# Bovine cytosolic IMP/GMP-specific 5′-nucleotidase: cloning and expression of active enzyme in *Escherichia coli*

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A cDNA coding for bovine cytosolic IMP/GMP-specific 5′-nucleotidase endowed with phosphotransferase activity was cloned from calf thymus RNA, by 5′ and 3′ rapid amplification of cDNA ends protocols (5′ and 3′ RACE). Two products were isolated: a 5′ RACE 1.6 kb fragment and a 3′ RACE 2.0 kb fragment, with an overlapping region of 505 bp, leading to a total length of approx. 2951 bp. The similarity in the coding region to that of the human 5′-nucleotidase cDNA sequence [Oka, Matsumoto, Hosokawa and Inoue (1994) Biochem. Biophys. Res. Commun. **205**, 917–922], indirectly identified as a 5′-nucleotidase, was 94 % and the deduced amino acid sequences were 99.5 % identical. The bovine cDNA sequence included the sequences codifying for six peptides obtained from 5′-nucleotidase/phosphotransferase purified from calf thymus. Northern blots of human mRNA species from different tissues showed a 3.6 kb mRNA expressed at equal levels in most tissues. The cDNA was cloned into a pET-28c expression vector and the protein obtained after induction had a molecular mass of 61 kDa under SDS/PAGE. It exhibited both 5′-nucleotidase and phosphotransferase activity, as well as immunological and kinetic properties similar to those of the enzyme purified from calf thymus. This is the first time that a fully active recombinant 5′nucleotidase has been described.

## INTRODUCTION

IMP/GMP-preferring cytosolic 5′-nucleotidase (5′N) is a widespread hydrolase endowed with phosphotransferase activity. The enzyme is expressed at a high level in cells and organs with a rapid turnover rate of nucleic acids or DNA synthesis. High enzyme activity has been measured in testis, spleen and lymphoblastoid cells and very low activity in skeletal muscle and erythrocytes [1,2]. 5′N has been purified from many eukaryotic sources; the various enzymes exhibit similar kinetic characteristics, substrate specificity and regulation, despite the large differences reported in the molecular mass [3]. The apparent molecular mass of the enzyme estimated by gel filtration ranges from 165 kDa for the *Artemia* enzyme [4] to 265 kDa for the pig lung enzyme purified in the presence of protease inhibitors [5], whereas the subunit molecular masses ranged from 51 to 62 kDa, indicating a tetrametric structure for 5′N. The enzyme is specific for IMP, GMP and their corresponding deoxyderivatives. It is activated by ATP, ADP, 2,3-bisphosphoglycerate, decavanadate and, to a smaller extent, by other nucleoside diphosphates and triphosphates. The enzyme is also inhibited by $P_i$. This inhibition is counteracted by ATP at physiological concentration and, to a smaller extent, by ADP [3,6,7].

In 1982 Worku and Newby [8] proposed that the hydrolysis of the monophosphate proceeds via the formation of an enzyme–phosphate intermediate, as with many phosphate-transferring enzymes; this hypothesis was based on kinetic evidence. The formation of a phosphorylated intermediate was recently demonstrated directly by trapping the $^{32}$P-labelled phosphoenzyme followed by electrophoresis and autoradiography [9]. Indirect evidence for an involvement of histidine and cysteine residues in the catalysis were also obtained [9]. The destiny of the phosphate bound to the active site of the enzyme depends on the presence of a nucleoside acceptor, e.g. inosine or deoxyinosine, and on the concentration of phosphate and ATP. In fact, at an adenylate energy charge close to 0.9 and at a millimolar phosphate concentration, 5′N behaves mainly as a phosphotransferase, its activity depending on the availability of a suitable nucleoside acceptor. Furthermore, under these conditions, because the nucleoside is a better phosphate acceptor than water, an increase in the overall reaction rate is observed [6]. The main metabolic role of 5′N in various organs and tissues has been matter of discussion and several hypotheses have been proposed that are related to the regulation of the intracellular IMP concentration [3]. Although the physiological relevance of the phosphotransferase activity associated with 5′N is unknown, its role in the phosphorylation *in vitro* and *in vivo* of anti-viral or anti-tumour purine prodrugs such as acyclovir, 2′,3′-dideoxyinosine, carbovir, 8-azaguanosine and deoxycoformycin has been studied intensely [10–12]. The abundance of 5′N is low in most tissues and the purified enzyme is very unstable. A recombinant enzyme source is therefore needed for the study of its structure and function [3,6,13]. Furthermore the identification of the sequence of the human cDNA by Oka et al. [14] relied on sequence similarity, and, to our knowledge, no functional recombinant 5′N has as yet been expressed. We cloned the cDNA for the bovine 5′N by using the information from the human cDNA sequence. The expression of the cloned bovine cDNA led to a protein with enzymic properties identical with the cytosolic 5′-nucleotidase/phosphotransferase purified from calf thymus [6].

