# Cloning and expression of a new cDNA from monocotyledonous plants coding for a diadenosine 5′,5‴-P$^1$,P$^4$-tetraphosphate hydrolase from barley (*Hordeum vulgare*)

Juri Churin[a], Bettina Hause[b], Ivo Feussner[b], Helmut P. Maucher[b], Kirstin Feussner[b], Thomas Börner[a], Claus Wasternack[b,*]

[a]*Institute of Biology, Division of Genetics, Humboldt-University Berlin, Chausseestr. 117, 10115 Berlin, Germany*
[b]*Institute of Plant Biochemistry Halle, P.O.B. 110432, 06018 Halle/Saale, Germany*

Received 13 June 1998

**Abstract From a cDNA library generated from mRNA of white leaf tissues of the ribosome-deficient mutant 'albostrians' of barley (*Hordeum vulgare* cv. Haisa) a cDNA was isolated carrying 54.2% identity to a recently published cDNA which codes for the diadenosine-5′,5‴-P$^1$,P$^4$-tetraphosphate (Ap$_4$A) hydrolase of *Lupinus angustifolius* (Maksel et al. (1998) Biochem. J. 329, 313–319), and 69% identity to four partial peptide sequences of Ap$_4$A hydrolase of tomato. Overexpression in *Escherichia coli* revealed a protein of about 19 kDa, which exhibited Ap$_4$A hydrolase activity and cross-reactivity with an antibody raised against a purified tomato Ap$_4$A hydrolase (Feussner et al. (1996) Z. Naturforsch. 51c, 477–486). Expression studies showed an mRNA accumulation in all organs of a barley seedling. Possible functions of Ap$_4$A hydrolase in plants will be discussed.**



*Key words:* *Hordeum vulgare*; Diadenosine 5′,5‴-P$^1$,P$^4$-tetraphosphate (Ap$_4$A) hydrolase; Cloning, overexpression

## 1. Introduction

Diadenosine 5′,5‴-P$^1$,P$^4$-tetraphosphate (Ap$_4$A) and related diadenosine oligophosphates (Ap$_n$A) occur ubiquitously in microbial, plant and animal organisms [1]. Ap$_n$A including Ap$_4$A are unspecifically formed by various aminoacyl-tRNA synthetases [2]. This contrasts to the occurrence of three distinct classes of enzymes cleaving specifically Ap$_4$A [3]. (i) An Ap$_4$A (symmetrical) pyrophosphohydrolase (Ap$_4$A → ADP+ADP, EC 3.6.1.41) was found in prokaryotes and the slime mould *Physarum polycephalum*. (ii) An Ap$_4$A (asymmetrical) pyrophosphohydrolase (Ap$_4$A → AMP+ATP, EC 3.6.1.17) has been described for *Schizosaccharomyces pombe* and all higher eukaryotes (invertebrates, vertebrates and plants) studied so far. (iii) A phosphorolytic cleavage occurs in yeast [4], protozoa [5] and cyanobacteria [6] by a reversible acting Ap$_4$A phosphorylase (Ap$_4$A+Pi ↔ ADP+ATP; EC 2.7.7.53).

Among the various implications proposed for a role of Ap$_4$A, most data of the last two decades support a function in cell proliferation [7,8]. The inverse correlation between the intracellular level of Ap$_4$A and the activity of Ap$_4$A-cleaving enzymes observed in several organisms [1] was thought to play a crucial role in Ap$_4$A function. However, a paradoxical increase of Ap$_4$A upon expression of its catabolic enzyme was also found [9], and for the Ap$_3$A/Ap$_4$A ratio a role was suggested in apoptosis of human cells [10]. Recent data implicate that Ap$_n$A are intracellular and extracellular signalling molecules maintaining and regulating vital cellular functions [8].

A breakthrough in understanding the role of Ap$_4$A and its catabolic enzymes came by the observation that the fragile histidine trial (FHIT) gene product, implicated to function in tumor suppression [11], shares sequence homology to the apaH1 gene. The apaH1 gene codes for the Ap$_4$A hydrolase of *Schizosaccharomyces pombe* [11,12]. However, FHIT was identified as an Ap$_3$A hydrolase [13]. FHIT proteins represent one branch of the HIT protein superfamily which is characterized by a His-X-His-X-His-XX-motif near the C-terminus, containing X as a hydrophobic amino acid [14].

