,** 4847
3. Abeygunawardana, C., Weber, D. J., Gittis, A. G., Frick, D. N., Lin, J., Miller, A.-F., Bessman, M. J., and Mildvan, A. S. (1995) *Biochemistry* **34,** 14997–15005
4. Lin, J., Abeygunawardana, C., Frick, D. N., Bessman, M. J., and Mildvan, A. S. (1997) *Biochemistry* **36,** 1199–1211
5. Lin, J., Abeygunawardana, C., Frick, D. N., Bessman, M. J., and Mildvan, A. S. (1996) *Biochemistry* **35,** 6715–6726
6. Mejean, V., Salles, C., Bullions, L. C., and Bessman, M. J. (1993) *Mol. Microbiol.* **11,** 323–330
7. Altschul, S. F., Gish, W., Meyers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **203,** 403–410
8. Barik, S. (1993) in *PCR Protocols: Current Methods and Applications* (White, B. A., ed) Vol. 15, pp. 277–286, Humana Press Inc., Totowa, NJ
9. Sarkar, G., and Sommer, S. S. (1990) *BioTechniques* **8,** 404–407
10. Ames, B. N., and Dubin, D. T. (1960) *J. Biol. Chem.* **235,** 769–775
11. Markham, R., and Smith, J. D. (1952) *Biochem. J.* **52,** 552–557
12. Bhatnagar, S. K., Bullions, L. C., and Bessman, M. J. (1991) *J. Biol. Chem.* **266,** 9050–9054
13. Frick, D. N., and Bessman, M. J. (1995) *J. Biol. Chem.* **270,** 1529–1534
14. Frick, D. N., Townsend, B. D., and Bessman, M. J. (1995) *J. Biol. Chem.* **270,** 24086–24091
15. O'Handley, S. F., Frick, D. N., Bullions, L. C., Mildvan, A. S., and Bessman, M. J. (1996) *J. Biol. Chem.* **271,** 24649–24654
16. Riordan, J. F., McElvaney, K. D., and Borders, C. L. J. (1977) *Science* **195,** 884–886
17. Hurtado, C., Ruiz, A., Sillero, A., and Sillero, M. A. G. (1987) *J. Bacteriol.* **169,** 1718–1723
18. Bhatnagar, S. K., and Bessman, M. J. (1988) *J. Biol. Chem.* **263,** 8953–8957
19. Weber, D. J., Bhatnagar, S. K., Bullions, L. C., Bessman, M. J., and Mildvan, A. S. (1992) *J. Biol. Chem.* **267,** 16939–16942
20. Guranowski, A., and Sillero, A. (1992) in *Ap4A and Other Dinucleoside Polyphosphates* (McClennan, A. G., ed) pp. 81–133, CRC Press, Inc, Boca Raton
21. Bhatnagar, S. K., Bullions, L. C., Lew, G., and Bessman, M. J. (1990)

J. Bacteriol. **172,** 2802–2803
22. Treffers, H. P., Spinelli, V., and Belser, N. O. (1954) *Proc. Natl. Acad. Sci. U. S. A.* **40,** 1064–1071
23. Kamath, A. V., and Yanofsky, C. (1993) *Gene* (*Amst.*) **134,** 99–102
24. Sakumi, K., Furuichi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H., and Sekiguchi, M. (1993) *J. Biol. Chem.* **268,** 23524–23530
25. Cai, J. P., Kakuma, T., Tsuzuki, T., and Sekiguchi, T. (1995) *Carcinogenesis* **16,** 2343–2350
26. Kakuma, T., Nishida, J., Tsuzuki, T., and Sekiguchi, M. (1995) *J. Biol. Chem.* **270,** 25942–25948
27. Yanofsky, C., Cox, E., C., and Horn, V. (1966) *Genetics* **55,** 274–281
28. Maki, H., and Sekiguchi, M. (1992) *Nature* **355,** 273–275
