Component analysis and characterization of a nuclear deoxyribonucleotidase

Kevin FORD, Jon WALTHO and David HORNBY*

Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, P.O. Box 594, Sheffield S10 2UH, U.K.

We have previously reported the identification of a novel activity residing in the nuclear fraction of mammalian cells that selectively binds and hydrolyses deoxyribonucleoside triphosphates. Incubation of this protein with [α - 32 P]dATP leads to the appearance of a retarded band relative to free dATP when the reaction is analysed on non-denaturing polyacrylamide gels. We now show that the retarded species comprises the product of dATP hydrolysis (dADP or dAMP) bound to an as yet unidentified species. We have termed this complex the 'product–nucleotide binding particle' or PNB^{*}. Through a combination of continuous elution polyacrylamide-gel electrophoresis and gel-filtration chromatography, we demonstrate that the hydrolytic

activity (dNTPase) is distinct from the radiolabelled species detected in gel-retardation experiments. T.l.c. confirms that the labelled product does not share R_f values associated with a range of mono-, di- and tri-phosphate deoxyribonucleotide standards, and gel-filtration experiments suggest a molecular mass for PNB^{*} of between 2.5 and 3 kDa. The ability of purified PNB^{*} to retain its nucleotide ligand after a number of denaturing processes suggests that the ligand is covalently bound. The recovery of dNTPase activity from both gel-filtration and ion-exchange chromatography reveals that the as yet unliganded PNB^{*} (or a precursor form) is associated with the dNTPase enzyme as part of the active complex, prior to addition of dATP.

INTRODUCTION

Many proteins have been described that bind deoxyribonucleoside triphosphates, the most obvious of which are the replication and repair enzymes associated with DNA. Other enzymes, such as those that catalyse phosphate transfer reactions, can often substitute dATP for the usual ATP substrate (Gorbalenya and Koonin, 1990). Others that utilize ATP more directly as an energy source can in some cases utilize dATP. The DNA helicases, for example, while preferentially hydrolysing ATP, can utilize other ribose and deoxyribose forms (Matson and Kaiser-Rogers, 1990), whereas ribonucleotide reductase, whose main activity is the reduction of ribonucleotide diphosphates to the deoxy form, is able to hydrolyse ATP and can recruit deoxyribonucleotides to an allosteric site (Reichard, 1988). A requirement for ATP has also been reported for the association of nucleoplasmin and high mobility group protein 1, although deoxyribonucleotides have been shown to fulfil the function equally well (Imamoto-Sonobe et al., 1988). Histones have recently been shown to possess ATPase activity (Oikarinen, 1991; Manermaa and Oikarinen, 1992), although no evidence exists for the utilization of deoxyribose forms of the nucleotides. None of the enzyme activities reported above utilize dNTPs as their primary and only substrate. The deoxyribonucleotidase (dNTPase) reported by us previously (Dalton et al., 1992) and further described in this paper is unique in that it binds and hydrolyses dNTPs independently of DNA (unlike polymerases or ligases), does not utilize ATP at all (unlike kinase enzymes and those mentioned above) and retains the hydrolysed nucleotide as a covalent adduct with one of its dissociated components. Extensive literature searches have revealed no reports of a protein with similar properties to this enzyme. Its apparent widespread distribution in eukaryotes (e.g. yeast, pig, rat, HeLa cells) suggests a fundamental role in cell metabolism and, given its marked preference for dNTPs, this is most likely to be in the area of DNA replication/repair.

We have previously shown that the use of the chemically identical radiolabel [γ - 32 P]dATP does not give rise to the characteristic retardation pattern seen with [α - 32 P]dATP, suggesting that the terminal phosphate at least is removed away from the retarded nucleotide. Moreover, we have shown that the activity is specific for deoxyribonucleotides, with sugar status rather than base identity being the main determinant of specificity. Initial speculation that the hydrolytic activity was also directly responsible for the nucleotide retardation seen in gel-mobility shifts has been clarified. We now report the results of further investigations using gel-filtration and continuous elution electrophoresis that resolve the 200 kDa hydrolytic activity and the dAMP/dADP peptide complex ('protein–nucleotide binding particle'; PNB^{*}) as separate species.