Abbreviations used: 5′N, IMP/GMP-preferring cytosolic 5′-nucleotidase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U73690.

## EXPERIMENTAL

### Materials

Calf thymus was obtained from the local slaughterhouse. Radiolabelled nucleotides, [$\alpha$-$^{35}$S]deoxyATP (1000 Ci/mmol), [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol), [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) and [1′,2′-$^{3}$H]deoxyguanosine (26 Ci/mmol), were from the Radiochemical Centre (Amersham). [8-$^{14}$C]Inosine (55 mCi/mmol), [8-$^{14}$C]adenosine (57 mCi/mmol), [8-$^{14}$C]deoxyadenosine (55 mCi/mmol) and [2-$^{14}$C]thymidine (47.7 mCi/mmol) were from Sigma. [U-$^{14}$C]Guanosine (1.75 mCi/mg) was from New England Nuclear. Calf spleen purine nucleoside phosphorylase and xanthine oxidase from milk were obtained from Sigma. Oligo(dT)-cellulose, Expand® long template PCR system (*Taq*/*Pwo* mixture of polymerases, 3.5 units/$\mu$l), Expand® high-fidelity PCR system (*Taq*/*Pwo* mixture of polymerases, 3.5 units/$\mu$l), 5′/3′ rapid amplification of cDNA ends (RACE) kit, High Pure PCR product purification kit and random primed DNA labelling kit were from Boehringer Mannheim. A Marathon kit, TaqStart antibody (7 $\mu$M, 1.1 $\mu$g/$\mu$l), human multi-tissue blot and TALON metal-affinity resin were purchased from Clontech. A TA cloning kit was purchased from Invitrogen. Isopropyl $\beta$-D-thiogalactoside, Wizard PCR Preps and restriction enzymes, were purchased from Promega. A Sequenase version 2.0 DNA-sequencing kit was purchased from Amersham–UBS. An RNA PCR kit was purchased from Perkin Elmer. Vector pET-28c and recombinant thrombin were purchased from Novagen.

### 5′N purification and sequence determination of proteolytic fragments

The enzyme was partially purified as described previously [6], with minor modifications. Briefly, the steps were as follows: crude extract from calf thymus; DE-52 chromatography; $(NH_4)_2SO_4$ precipitation; chromatography on pentyl-agarose; gel filtration on Sephacryl S-300; Dye-ligand chromatography on Matrix Green A; affinity chromatography on ADP-agarose. The final SDS/PAGE [14 % (w/v) gel] was performed by the method of Laemmli [15]; the gel was then blotted on a PVDF membrane and stained with Coomassie Blue. The band corresponding to 59 kDa and identified by a parallel reaction with antibodies specific for 5′N [9] was cut out and digested with trypsin. The peptide sequence was determined by automatic Edman degradation with a Porton Instrument 2090 microsequencing system.

### Purification of mRNA from calf thymus

Calf thymus was used to purify total RNA by the method of Chomczynski and Sacchi [16]; this bulk RNA was used to purify poly(A)$^+$ RNA by affinity chromatography on oligo(dT)-cellulose as described by Sambrook et al. ([17], vol. 1, pp. 7.25–7.29).