29. Kamiya, H., and Kasai, H. (1995) *J. Biol. Chem.* **270,** 19446–19450
30. Thelander, L., and Reichard, P. (1979) *Annu. Rev. Biochem.* **48,** 133–158
31. McCreanor, G., and Bender, D. (1983) *Biochim. Biophys. Acta* **759,** 222–228
32. Glaser, L. (1965) *Biochim. Biophys. Acta* **101,** 6–15
33. Sonnino, S., Carminatti, H., and Cabib, E. (1966) *Archiv. Biochem. Biophys.* **116,** 26–33
34. Lazewska, D., Starzynska, E., and Guranowski, A. (1993) *Protein Exp. Purif.* **4,** 45–51
35. Thorne, N. M. H., Hankin, S., Wilkinson, M. C., Nunez, C., Barraclough, R., and McLennan, A. G. (1995) *Biochem. J.* **311,** 717–721
36. Hankin, S., Wintero, A. K., and McLennan, A. G. (1997) *Int. J. Biochem. Cell Biol.* **29,** 317–323
37. Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., and Randerath, K. (1966) *Biochem. Biophys. Res. Commun.* **24,** 91–97
38. Baxi, M. D., and Vishwanatha, J. K. (1995) *J. Pharmacol. Toxicol. Methods* **33,** 121–128
39. McLennan, A. G. (ed) (1992) *Ap4A and Other Dinucleoside Polyphosphates*, CRC Press, Inc., Boca Raton, FL
40. Barnes, L. D., Garrison, P. N., Siprashvili, Z., Guranowski, A., Robinson, A. K., Ingram, S. W., Croce, C. M., Masataka, O., and Huebner, K. (1996) *Biochemistry* **35,** 11529–11535
41. Ohta, M., Inoue, H., Cotticelli, M. G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P, Druck, T., Croce, C. M., and Huebner, K. (1996) *Cell* **84,** 587–597
42. Sozzi, G., Veronese, M. L., Negrini, M., Baffa, R., Cotticelli, M. G., Inoue, H., Tornielli, S., Pilotti, S., De Gregorio, L., Pastorino, U., Pierotti, M. A., Ohta, M., Huebner, K., and Croce, C. M. (1996) *Cell* **85,** 17–26
43. Ueda, K., and Hayaishi, O. (1985) *Annu. Rev. Biochem.* **54,** 73–100
44. Moss, J., and Zahradka, P. (eds) (1994) *Mol. Cell. Biochem.* **138**
45. Olivera, B. M., Hughes, K. T., Cordray, P., and Roth, J. R. (1989) in *ADP-Ribose Transfer Reactions* (Jacobson, M. K., and Jacobson, E. L., eds) pp. 353–360, Springer-Verlag, New York
46. Jacobson, E. L., Cervantes-Laurean, D., and Jacobson, M. K. (1994) *Mol. Cell. Biochem.* **138,** 207–212
47. McDonald, L. J., and Moss, J. (1994) *Mol. Cell. Biochem.* **138,** 221–226
48. Vlassara, H., Brownlee, M., and Cerami, A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 5588–5592
49. Bucala, R., and Cerami, A. (1992) *Adv. Pharmacol.* **23,** 1–34
50. Kaneto, H., Fujii, J., Myint, T., Miyazawa, N., Islam, K. N., Yamasaki, Y., and Taniguchi, M. (1996) *Biochem. J.* **320,** 855–863
51. Cervantes-Laurean, D., Jacobson, E. L., and Jacobson, M. K. (1996) *J. Biol. Chem.* **271,** 10461–10469
52. Fernandez, A., Ribeiro, J. M., Costas, M. J., Pinto, R. M., Canales, J., and Cameselle, J. C. (1996) *Biochim. Biophys. Acta* **1290,** 121–127
53. Cleland, W. W. (1979) *Methods Enzymol.* **63,** 103–139