MATERIALS AND METHODS

Preparation of nuclear extract from pig liver

Nuclear extract was prepared as described (Dalton et al., 1992) with the following modifications. Prior to initial homogenization the tissue was shredded using a manually operated rotary food mincer in order to improve subsequent homogenization efficiency. The buffer used for homogenization contained 1.4 M sucrose, 80 mM NaCl, 1 mM EDTA, 20 mM Hepes, pH 7.9, 2 mM dithiothreitol, 0.5 mM spermidine and 0.15 mM spermine. The homogenate was then centrifuged on to a 1.8 M sucrose cushion as described by Saluz and Jost (1987). The nuclear pellet was homogenized using 10–15 strokes of a hand-held glass homogenizer in a buffer containing 20 mM Hepes, pH 7.9, 3% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 0.5 mM phenylmethanesulphonyl fluoride according to the method of Dignam et al. (1983). The nuclear homogenate was transferred to a beaker and stirred gently on ice for 30 min. After clarification at 25000 g (Sorval, SS34 rotor), the clear aqueous layer was removed and stored. The pellet containing ruptured nuclei was washed with 3–4 ml of nuclei homogenization buffer

Abbreviation used: PNB^{*}, product–nucleotide binding particle.

* To whom correspondence should be addressed.

for 10 min with mild agitation. The resulting cloudy solution, which contains most of the dNTPase activity, was retained and dialysed against 20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 100 mM KCl, 2 mM MgCl_2 , 2.2 mM EDTA and 0.5 mM phenylmethanesulphonyl fluoride.

dATPase assay

The standard dNTP utilization assay reaction contained 1 μl (0.2 μg) of protein extract and 0.06 pmol of [α - ^{32}P]dATP (3000 Ci/mmol; Amersham) in a final buffer solution comprising 20 mM Hepes, pH 7.9, 3.3% (v/v) glycerol, 100 mM KCl and 1 mM EDTA. Incubation was for 30 min at room temperature. Samples were subsequently loaded on to a 4% (19:1, acrylamide/bisacrylamide) 1 $\times$ TBE (0.1 M Tris, 80 mM boric acid, 2.5 mM EDTA) polyacrylamide gel and run in 1 $\times$ TBE electrophoresis buffer at 200 V for 2 h. Gels were then dried under vacuum and exposed to X-ray film overnight at -70°C . DNA molecular size markers were end-labelled with [α - ^{32}P]dATP using the reaction catalysed by the Klenow fragment of DNA polymerase I, as described in Sambrook et al. (1989).

Ion-exchange chromatography

Typically, 5–10 ml of nuclear extract were loaded on to a 7 ml Q-Sepharose (Pharmacia) Fast Flow column pre-equilibrated in 20 mM Hepes, pH 7.9, and 10% (v/v) glycerol. After subsequent washing with 3–5 column volumes of buffer, protein was eluted with a linear gradient of 0–2 M KCl in pre-equilibration buffer over a volume of 50 ml. Active fractions were pooled, dialysed against a solution of 20 mM Hepes, pH 7.9, and 10% (v/v) glycerol, and concentrated via reverse dialysis against solid poly(ethylene glycol) (molecular mass 15–20 kDa) to a final volume of 0.5 ml.

Gel-filtration chromatography

Pooled fractions (200 μl vol.) from initial Q-Sepharose fractionation were applied to a Superdex 75 (Pharmacia) gel-filtration column, pre-equilibrated in 20 mM Hepes, pH 7.9, 5% glycerol and 100 mM KCl. Component analysis of the dNTPase activity was performed similarly. Typically, 100 μl of crude protein fraction was preincubated with 1 μl of [α - ^{32}P]dATP for 1 h at room temperature. The sample was then loaded directly on to a Superdex 75 gel filtration column. Collected fractions were run directly on a non-denaturing gel which was then dried down and exposed to X-ray film at -70°C overnight. Control fractions resulting from the application of crude extract in the absence of preincubation with [α - ^{32}P]dATP were assayed for dNTPase activity as described.