### Reverse transcriptase (RT) reactions

RT reactions were set up in accordance with the manual provided with the kits used.

### PCR amplification of 5′nucleotidase/phosphotransferase cDNA ends (5′ RACE and 3′ RACE)

Two primers (Table 1), deduced from the nucleotide sequence of human enzyme [14], were used in combination with an adaptor primer AP1 from Marathon kit, to amplify 5′N cDNA. 5′N-P1F (sense primer) was used in combination with 0AP1 in a 3′ RACE protocol, whereas a 5′N-P1R (anti-sense primer) and AP1 were used for 5′ RACE protocol. An adaptor-ligated cDNA library, obtained as described in the Marathon kit manual, was used as a template. Cycling parameters were identical for 5′- and 3′ RACE: (1) 94 °C for 1 min; (2) 94 °C for 30 s; (3) 60 °C for 30 sec; (4) 68 °C for 3 min; steps 2–4 repeated for a total of 10 cycles; (5) 94 °C for 30 s; (6) 60 °C for 30 s; (7) 68 °C for 3 min plus 20 s per cycle; steps 5–7 repeated for a total of 20 cycles; (8) 68 °C for 10 min. Each reaction mixture contained, in a total volume of 50 $\mu$l, 0.7 $\mu$l of DNA polymerase (*Taq*/*Pwo* mix from Expand® long template PCR system) and 0.3 $\mu$l of TaqStart antibody. The buffer was 50 mM Tris/HCl (pH 9.2 at 25 °C)/16 mM $(NH_4)_2SO_4$/1.75 mM $MgCl_2$.

**Table 1 Primers used in PCR reactions**

| Name | Sequence | Notes |
|---|---|---|
| 5′N-P1F | 5′-GGGAGCCAAGGGAAAAGACATT-3′ | Position in the human sequence: 1017–1038 |
| 5′N-P1R | 5′-CCGGGTGGCAAGAGGAGACT-3′ | Position in the human sequence: 1521–1502 |
| 5′N-PFNheI | 5′-CCGCTAGCATGACAACCTCCTG-3′ | 3–8, *Nhe*I 9–22, 5′-coding region |
| 5′N-$P_{tot}$R | 5′-CCGAATTCTCACTCCTCCTCTTC-3′ | 3–8, *Eco*RI 9–23, 3′-coding region |
| AP1 | 5′-CCATCCTAATACGACTCACTATAGGGC-3′ | Provided with Marathon kit |

### Cloning of 5′ and 3′ RACE products in pCR®II plasmid

After the PCR reaction described in the previous section, 1 unit of *Taq* polymerase was added to the mixture and incubated for 10 min at 72 °C. DNA was then extracted immediately with an equal volume of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.), and precipitated with a 0.1 vol. of 3 M sodium acetate and 2 vol. of ethanol. The resuspended pellet was purified from a 1 % (w/v) low-melting agarose gel. The recovered 5′ and 3′ RACE products were then ligated overnight at 14 °C with the pCR®II vector (molar ratio of plasmid to insert, 1:1) supplied with the TA cloning kit. The ligation mixture was used to transform competent inv$\alpha$F′ cells. The cDNA sequence was determined by the Sanger dideoxynucleotide method with a Sequenase version 2.0 DNA-sequencing kit.

### Northern-blot analysis

A 1.1 kb fragment corresponding to the 5′-terminus of the open reading frame (ORF) and a 700 bp fragment corresponding to the 3′-terminus were labelled with $^{32}$P (random primed DNA-labelling kit), and used to probe a human multi-tissue blot to determine the amount of 5′N mRNA in various tissues.