The occurrence of HIT homologous sequences in man, plants, cyanobacteria and fungi [15] and identification of the putative tumor suppressor FHIT in man as an Ap$_3$A hydrolase [13] implies an important role of this gene product in cell proliferation. However, all cDNAs coding for an Ap$_4$A hydrolase known so far from man [16], pig [17] and lupin [18] lack a HIT domain. In contrast, they exhibit homology to a nucleotide binding site defined for prokaryote Mut-T proteins [19]. This occurrence of Ap$_n$A hydrolases carrying either a HIT domain or a Mut domain as nucleotide binding site in different branches of the eukaryotes prompted us to inspect a monocotyledonous plant. For that we have chosen the white leaf tissues of the '*albostrians*' mutant of barley (*Hordeum vulgare* cv. Haisa). Here, relative abundance of rare mRNAs was expected due to a lack of synthesis of plastid proteins by the absence of plastid ribosomes [20].

In this paper, we show the cloning and overexpression of an Ap$_4$A hydrolase from a monocotyledonous plant. This enzyme shares 54.2% identity to a cDNA coding for the lupin Ap$_4$A hydrolase [18] and is expressed in all organs of barley seedlings.

## 2. Material and methods

### 2.1. Plant material

Seeds of barley (*Hordeum vulgare* cv. Salome and cv. Haisa, mutant line *albostrians*) were grown under green house conditions at 16 h light (150 μmol photons cm$^{-2}$ s$^{-1}$) and 8 h dark at 24°C and 60% relative humidity for seven days. Seeds of 'Salome' were germinated on wet filter paper in Petri dishes in the dark for three days. Corresponding tissues were collectively sampled by freezing in liquid nitrogen.

*Corresponding author. Fax: (49) (345) 5582-162.
E-mail: cwastern@ipb.uni-halle.de

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank with accession number Z99996.


PII: S0014-5793(98)00819-9

*2.2. Construction of the cDNA library and isolation of the cDNA*

The cDNA expression library from white tissues of 7-day-old barley leaves of the *albostrians* mutant line was constructed as described [21]. Screening was performed with a labeled probe containing part of the wheat cab1-gene promoter [22]. The library was plated at $2\times10^4$ p.f.u. per 137-mm plate and screened essentially as described [23]. Recombinant phage expressing proteins able to bind to the probe were further purified by additional rounds of screening.

*2.3. Overexpression and activity tests*

A barley $Ap_4A$ hydrolase cDNA clone named 1 (337) was digested and inserted into the *Sal*I and *Hin*dIII sites of the pQE-30 vector. This