Continuous elution electrophoresis

Crude nuclear extract (1–2 ml) was incubated with 25 μl of [α - ^{32}P]dATP for 2 h at room temperature. This volume was loaded directly on to a 4%, 1 $\times$ TBE, non-denaturing polyacrylamide gel, mounted in a Bio-Rad model 491 Prep Cell. Elution buffer contained 20 mM Hepes, pH 7.9, 2% glycerol and 100 mM KCl. The gel was run at 200 V for 3–4 h with buffer circulation. The elution buffer flow rate was 0.2 ml/min. Fractions were loaded directly on to a non-denaturing gel.

T.l.c. analysis

Aliquots (2–3 μl) of either nucleotide standard or PNB P^* (derived from continuous elution electrophoresis) were spotted on to a poly(ethylene imine) cellulose t.l.c. sheet. This was allowed to air dry and then placed in a suitable volume of solvent (0.2 M ammonium bicarbonate). Chromatography was continued until the solvent front had reached two-thirds the way up the sheet. After subsequent air drying, the chromatogram was exposed to long-wavelength u.v. irradiation to identify deoxy-nucleotide and ribonucleotide standards (mono-, di- and tri-phosphates) and subjected to autoradiography overnight at -70°C in order to visualize the position of the labelled species relative to the standards. After initial standard calibration, purified PNB P^* and nucleotide standards were overlaid on the t.l.c. plate prior to running, to ensure accuracy in mobility comparisons.

N.m.r.

^{31}P n.m.r. experiments were recorded on a Bruker AMX500 spectrometer using an inverse detection probe with the X-coil tuned to 202.46 MHz. The sample temperature was at 298 K. The optimal signal-to-noise ratio was obtained using a 16 μs pulse. Data were acquired for 0.4 s over a spectral width of 98.8 p.p.m. Typically, 1024 transients were recorded per spectrum. ^1H decoupling during acquisition was found not to be necessary in terms of the extra resolution it afforded.

RESULTS

The nucleotide product of dATP hydrolysis has an anomalous electrophoretic mobility

Incubation of pig liver nuclear extract with [α - ^{32}P]dATP leads to dATP hydrolysis and retention of the dADP or dAMP remnant by an as yet unidentified component of the dNTPase activity. The results of such an incubation can be visualized by application to a non-denaturing polyacrylamide gel as shown in Figure 1. The position of the retarded remnant nucleotide is indicated by an arrow. Comparison to DNA molecular size standards reveals the dNTP derived band to be running at approx. 200 bp. This represents a significant retardation of radiolabel. An oligonucleo-

Figure 1 Electrophoretic mobility of PNB P^*

The mobility of PNB P^* , resulting from the incubation of crude extract with [α - ^{32}P]dATP (see the Materials and methods section) was compared to DNA size markers by non-denaturing PAGE. Lanes contain: 1, 1 ng of 16 bp DNA oligonucleotide; 2, 1 ng of 245 bp DNA fragment; 3, standard dNTPase assay as described in the text. The arrow indicates PNB P^* .

Figure 2 N.m.r. spectra of the products of dATP hydrolysis

(a) ^{31}P n.m.r. spectra for 2 mM dATP (A), 2 mM dADP (B) and 2 mM dAMP (C). (b) ^{31}P n.m.r. monitoring of a time course experiment containing 50 μl of enriched enzyme, 2 mM dATP and 50 μl of $^2\text{H}_2\text{O}$ in a final volume of 500 μl . The reaction was monitored at intervals of 1.5 h (A), 12 h (B), 24 h (C) and 72 h (D).

tide marker (16 bp duplex; lane 1) runs at the foot of the gel, while free dATP migrates off the gel totally. The retardation characteristics of the dNTPase product cannot be compared directly with the size of the DNA markers and this experiment only serves to indicate the relative extent of retardation of the radiolabel compared with free dATP.