### Expression and characterization of recombinant 5′N

Once the sequence of the expressed region had been determined, two new primers were synthesized (Table 1), 5′N-FNheI, and 5′N-$P_{tot}$R. The first contained an *Nhe*I site followed by the beginning of the ORF; the second contained the end of the ORF followed by an *Eco*RI cleavage site. These primers were used in a RT–PCR reaction. The PCR protocol was: (1) 94 °C for 30 s; (2) 94 °C for 10 s; (3) 54 °C for 10 s; (4) 72 °C 1 min; steps 2–4 repeated for a total of three cycles; (5) 94 °C for 10 s; (6) 64 °C for 10 s; (7) 72 °C for 1 min plus 5 s per cycle; steps 5–7 repeated for a total of 30 cycles; (8) 72 °C for 7 min. The reaction mixture (master mix) contained, in a total volume of 49 $\mu$l, 1 $\mu$l of RT product as the template, 1.5 mM $MgCl_2$, each dNTP at 200 $\mu$M, each primer at 0.2 $\mu$M. DNA polymerase (0.7 $\mu$l) (a *Taq*/*Pwo* mix from a high-fidelity PCR system) were incubated for 10 min at room temperature with 0.3 $\mu$l of TaqStart antibody and then

added to the master mix just before starting the PCR reaction. The PCR product was purified; after cleavage with *Nhe*I and *Eco*RI, it was ligated to *Nhe*I/*Eco*RI-digested pET-28c DNA at 25 °C for 1 h. The ligation mixture was then transformed into Jm109 competent cells. This vector provides a histidine tag, used for a single-step purification, followed by a thrombin recognition site fused to the N-terminus of the recombinant protein. The 5′N construct was then transformed into both BL21(DE3) and BL21(DE3)pLysS cells [18]. The expression of the recombinant protein was performed, in Luria–Bertani medium containing 1 mM isopropyl $\beta$-D-thiogalactoside, at 37 °C for 3 h. Bacteria were harvested and resuspended in 20 mM Tris/HCl (pH 8.0)/100 mM NaCl/0.5 % Triton X-100/0.5 mM PMSF (lysis buffer) in a ratio of 1 g of bacteria to 20 ml of buffer. Lysis was achieved by freeze–thawing the cells in the presence of 2 mg/ml lysozyme. Lysates were then centrifuged at 35000 ***g*** for 90 min. The supernatant was loaded on a column containing TALON resin equilibrated in the lysis buffer. The loading step was repeated once. The resin was then washed with 10 column volumes of lysis buffer and then with 100 mM NaCl/30 mM imidazole in 20 mM Tris/HCl, pH 8.0 (five to ten column volumes). The recombinant 5′N was eluted from the column with 400 mM imidazole in 50 mM Tris/HCl, pH 7.4. The tubes used to collect purified protein contained dithiothreitol in 50 mM Tris/HCl, pH 7.4, to give a final dithiothreitol concentration of 2 mM. The molecular mass of the native recombinant protein was determined by gel filtration on Sephacryl S-300, whereas the size of the recombinant protein was determined by SDS/PAGE.

### Enzyme assay

5′N activity was measured as described previously [13]. One unit of enzyme activity is the amount of enzyme required to convert 1 $\mu$mol of substrate to product/min under the assay conditions.

## RESULTS

### Purification of 5′N and sequence determination of proteolysis fragments

As described previously, the purified enzyme preparation contained two major polypeptides of 59 and 54 kDa. Both peptides cross-reacted with anti-5′N antibodies; moreover they exhibited both nucleotidase and phosphotransferase activities [9]. The 59 kDa peptide was recovered after electrophoresis and immunoblotting, then digested with trypsin; the resulting peptides were separated by reverse-phase liquid chromatography. Six peptides were sequenced (Figure 1). All the peptides were found in the deduced amino acid sequence of the human 5′N cDNA [14].

### Cloning and sequencing of calf 5′N cDNA

Purified calf poly(A)$^+$ RNA was used to produce total double-stranded cDNA by an RT reaction primed with the Marathon cDNA-synthesis primer. After the creation of blunt ends with a T4 DNA polymerase, a DNA adaptor provided with the kit was ligated to both ends of the cDNA by a T4 DNA ligase. This adaptor-ligated cDNA library was used as a template for 5′ and 3′ RACE PCR reactions. Both reactions were primed with a gene-specific internal primer (P1R for 5′ RACE and P1F for 3′ RACE), deduced from the human sequence, and with the Marathon adaptor primer (AP1) provided. The obtained amplification products were cloned in a pCRII vector and sequenced. The 5′ RACE PCR product was 1.6 kb (including the AP1 sequence) and the 3′ RACE PCR product was 2 kb (including the cDNA-synthesis primer and AP1 sequences). Their sequences showed an overlapping region of 505 bp.