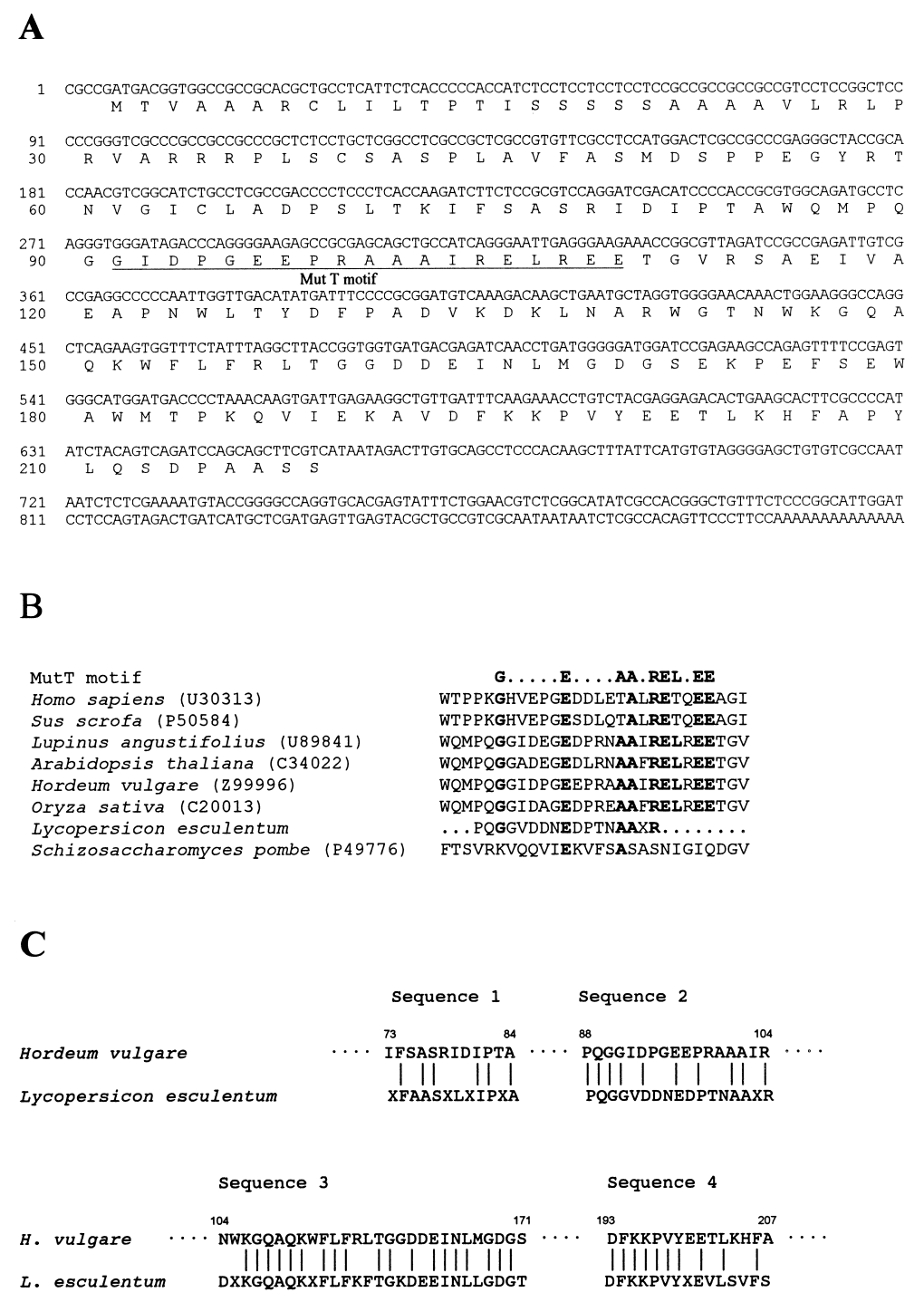








Fig. 1. Primary structure of clone 1 (337). A: Nucleotide sequence and derived amino acid sequence of clone 1 (337). Nucleotides and amino acid residues are numbered on the left. The Mut-T motif known for nucleotide pyrophosphatases is underlined. B: Sequence comparison within the Mut-T family. The essential amino acid residues of the Mut-T motif are given in bold letters together with the corresponding amino acids within identified $Ap_4A$ hydrolase and homologous sequences, respectively, indicated by the accession number within brackets. The peptide sequence of *Lycopersicon esculentum* was taken from [26], and corresponds to sequence 2 of C. C: Comparison of peptide sequences of tomato $Ap_4A$ hydrolase purified recently [26] with corresponding amino acid sequences deduced from the cDNA isolated for barley $Ap_4A$ hydrolases and shown in A. The position of each barley peptide within the sequence shown in A is given by the corresponding number of the terminal amino acids.

construct was used to transform *E. coli* M15. The cells were grown overnight at 37°C in 20 ml LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Four ml of this culture were added to 200 ml of the same medium. The cells were grown at 37°C to a cell density of $A_{600}$=0.7. Then, the culture was incubated in the presence of 2 mM IPTG at 8°C for 16 h. The cells corresponding to 1 l culture medium were collected, suspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, containing 10% (v/v) glycerol, 0.1% Tween 20, 0.5 M NaCl), and then disrupted using a sonifier tip with 5 pulses each of 30 s as described [24]. The cell debris was removed by centrifugation (12 000×*g* for 15 min). The supernatant was applied onto 0.5 ml of a Ni-chelate agarose column (Qiagen, Germany) equilibrated with lysis buffer. Unbound protein was washed from the column with 5 ml of lysis buffer, pH 6.3, and the recombinant protein was eluted with 2 ml of lysis buffer, pH 3.6, containing 100 mM EDTA.

$Ap_4A$ hydrolase activity (40 ng affinity purified protein) was assayed in 10 μl 100 mM Tris-HCl buffer, pH 8.0, containing 5 mM $MgCl_2$, 0.1 mM DTT and 0.5 mM $Ap_4A$ at 30°C for 3–12 min. The reaction was stopped by spotting the reaction mixture onto a TLC plate coated with silica gel containing a fluorescent indicator (Merck, Germany). Plates were developed in dioxane/ammonia/$H_2O$ (6:1:5, by volume) and the products visualized under UV light.