The results of a time course incubation of partially purified dNTPase (derived from a nuclear wash step after nuclear lysis) with dATP monitored by ^{31}P n.m.r. spectroscopy are shown in Figure 2(b). The main features of the spectra are the emergence of characteristic dADP signals coupled to the appearance of a free phosphate signal at 2 p.p.m. and a decrease in signals seen for α -, β - and γ -phosphates derived from the dATP species. Significantly, no corresponding dAMP signal was observed at the same time point (12 h), but it became evident and progressively stronger as the time course continued. At the end point of the experiment all the dATP had been converted to dADP, dAMP and free phosphate, as determined using the standard spectra shown in Figure 2(a). Slight variations in characteristic peak positions in Figure 2(b) are most probably attributable to a change in pH over the course of the experiment. The action of contaminant proteins such as phosphatases cannot be excluded at this point, however, due to the impure nature of the enzyme preparation. However, this approach offers an opportunity to monitor the hydrolytic activity of the dNTPase and should be a

Figure 3 Q-Sepharose ion-exchange chromatography

(a) Elution profile of crude pig liver extract fractionated by Q-Sepharose chromatography. (b) PNBp* formation was monitored via non-denaturing PAGE of each fraction after preincubation with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (see the Materials and methods section). Fraction numbers are given above the corresponding lanes of the gel. Only the relevant portion of the non-denaturing gel is shown, i.e. the region containing the PNBp* signal, to allow a degree of alignment with the elution profile. Buffer B is equilibration buffer containing 2 M KCl (see the Materials and methods section).

useful adjunct to the gel-retardation assays when homogeneous preparations become available.

The dNTPase enzyme can be resolved into activity and product components

In order to determine whether the dNTPase enzyme was directly responsible for retardation of the hydrolysed nucleotide, a number of gel-filtration experiments were performed on partially pure enzyme fractions, as described below.

Figure 3(a) shows the absorbance profile for the fractionation of crude extract using Q-Sepharose ion-exchange chromatography. Fractions were assayed for dNTPase activity as described in the Materials and methods section. Figure 3(b) shows the relevant portion of the resulting non-denaturing gel; the characteristic dNTPase signal is arrowed. Activity was eluted in fractions 20/21 over a fairly narrow range of salt concentration.

Figure 4(a) shows the absorbance profile obtained by gel filtration on a Superdex 75 (Pharmacia) column of partially purified enzyme derived from the Q-Sepharose fractionation described above. Figure 4(b) shows the corresponding distribution of dNTPase activity associated with the column fractions. The activity resides predominantly in the excluded volume, fractions 19/20, suggesting a size in excess of 100 kDa. This value is in agreement with earlier reported values of about 200 kDa derived from h.p.l.c. gel-filtration studies (Dalton et al., 1992). Incubation of crude extract with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ followed by gel filtration gave the profile shown in Figure 4(c). All of the radioactivity applied to the column in the extract sample was

Figure 4 Gel filtration on Superdex 75

(a) Superdex 75 gel-filtration profile of fractions derived from Q-Sepharose chromatography as detailed in Figure 3. (b) PNB⁺ formation was monitored via non-denaturing PAGE of each fraction after preincubation with [α - 32 P]dATP (see the Materials and methods section). Fraction numbers assayed are given above the relevant lane of the gel. Only the relevant portion of the non-denaturing gel is shown, i.e. the region containing the PNB⁺ signal, to allow a degree of alignment with the elution profile. (c) Superdex 75 gel-filtration profile of crude extract after preincubation with [α - 32 P]dATP. The dotted line represents the profile obtained for gel-filtration molecular mass markers (Bio-Rad). These peaks correspond to: 1, protein aggregates; 2, γ -globulin (158 kDa); 3, ovalbumin (44 kDa); 4, myoglobin (17 kDa); 5, vitamin B₁₂ (1.35 kDa). (d) Non-denaturing PAGE of fractions derived from gel filtration of [α - 32 P]dATP preincubated extract on a Superdex 75 column. Fraction numbers are given above each lane of the gel.