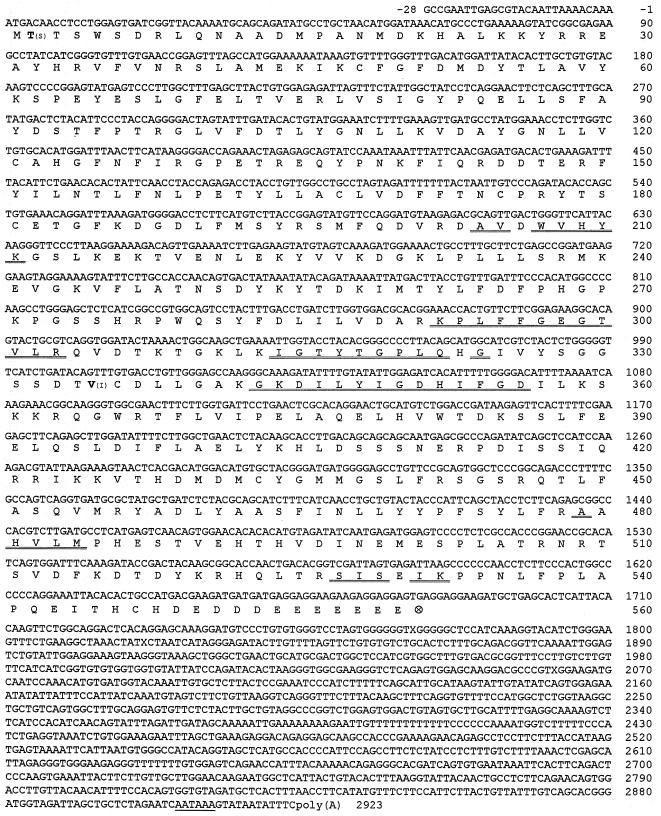


**Figure 1 cDNA and predicted amino acid sequence of bovine 5′N**

Both the deduced amino acid and nucleotide sequences are shown. cDNA numbering starts from the predicted initiation methionine residue. Amino acid substitutions with respect to human 5′N are indicated in bold letters; the corresponding residue in the human sequence is shown in parentheses. The sequences determined for tryptic fragments from the purified calf protein are doubly underlined. The possible polyadenylation signal is underlined. The symbol ⊗ indicates the predicted TGA stop codon.

The full-length cDNA sequence of bovine 5′N is shown in Figure 1, together with the deduced amino acid sequence. The ORF started at the first ATG (nt 1–3) and terminated with a TGA stop codon at nt 1681–1683. This ORF encodes for a polypeptide of 560 amino acid residues with a calculated molecular mass of 64848 Da and an isoelectric point of 6.01. The sequence of bovine 5′N showed a very high degree of similarity to the human cDNA sequence [14]: approx. 94 % of the nucleotide sequences of the coding region and 99.5 % of the deduced amino acid sequences were identical. Only two amino acid conservative substitutions (Thr-2 for Ser and Val-335 for Ile) were found. The human sequence also displayed an additional glutamic residue at the C-terminus with respect to the bovine enzyme. The sequence of six peptides obtained from the proteolytic digestion of the 5′N purified from calf thymus was in complete agreement with the deduced peptide sequence. The 3′-untranslated region was 1240 bp long and contained a polyadenylation signal at position 2906 followed by the poly(A)$^+$ tail. When we searched the GeneBank database we noticed that the cDNA sequence from nt 1 to 1065 showed no similarities to other known sequences besides human 5′-nucleotidase and an expressed sequence tags sequence from rat, whereas the search made with the second part of the cDNA (from nt 1060 to 2923) showed many sequences displaying some degree of similarity. A large number of them contained Alu sequences, but one, corresponding to the the N-terminal region of the surfactant protein