### 2.4. Extraction of RNA and proteins as well as Northern and immunoblot analysis

Simultaneous extraction of proteins and total RNA was performed as described previously [25]. Immunoblot analysis was performed by using polyclonal rabbit antibody raised against purified tomato $Ap_4A$ hydrolase in a dilution of 1:500 [26]. For Northern blot analysis RNA electrophoresis, RNA transfer and hybridization with [α-$^{32}$P]-dATP-labelled cDNA insert was performed as described [27] using 20 μg total RNA per lane.

### 2.5. Southern blot analysis and protein sequencing

For Southern blot analysis 10 μg of genomic DNA were digested with the restriction enzymes *Eco*RI, *Hin*dIII and *Xba*I followed by agarose gel electrophoresis. Southern blotting and hybridizations were performed as described [28]. Sequencing of peptides of the tomato $Ap_4A$ hydrolases was performed as described [26].

## 3. Results and discussion

A cDNA expression library, made from poly(A)$^+$-mRNA of white tissues of the ribosome-deficient mutant '*albostrians*' of barley (*Hordeum vulgare* cv. Haisa) [20], was used for a South Western screening with a fragment of the wheat cab1 promoter. One of the isolated full length cDNA clones designated as 1 (337) showed a 5′-flanking region of 5 bp, a 3′-untranslated region of 241 bp, and an open reading frame encoding for 218 amino acids (Fig. 1A). Surprisingly, the comparison of the nucleotide and the deduced amino acid sequence with sequences of data bases (EMBL, Heidelberg, Germany) using Needleman and Wunsch algorithm revealed 54.2% identity to the recently published $Ap_4A$ hydrolase of *Lupinus angustifolius* [18]. On amino acid level the clone exhibited 43.2%, 45.8%, 43.9% and 75.5% similarity, respectively, to sequences of $Ap_4A$ cleaving enzymes known from man, *Sus scrofa*, *Schizosaccharomyces pombe* and *Lupinus angustifolius*, respectively, and about 82% similarity to partial sequences from *Arabidopsis thaliana* and *Oryza sativa*. The Mut-T sequence motif which is a characteristic feature of pyrophosphate releasing nucleotide triphosphatases of eubacteria, eukaryotes and viruses [19], occurs also in the barley cDNA clone 1 (337) (Fig. 1A). Interestingly, a peptide sequence of the recently published $Ap_4A$ hydrolase of tomato cells grown in suspension [26] corresponds to a part of this Mut-T motif (Fig. 1B). The essential amino acid residues of this motif could be found in all sequences carrying similarities to so far identified asymmetrical $Ap_4A$ hydrolases in eukaryotes except *S. pombe* (Fig. 1B), whereas the symmetrically cleaving $Ap_4A$ hydrolases of prokaryotes and the fungal $Ap_4A$ phosphorylases lack this Mut-T domain [16]. This lack of a Mut-T motif in $Ap_4A$ hydrolase of *S. pombe* and fungal $Ap_4A$ phosphatases is accompanied by the occurrence of another motif for nucleotide binding, the HIT-motif [15], a characteristic of the human FHIT gene product [29], which was identified as $Ap_3A$ hydrolase [13]. The recently isolated cDNA of a lupin $Ap_4A$ hydrolase [18] and the barley cDNA presented here (Fig. 1A), both of them carrying a Mut-T motif, also lack a HIT motif. This exclusive occurrence of the Mut-T motif or the HIT motif in distinct $Ap_nA$ cleaving enzymes coincides with distinct differences in enzymatic properties of the encoded proteins [18] suggesting that there are evolutionary branches of these enzymes.

The fact that we isolated the barley cDNA by South Western screening with a fragment of the wheat cab1 promoter

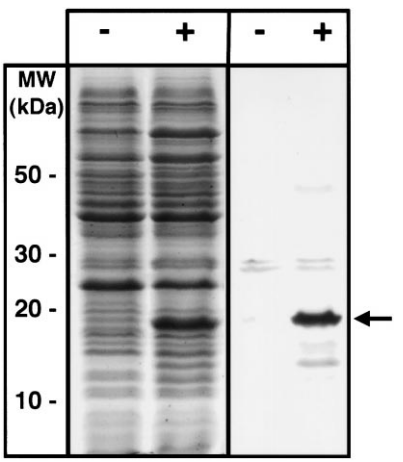


Fig. 2. Expression of clone 1 (337) in the His-tag expression vector pQE30 transformed in *E. coli* and identification of the recombinant protein by immunoblot analysis with a rabbit polyclonal antibody raised against purified tomato $Ap_4A$ hydrolase [26]. Expression was performed with cells lacking (−) or carrying (+) the vector. In the Coomassie-stained gels (left panel) and in the immunoblot (right panel) an abundant protein was detected at 19 kDa (arrow). For electrophoresis 40 μg total *E. coli* protein extract was loaded.