found in a group of six fractions at the low-molecular-mass end of the profile, with each fraction containing the same amount of radioactive material. Direct application of these fractions to a non-denaturing gel (Figure 4d) revealed that PNB⁺ is present in only some of these fractions (nos. 26–28), with the rest containing free dATP. PNB⁺ was not detected in the excluded fractions, where dNTPase activity is known to reside (results not shown). Calibration of the column with molecular mass markers (Figure 4c; dotted profile) suggests a molecular mass for PNB⁺ of 2.5–3 kDa. (Under these conditions, dAMP, dADP and dATP standards run anomalously at equivalent molecular masses centred at approx. 1.6 kDa.) The size discrepancies between the

Figure 5 Gel-retardation analysis of fractions derived from continuous gel electrophoresis of pig extract preincubated with [α - 32 P]dATP

Crude extract was pre-incubated with [α - 32 P]dATP for 120 min and then fractionated by continuous elution gel electrophoresis (see the Materials and methods section). Eluted fractions were assayed for the presence of PNB⁺ by direct application to a non-denaturing gel, the autoradiograph of which is shown. The arrow above the control lane, C, denotes increasing fraction numbers in the order that they eluted from the preparative gel. The lane marked with a black triangle represents the fraction used in subsequent t.l.c. and denaturing experiments. PNB⁺ is arrowed on the right hand side of the Figure.

activity itself and the labelled product indicate that they are distinctly separate aspects of the dNTPase reaction, i.e. the hydrolytic activity itself is not directly responsible for retardation of the hydrolysed nucleotide.

The product of dNTPase hydrolysis comprises dAMP/dADP bound to an unidentified species

In order to confirm that the dNTPase signal was not simply an anomalous electrophoretic migration of, for example, free dADP or dAMP, especially in the light of the small degree of overlap found in elution profiles between nucleotide standards and PNB⁺ fractions during gel-filtration analysis, t.l.c. was performed on purified samples.

The use of continuous elution PAGE has facilitated the isolation of PNB⁺ away from free dATP and the majority of contaminating proteins. Figure 5 shows the results of gel-retardation analysis of fractions derived from continuous elution gel electrophoresis of pig extract preincubated with [α - 32 P]dATP. PNB⁺ is evident in a small number of grouped fractions and is well defined relative to the low background of non-specifically retarded dATP, suggesting uniformity of size and charge. SDS/PAGE of continuously eluted fractions failed to resolve PNB⁺ away from the dye front (results not shown).

PNB⁺ purified in the above manner was applied to a poly(ethylene imine) cellulose sheet and subjected to t.l.c. (In the absence of readily available radiolabelled dADP or dAMP, direct mobility comparison via gel-retardation assay was not possible.) The results shown in Figure 6 reveal a marked difference in mobility between the dATP, dADP and dAMP standards, the ATP and ADP standards and that of PNB⁺, and confirm that this labelled product is not simply the result of dephosphorylation of dATP and/or conversion to the ribose forms.

The dAMP/dADP nucleotide is covalently bound as part of PNB⁺

The ability of the retardation signal to be maintained throughout the purification suggests a strong interaction between the dAMP/dADP moiety within PNB⁺. Moreover, retardation assays can be run in 1% SDS with no loss of the radioactive signal (K. Ford and D. Hornby, unpublished work). In an effort

Figure 6 T.L.C. of purified PNBp* and nucleotide standards

T.L.C. was performed on poly(ethylene imine)-cellulose as described. The reaction contained 3 μ l of continuously eluted PNBp* plus 2 μ l of a dATP/dADP/dAMP standard nucleotide mixture. This was spotted at the origin of the sheet prior to running. The result shown is an autoradiograph. Deoxyribonucleotide (and ribonucleotide) standards were visualized by u.v. shadowing and their positions have been marked.