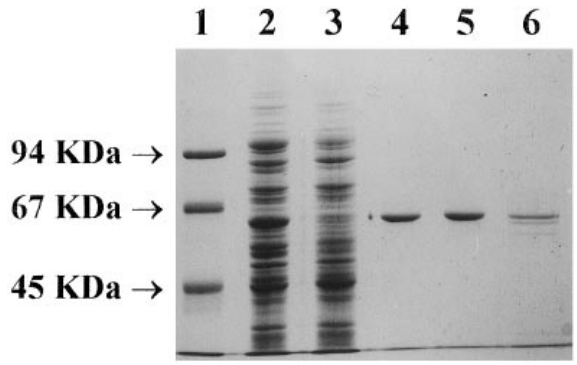


**Figure 2 SDS/PAGE of recombinant bovine 5′N purified by TALON ion metal-affinity chromatography**

Lane 1, molecular mass standards; lane 2, 10 $\mu$l of crude cell lysate; lane 3, 10 $\mu$l of the flow-through fraction; lanes 4 and 5, 10 and 15 $\mu$l of the imidazole eluate respectively; lane 6, 10 $\mu$l of the purified recombinant 5′N cleaved overnight with thrombin.

A recognition protein (SPAR) cloned from pig [19], showed 100 % identity with the C-terminal sequence of 5′N. The overlapping region covered the last 132 amino acids of 5′N and extended into the 3′-untranslated region. The reason for this apparently 'split-gene' behaviour of the 5′N cDNA is not clear but the results indicate that there might have been a gene fusion event that has led to the current architecture of this protein coding sequence.

### Northern blot analysis

A Northern blot containing mRNA from eight different human tissues was probed with both bovine 5′-terminal 1065 bp and 3′-terminal 666 bp DNA fragments. A 3.6 kb mRNA was observed in all the tissues tested with both probes, but at somewhat varying levels in different tissues ([14] and R. Pesi, S. Allegrini, M. G. Tozzi and S. Eriksson, unpublished work).

### Expression and purification of bovine 5′N in *Escherichia coli*

The RT reaction followed by the PCR reaction primed with 5′N-FNheI and 5′N-$P_{tot}$R was repeated several times in different conditions, for both RT and PCR protocols, obtaining the isolation of a cDNA containing a 61 bp insertion after $G^{175}$. The sequence of this insertion included a stop codon leading to the premature end of the peptide synthesis. When we used a modified RT reaction (5′/3′ RACE kit) performed at 52 °C instead of 45 °C, we were finally able to isolate one clone out of 14 that contained a cDNA of the expected length. The correct cDNA for bovine 5′N was then transfected into BL21(DE3) and BL21 (DE3)pLysS cells. The presence of recombinant protein in various colonies was determined by SDS/PAGE analysis. The colonies that showed a polypeptide of the expected size were used to prepare a crude extract as described in the Experimental section. The crude extract from BL21(DE3) showed phosphotransferase activity (2.4 units/mg), but no activity was found in lysates from BL21(DE3)pLysS cells. Unfortunately the amount of recombinant protein in the crude extract was lower than that observed in the extract from total cells (results not shown), thus indicating that a large part of the 5′N probably forms inclusion bodies.

The purification of recombinant protein by ion metal-affinity chromatography resulted in a single polypeptide of 61 kDa under SDS/PAGE (Figure 2). The histidine tag and the thrombin cleavage site fused at the N-terminal of the expressed peptide account for approx. 2–3 kDa. Thrombin cleavage, performed overnight at 10 °C, led to the partial digestion of the recombinant 5′N with the formation of a 59 kDa fragment (line 6 in Figure 2). The purified protein, both before and after thrombin cleavage, cross-reacted with the 5′N antibodies (results not shown). The purified enzyme showed both nucleotidase and phosphotransferase activities and exhibited a high degree of instability. Recombinant enzyme was stabilized at 4 °C in the presence of 2 mM dithiothreitol along with either 11 % (w/v) $(NH_4)_2SO_4$ or 50 % (v/v) glycerol. After 1 month, 80 % of the activity was still present under both conditions. The purified recombinant 5′N used to determine the kinetic parameters described in the next section had a specific activity of 83.3 $\mu$mol/min per mg of protein measured as IMP hydrolase.

**Table 2 Phosphate donors of recombinant 5′N**

Nucleoside phosphotransferase activity in the presence of 2 mM of each nucleoside monophosphate was measured as described by Tozzi et al. [13] with 1.4 mM [8-$^{14}$C]inosine as phosphate acceptor.

| Phosphate donor | Human purified 5′N [13] | Bovine purified 5′N [6] | Recombinant 5′N |
|---|---|---|---|
| 5′-dIMP | 100 | 100 | 100 |
| 5′-IMP | 78 | 85 | 95.6 |
| 5′-GMP | 82 | 64 | 94.3 |
| 5′-dGMP | 76 | 68 | 86.7 |
| 5′-UMP | 26 | 31 | 35.5 |
| 5′-CMP | 10 | 14 | 21.5 |
| 5′-AMP | 11 | 9 | 19.1 |
| 5′-dAMP | 3 | 4 | 7.7 |
| 3′-UMP | 1.5 | 3 | 2.5 |
| 3′-IMP | 0.3 | 5 | 1.3 |

### Substrate specificity and regulation of recombinant 5′N

The nucleotidase and phosphotransferase activities of the recombinant protein were determined with various phosphate donors (Table 2) and several nucleosides as phosphate acceptors (Table 3). The substrate specificity of recombinant 5′N was similar to that of the enzyme purified from both human colon carcinoma [13] and calf thymus [6]. 6-Hydroxypurine nucleosides and deoxynucleosides were the best phosphate donors, and inosine seemed to be the best substrate for the phosphotransferase reaction. Furthermore the cloned enzyme was activated by ATP and inhibited by phosphate under the same conditions described for the enzyme purified from different sources [3,6,13,20] (results not shown).

**Table 3 Phosphate acceptors of recombinant 5′N**

Nucleoside phosphotransferase activity in the presence of 1.4 mM of different labelled nucleosides was measured as described by Tozzi et al. [13], with 2 mM IMP as phosphate donor.

| Phosphate acceptor | Human purified 5′N [13] | Bovine purified 5′N [6] | Recombinant 5′N |
|---|---|---|---|
| Inosine | 100 | 100 | 100 |
| Guanosine | 16 | 28 | 28 |
| Deoxyguanosine | 0 | 0 | 5.4 |
| Adenosine | 13 | 2 | 1.8 |
| Deoxyadenosine | 1.7 | 1 | 1.2 |

## DISCUSSION

The nucleotide sequences coding for the human and the bovine enzyme showed a very high degree of similarity, i.e. 94 % in the coding region. It is not possible to compare directly the untranslated region because of several insertions or deletions but there are several 'Alu-like' sequences in the 3′-untranslated region, which may be involved in the unexpected occurrence of part of the surfactant protein A recognition protein sequence [14] in the C-terminal domain of the 5′N cDNA. The functional role of this domain in the 5′N protein is not known. The amino acid sequence deduced from the ORF showed two amino acid substitutions and one insertion. Studies on the frequency of amino acid mutation demonstrated that Val/Ile and Thr/Ser are among the most common substitutions observed in various classes of polypeptide chain [21]. When the sequences of the six peptides obtained from trypsin digestion of purified bovine 5′N were compared with the deduced coding sequence there was complete identity.

Northern blot analysis of human mRNA with bovine 5′-terminal cDNA showed a single band of 3.6 kb in all tissues; the length of this transcript agrees with that described by Oka et al. [14]. This transcript is 600 bp longer than the cloned cDNA (approx. 3 kb). We do not know whether the 600 bp missing from the cDNA is due to an additional polyadenylation signal at the 3′-terminus or to an extended untranslated 5′-terminus. The presence of the transcript in all tissues is a further indication of the widespread distribution of the enzyme ([3], and R. Pesi, S. Allegrini, M. G. Tozzi and S. Eriksson, unpublished work).