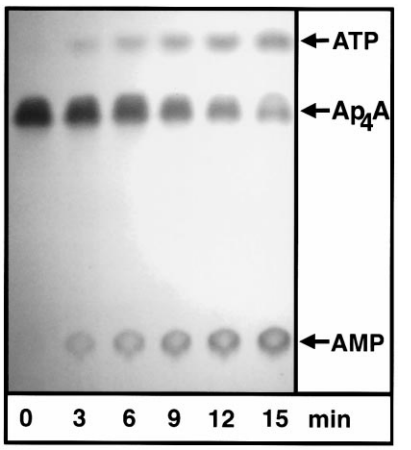


Fig. 3. Activity test on $Ap_4A$ hydrolase using crude extracts of *E. coli* overexpressing $Ap_4A$ hydrolase protein as shown in Fig. 2. Time dependent formation of the products 5′-ATP and 5′-AMP is shown after TLC as described elsewhere [26].

could be due to the ability of cab1 sequences to bind at nucleotide binding sites such as the Mut-T domain.

In addition to the peptide sequence 2 of the tomato $Ap_4A$ hydrolase [26] (Fig. 1B), further sequences of this enzyme collected here were homologous to the deduced amino acid sequence of the barley cDNA (Fig. 1C). Based on these homologies, it was suggested that the isolated barley cDNA may encode an $Ap_4A$ hydrolase. Therefore, overexpression was performed as a final proof using the pQE30 vector in *E. coli* M15 with a His tag fused to the N-terminal part of the protein. After low temperature cultivation and affinity chromatography, in vector-containing cells a newly formed protein of a molecular mass of 19 kDa accumulated which cross-reacted with a polyclonal rabbit antibody raised against purified tomato $Ap_4A$ hydrolase (Fig. 2). Thus, this molecular mass corresponds to that found for the tomato enzyme (20 kDa) [26] and the lupin enzyme (19 kDa) [18]. In crude extracts of *E. coli* cells exhibiting this immunoreactive protein of 19 kDa, $Ap_4A$ hydrolase activity was detected in terms of TLC-detectable formation of 5′-AMP and 5′-ATP using $Ap_4A$ as a substrate (Fig. 3). Based on sequence homology as well as immunological and enzymatic properties upon overexpression we suppose that the cDNA shown in Fig. 1A codes for an asymmetrically cleaving $Ap_4A$ hydrolase which is characteristic for higher eukaryotes. Upon *Eco*RI, *Hin*dIII and *Xba*I digest of genomic DNA of barley we detected one fragment in the Southern blot analysis using the cDNA clone 1 (337) as probe (Fig. 4) indicating the occurrence of one gene locus.

In order to inspect expression of this barley $Ap_4A$ hydrolase, we analyzed several tissues of seedlings as well as various stress conditions. $Ap_4A$ hydrolase mRNA and protein was detectable within all organs of 3-day-old barley seedlings, in tissues known to carry rapidly dividing cells as well as tissues known to be fully differentiated (Fig. 5). Jasmonate known to be a signal of various stress responses of barley leaves [30] did not change the low constitute amount of $Ap_4A$ hydrolase in leaves, and an in situ hybridization failed presumably due to low abundance of the transcript. Rapidly growing cells like tobacco cells grown in suspension may help to elucidate the role of this enzyme and its substrate $Ap_4A$ in plants. The disappearance of the $Ap_4A$ hydrolase protein from tomato cells during mitosis [31], and control of timing of cell division of *E. coli* by $Ap_4A$ [32], suggest a link to cell proliferation. Recent crystallographic [33], biochemical [34], and genetic studies [35] on the FHIT protein and the FHIT-$Ap_4A$/$Ap_3A$ substrate complexes of a human cancer gave arguments that not the $Ap_4A$/$Ap_3A$ hydrolysis but the complex itself may function as a signal to terminate the cell cycle. In this context the ratio of $Ap_4A$ and $Ap_3A$ as well as other $Ap_nA$ were suggested to function as signals in eukaryotes [8].