Figure 7 Stability of the interaction between dAMP/dADP and PNBp*

(a) The standard dNTPase assay was performed either after (lane 1) or before (lane 2) heat treatment of crude extract, and the presence of PNBp* was assayed by non-denaturing PAGE. Treatment was at 75 °C for 10 min. (b) The stability of purified PNBp* was assayed via non-denaturing PAGE. PNBp* derived from continuous elution gel electrophoresis was subjected to the following conditions prior to electrophoresis. Lane C contains a standard dNTPase assay using 1 μ l of crude extract. Lane 1, PNBp* (3 μ l) preincubated in 0.5% SDS; 2, as lane 1 plus 10 μ g of proteinase K; 3, PNBp* (3 μ l) preincubated with 10 μ g of Pronase; 4, PNBp* recovered from the aqueous phase of a single phenol extraction of continuously eluted sample; 5, PNBp* heated to 75 °C for 10 min prior to electrophoresis; 6, PNBp* preincubated with 30 μ M dATP. All preincubations were for 30 min at 37 °C prior to electrophoresis. The arrow indicates PNBp*.

to address the nature of this interaction, a number of key experiments were performed.

Heat denaturation of enzyme preparations (75 °C, 10 min) abolished activity (Figure 7a, lane 1). However, similar heat treatment of the extract after preincubation with [α - 32 P]dATP revealed that PNBp* is insensitive to heat denaturation (Figure 7a, lane 2). Purified PNBp* isolated using continuous elution electrophoresis was subjected to a number of denaturing processes, as detailed in the legend to Figure 7(b), prior to gel electrophoresis. Its resistance to 0.5% SDS, proteinase K digestion in 0.5% SDS and to phenol extraction suggests it has a small compact structure. Purified PNBp* shows a similar resistance to heat treatment as does PNBp* in crude extract, suggesting that heat insensitivity is genuine and not the result of stabilization by non-specifically associating proteins. Preincubation of PNBp* with excess unlabelled dATP (30 μ M final concentration; Figure 7b, lane 6) prior to electrophoresis had no effect on the intensity or mobility of the retarded band. All the above results are in accordance with covalent retention of the hydrolysed nucleotide within the PNBp* complex.

DISCUSSION

The initial assignment of the enzyme activity reported by us previously (Dalton et al., 1992) as a deoxyribonucleotidase now appears a somewhat simplistic view in the light of our recent findings, which are discussed below. Traditionally deoxyribonucleotidases, such as those found in human erythrocytes for example (Paglia et al., 1984), selectively dephosphorylate deoxyribonucleotide monophosphates, e.g. dAMP, in a straightforward hydrolysis reaction. Clearly the dNTPase reported here does not fall precisely into this category. A possible reaction scheme for dNTPase activity is shown in Scheme 1.

We have shown that, during the course of its reaction, the dNTPase activity resolves ostensibly into two components. The first is a protein complex that has previously been shown to be approx. 200 kDa in size and is responsible for the hydrolysis of dNTP. The second component, PNBp*, results from incubation of the dNTPase fraction with [α - 32 P]dATP (or dNTPs in general). The identity of the PNBp* component is as yet unknown. Previous experiments have shown it to be resistant to DNAase, RNAase and S1 nuclease, suggesting that it is not a polynucleotide (Dalton, 1991). PNBp* also partitions into the aqueous phase when subjected to chloroform/methanol/HCl extraction, suggesting that it is non-lipid in nature (K. Ford and D. Hornby, unpublished work). Addition of lectins to the dNTPase assay has demonstrably failed to either abolish PNBp* formation or supershift the PNBp* species (Dalton, 1991). The latter result suggests that PNBp* is not a polysaccharide, although the specificity of lectins for sugar is not sufficiently broad to eliminate this possibility. One last possibility is that

Scheme 1 A proposed reaction schematic for dNTPase activity.

E + S represents the 200 kD hydrolytic activity, where S represents unliganded PNBp*. E-S $\sim$ dADP is an intermediate state in the reaction that is not observed experimentally and therefore may exist for only a short time. The resulting dissociated species S $\sim$ dADP (or dAMP), represents PNBp*.

PNBP* is peptidic in nature. Since PNBP* can currently only be identified by autoradiographic methods, the existence of unliganded PNBP* (or a precursor) has yet to be ascertained.