Expression of 5′N in the BL21(DE) *E. coli* strain with the pET-28c bacterial vector resulted in a high concentration of active soluble enzyme, although a high amount of expressed protein was also present as inclusion bodies. The presence of a histidine tag at the N-terminus allowed one-step purification by ion metal-affinity chromatography, giving a pure protein with a native molecular mass of approx. 300 kDa (results not shown). SDS/PAGE of the final preparation showed a single band of 61 kDa. After thrombin cleavage of the histidine tag sequence (approx. 2–3 kDa), the polypeptide showed a molecular mass identical with that of 5′N purified from calf thymus [2]. The molecular mass calculated from the amino acid sequence was 64848 Da. Even though many different events, besides the molecular mass, might be responsible for the electrophoretic behaviour of a denatured polypeptide in an SDS/polyacrylamide gel, this discrepancy might well be due to proteolysis during enzyme preparation. Studies are in progress to determine the sequence of the C-terminus of purified recombinant protein to verify the hypothesis of a proteolytic cleavage. However, proteolysis at the N-terminus of the protein can be ruled out because of the histidine tag used for the purification.

Recombinant protein cross-reacted with antibodies specific for 5′N and showed a high capacity both to hydrolyse mononucleotides and to transfer the phosphate from nucleoside monophosphate donors to nucleoside acceptors. We can thus conclude that the bovine cDNA encodes a 5′-nucleotidase/phosphotransferase. The hydrolysing specific activity we obtained, 83.3 $\mu$mol/min per mg of protein, was higher than that reported for other sources, which ranged from a minimum of 1.1, for 5′N purified from *Artemia* [4], to a maximum of 37 $\mu$mol/min per mg for the chicken liver enzyme [22]. Discrepancies between the specific activities can probably be ascribed to the carry-over, during long purification procedures, of inactive protein. In the easy one-step purification of the recombinant nucleotidase this phenomenon is certainly decreased. We found that the substrate specificity measured both with different donors and acceptors was in complete agreement with that shown for the enzyme purified from different sources. Moreover the effects exerted on recombinant 5′N by ATP and $P_i$ were the same as those observed with both mammalian and avian enzymes [3].

The very high degree of similarity found between the human and bovine enzyme, also described earlier for the human and the avian enzymes [14], explains why, although the 5′N purified from different sources are sometimes different in size, they nevertheless display very similar kinetic and molecular characteristics. The rate of change of amino acids of 5′N in human and bovine (about 200 million years apart in evolution) or in human and chicken (about 300 million years apart) [14] is comparable with the rate observed for the very highly conserved protein, e.g. histones, actin and tubulin. This high level of similarity, together with the widespread distribution of the enzyme in various organisms and tissues [1,2], suggests that 5′N plays a central role in the cell, probably related to its ability to hydrolyse IMP and GMP, thus determining the destiny of the purine ring, which after dephosphorylation is free to abandon the cell.

The availability of large amounts of purified and active protein will increase the knowledge of the kinetic and regulatory properties of 5′N. The possibility of expressing mutants of the enzyme will also contribute greatly to the understanding of its role in the cellular metabolism and activation of antiviral and anticancer nucleoside analogues.

We thank Liya Wang, Anita Herrström Sjöberg, Jianghai Wang and Jin ping Li for their valuable suggestions, Professor Marcella Camici for critical reading of the manuscript, and Catarina Ljungcrantz and Simone Golfarini for expert technical assistance. This work was supported by the following grants: Swedish National Board of Technical Development; Medivir AB, Huddinge; EU Commission BMH4-CT96-0479; Svenska Institutet; Blanceflor Boncompagni-Ludovisi, född Bilds Foundation, Sweden.

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Received 17 February 1997/23 June 1997; accepted 8 July 1997