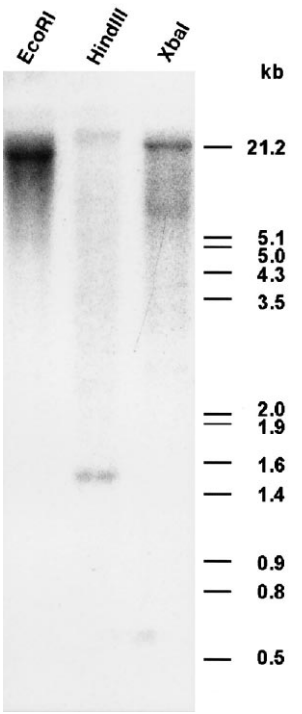


Fig. 4. Southern blot analysis of barley genomic DNA with the $Ap_4A$ hydrolase cDNA probe 1 (337). 10 μg genomic DNA was digested with the restriction endonucleases *Eco*RI, *Hin*dIII and *Xba*I, fractionated by agarose gel electrophoresis and transferred onto a nylon membrane. The blot was hybridized with [α-$^{32}$P]-dATP-labeled cDNA probe 1 (337) as described in Section 2. Size markers are indicated in kb on the right margin.

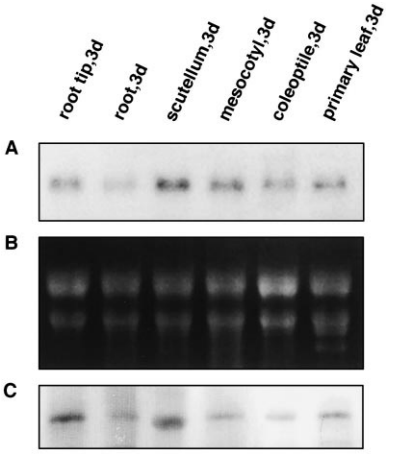


Fig. 5. Accumulation of $Ap_4A$ hydrolase mRNA (A) and $Ap_4A$ hydrolase (C) in different tissues of 3-day-old barley (*Hordeum vulgare* cv. Salome) seedlings. 20 μg total RNA was loaded per lane. Loading was checked by ethidium bromide staining (B). For C 5 μg total protein extract was loaded and subjected to immunoblot analysis.

*Acknowledgements:* We thank S. Krüger, B. Degner and B. Ortel for excellent technical assistance, Dr. A. Otto for determining the internal peptides 1, 3 and 4 of $Ap_4A$ hydrolase shown in Fig. 1C, and C. Dietel for typing the manuscript. Seeds of *albostrians* barley were kindly provided by G. Küntzel (Gatersleben) and M. Meixner (Berlin). We are thankful to W.R. Hess for establishing the cDNA library. Work in the lab of T.B. was supported by the Fonds der Chemischen Industrie, Frankfurt.

## References

[1] Remy, P. (1992) in: $Ap_4A$ and Other Dinucleoside Polyphosphates (McLennan, A.G., Ed.) pp. 151–204, CRC Press, Boca Raton, FL.
[2] Kisselev, L.L. and Wolfson, A.D. (1992) Prog. Nucleic Acid Res. Mol. Biol. 48, 83.
[3] Guranowski, A. and Sillero, A. (1992) in: $Ap_4A$ and Other Dinucleoside Polyphosphates (McLennan, A.G., Ed.) pp. 81–133, CRC Press, Boca Raton, FL.
[4] Guranowski, A. and Blanquet, S. (1985) J. Biol. Chem. 260, 3542–3547.
[5] Guranowski, A., Starzynska, E., Gzik, L., Langston, S.P.,

Brown, P. and Blackburn, G.M. (1995) Nucleosides Nucleotides 14, 731–734.
[6] McLennan, A.G., Mayers, E. and Adams, D.G. (1996) Biochem. J. 320, 795–800.
[7] McLennan, A.G. (Ed.) (1992) $Ap_4A$ and Other Dinucleoside Polyphosphates, CRC Press, FL.
[8] Kisselev, L.L., Justesen, J., Wolfson, A.D. and Frolova, L.Y. (1998) FEBS Lett. 427, 157–163.
[9] Avila, D.M., Robinson, A.K., Kaushal, V. and Barnes, L.D. (1991) J. Bacteriol. 173, 7875–7880.
[10] Vartanian, A., Prudovsky, I., Suzuki, H., Pra, I.D. and Kisselev, L. (1997) FEBS Lett. 415, 160–162.
[11] Ohta, M., Inoue, H., Coticelli, 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.
[12] Sozzi, G., Veronese, M.L., Negrini, M., Baffa, R., Coticelli, M.G., Inoue, H., Tornielli, S., Pilotti, S., DeGregorio, L., Pastorino, U., Pierotti, M.A., Ohta, M., Huebner, K. and Croce, C.M. (1996) Cell 85, 17–26.
[13] Barnes, L.D., Garrison, P.N.G., Siprashvili, Z., Guranowski, A., Robinson, A.K., Ingram, S.W., Croce, C.M., Ohta, M. and Huebner, K. (1996) Biochemistry 35, 11529–11535.
[14] Seraphin, B. (1992) DNA Sequence 3, 177–179.
[15] Huang, Y., Garrison, P.N. and Barnes, L.D. (1995) Biochem. J. 312, 925–932.
[16] Thorne, N.M.H., Hankin, S., Wilkinson, M.C., Nuñez, C., Barraclough, R. and McLennan, A.G. (1995) Biochem. J. 311, 717–721.
[17] Hankin, S., Wintero, A.K. and McLennan, A.G. (1996) Biochem. J. 312, 925–932.
[18] Maksel, D., Guranowski, A., Ilgoutz, S.C., Moir, B., Blackburn, G.M. and Gyler, K.R. (1998) Biochem. J. 329, 313–319.
[19] Koonin, E.V. (1993) Nucleic Acids Res. 21, 4847.
[20] Hess, W.R., Prombona, A., Fieder, B., Subramanian, A.R. and Börner, T. (1994) EMBO J. 12, 563–571.
[21] Hess, W.R. and Börner, T. (1997) Endocytobiosis Cell Res. 12, 95–98.
[22] Lamppa, G.K., Morelli, G. and Chua, N.H. (1985) Mol. Cell. Biol. 5, 1370–1378.
[23] Garabedian, M.J., Labaer, J., Liu, W.H. and Thomas, J.R. (1993) in: Gene Transcription, A Practical Approach, pp. 243–293, IRL Press at Oxford University Press, Oxford.
[24] Shirano, Y. and Shibata, D. (1990) FEBS Lett. 271, 128–130.
[25] Hause, B., Demus, U., Teichmann, C., Parthier, B. and Wasternack, C. (1996) Plant Cell. Physiol. 37, 641–649.
[26] Feussner, I., Guranowski, A., Kostka, S. and Wasternack, C. (1996) Z. Naturforsch. 51c, 477–486.
[27] Leopold, J., Hause, B., Lehmann, J., Graner, A., Parthier, B. and Wasternack, C. (1996) Plant Cell Environ. 19, 675–684.
[28] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY.
[29] Brenner, C., Garrison, P., Gilmour, J., Peisach, D., Ringe, D., Petsko, G.A. and Lowenstein, J.M. (1997) Nat. Struct. Biol. 4, 231–238.
[30] Wasternack, C. and Parthier, B. (1997) Trends Plant Sci. 2, 302–307.
[31] Hause, B., Feussner, I. and Wasternack, C. (1997) Bot. Acta 110, 452–457.
[32] Nishimura, A., Moriya, S., Ukau, H., Nagai, K., Wachi, M. and Yamada, Y. (1997) Genes Cells 2, 401–413.
[33] Brenner, C., Pace, H.C., Garrison, P.N., Ribonson, A.K., Rosler, A., Liu, X.-H., Blackburn, G.M., Croce, C.M., Huebner, K. and Barnes, L.D. (1997) Protein Eng. 12, 1461–1463.
[34] Pace, H.C., Garrison, P.N., Robinson, A.K., Barnes, L.D., Draganescu, A., Rösler, A., Blackburn, G.M., Siprashvili, Z., Croce, C.M., Huebner, K. and Brenner, C. (1998) Proc. Natl. Acad. Sci. USA 95, 5484–5489.
[35] Siprashvili, Z., Sozzi, G., Barnes, L.D., McCue, P., Robinson, A.K., Eryomin, V., Sard, L., Tagliabue, E., Greco, A., Fusetti, L., Schwatz, G., Pierotti, M.A., Croce, C.M. and Huebner, K. (1997) Proc. Natl. Acad. Sci. USA 94, 13771–13776.