We have demonstrated that dNTPase activity only resides in the gel-filtration exclusion volume and not in the 2.5–3 kDa range defined by PNBP*. The complete absence of any retarded band in gel-shift assays of the low-molecular-mass fractions suggests that, should unliganded PNBP* exist outside its usual location as part of the dNTPase active complex, then it has no dATP binding or hydrolysing activity associated with it as an independent protein.

The extent of retardation of hydrolysed nucleotide (dAMP or dADP) is significant relative to the mobility of free dATP. Comparison with DNA size markers, for example (Figure 1), shows that the retarded nucleotide migrates to the approximate position of a 200 bp DNA fragment. T.l.c. analysis shows marked differences in mobility between the nucleotide standards and PNBP* sample (Figure 6), demonstrating that the labelled species cannot be the product of simple hydrolysis of dATP down to dADP or dAMP, but represents a more complex association of hydrolysed nucleotide with an as yet unidentified component. The resistance of PNBP* to heat, phenol extraction and proteolysis certainly suggests both a covalent attachment of the α -phosphate moiety at minimum and an unusual tertiary structure for the complex as a whole.

Covalent retention of the hydrolysed nucleotide would seem to exclude a role for the dNTPase in dNTP pool maintenance on the grounds that PNBP* can then only be viewed as a dead-end complex and its formation would be energetically unfavourable. The nuclear location of the dNTPase combined with the relatively large concentrations of dNTPs found in the cytosol of actively replicating cells reinforces this view. A role for the dNTPase in either facilitated nucleobase or nucleotide/nucleoside transport (for review, see Plagemann et al., 1998), or in nucleotidase activity, is similarly excluded.

Given the possible association of the dNTPase activity with

the solid phase of the nucleus (most of the dNTPase activity is recovered from agitation of the nuclear pellet after homogenization and centrifugation), PNBP* is an attractive candidate for a component in a signal transduction pathway which may respond to elevated dNTP levels such as those which occur at the onset of DNA replication. The small size of the complex would facilitate a passive diffusion process across the nuclear membrane, although it has been shown that small proteins up to 40 or 50 kDa in size are able to equilibrate across the nuclear envelope (for reviews see Goldfarb, 1989; Hanover, 1992). We are currently purifying PNBP* to enable a more thorough analysis.

We thank the SERC for their support of this work through the award of a postdoctoral fellowship to K.F. The Krebs Institute has been designated by the SERC as a centre for studies in molecular recognition.

REFERENCES

- Dalton, A. (1991) Ph.D. Thesis, University of Sheffield, U.K.
- Dalton, A., Hornby, D., Langston, S. and Blackburn, G. (1992) *Biochem. J.* **287**, 871–879
- Dignam, J., Lebovitz, R. and Roeder, R. (1983) *Nucleic Acids. Res.* **11**, 1475–1489
- Goldfarb, D. (1989) *Curr. Opin. Cell Biol.* **1**, 441–446
- Gorbalenya, A. and Koonin, E. (1990) *J. Mol. Biol.* **213**, 583–592
- Hanover, J. (1992) *FASEB. J.* **6**, 2288–2295
- Imamoto-Sanobe, N., Yoneda, Y., Iwamoto, R., Sugawa, H. and Uchida, T. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3426–3430
- Manermaa, R.-M. and Oikarinen, J. (1992) *Biochem. Biophys. Res. Commun.* **182**, 309–317
- Matson, S. and Kaiser-Rogers, K. (1990) *Annu. Rev. Biochem.* **59**, 289–329
- Oikarinen, J. (1991) *FEBS Lett.* **294**, 6–10
- Paglia, D., Valentine, W. and Brockway, R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 588–592
- Plagemann, P., Wohlhueter, R. and Woffendin, C. (1988) *Biochim. Biophys. Acta* **947**, 405–443
- Reichard, P. (1988) *Annu. Rev. Biochem.* **57**, 349–375
- Saluz, H. and Jost, J. (1987) *A Laboratory Guide to Genomic Sequencing*, pp. 28–29, Birkhauser, Basel and Boston
- Sambrook, J., Fritsch, E. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY