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Bluetongue virus core protein VP4 has nucleoside triphosphate phosphohydrolase activity

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The intact virion of bluetongue virus comprises ten segments of dsRNA enclosed in two concentric protein capsids. The core, which is transcriptionally active, includes three minor proteins (VP1, VP4 and VP6) which are considered to be the candidates for the core-associated enzymes that transcribe and modify full-length mRNA copies for each of the ten genome segments. Using purified recombinant VP4 protein and core-like particles containing VP4, in this report it is demonstrated that VP4 has nucleoside triphosphatase (NTPase) activity. VP4 is a non-specific NTPase that hydrolyses four types of ribonucleoside triphosphate (NTP) to the corresponding nucleoside diphosphate. The substrate preference was GTP ATP UTP CTP. NTP hydrolysis by VP4 was maximal when the Mg^{2} or Ca^{2} ion concentrations were 4 mM or 6 mM, respectively. The presence of single-stranded polynucleotides poly(A), poly(U) and poly(C) had little effect on the NTPase activity. Although the enzyme exhibited a broad temperature optimum around 40 °C, the pH optimum was sharp, between pH 7·5 and 8. The K_m and V_{max} of ATP hydrolysis were calculated to be 0·25 0·05 μM ATP and 55 4 pmol ATP hydrolysed min ^{1} μg ^{1}, respectively. The K_m was affected by the addition of poly(A) to only a small extent in contrast to the V_{max}, which was increased by at least twofold.

Introduction

Nucleoside triphosphate phosphohydrolase (NTPase) activity is important for transcription (Lowery & Richardson, 1977), RNA processing (Schwer & Guthrie, 1991) and translation (Grifo *et al.*, 1984). Two vaccinia virus enzymes packaged inside the infectious virus particle have nucleic acid-dependent NTPase activities that were among the first to be reported (Paoletti & Moss, 1974). Since then NTPase activities have been found to be associated with the gene products of RNA viruses such as vesicular stomatitis virus (Testa & Banerjee, 1979), yellow fever virus (Warrener *et al.*, 1993) and Japanese encephalitis virus (Kuo *et al.*, 1996).

NTPase activities have also been identified for the gene products of dsRNA viruses such as reovirus (Borsa *et al.*, 1970; Kapuler *et al.*, 1970; Noble & Nibert, 1997) and bacteriophage ϕ6 (Gottieb *et al.*, 1992; Paatero *et al.*, 1995). In reovirus the NTPase activity was shown to be associated with purified cores and assigned to the λ1 protein, the viral guanylyltransferase. For bluetongue virus (BTV) recent studies have shown that the virion guanylyltransferase is the minor virion protein VP4 (Costas *et al.*, 1998).

The intact virion of BTV comprises ten segments of dsRNA enclosed by two concentric capsids involving seven proteins (VP1–VP7). Virions can be partially disassembled *in vitro* by removing the two outer capsid proteins (VP2 and VP5) to give transcriptionally active core particles. The core includes two major structural proteins (VP3 and VP7) and three minor proteins (VP1, VP4 and VP6). These minor proteins are considered to be the candidates for the core-associated enzymes that transcribe and modify full-length mRNA copies for each of the ten RNA segments. A recombinant baculovirus-expressed VP1 protein has been shown in *in vitro* assays to catalyse the synthesis of poly(A) when provided with a poly(U) template and an oligo(A) primer (Urakawa *et al.*, 1989). The second minor core protein, VP6, is an ATP-dependent RNA helicase that catalyses the unwinding of 3′-overhang, 5′-overhang or blunt-ended duplex RNAs in the presence of divalent cations and ATP (Staueber *et al.*, 1997). The third minor core protein, VP4, which covalently binds NTPs (and

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dNTPs except dCTP), has been shown to have guanylyltransferase activity (LeBlois *et al*., 1992; Costas *et al*., 1998). Recently, we have demonstrated that baculovirus-expressed, purified VP4 exists as dimers in solution, and that a leucine zipper at the carboxy terminus is responsible for the dimerization and encapsidation of VP4 within the co-expressed core-like particles (CLPs) composed of VP3 and VP7 (Ramadevi *et al*., 1998).

In this report we have used purified recombinant VP4 protein to demonstrate that it has NTPase activity in addition to its ability to cap mRNA. In order to characterize the activity we have determined the K_m and V_{max} values of the enzyme and investigated its substrate specificity.

Methods

■ **Viruses and cells.** *Spodoptera frugiperda* cells (Sf9) cells were grown in TC100 (Gibco BRL) medium supplemented with 10% (v/v) foetal calf serum and incubated either in suspension or in monolayer cultures at 28 °C. Recombinant *Autographa californica* nuclear polyhedrosis virus that expresses the VP4 of BTV serotype 10 (AcBTV10VP4) was propagated in suspension culture as described previously (LeBlois *et al*., 1992).

■ **Purification of VP4 from recombinant baculovirus-infected Sf9 cells.** The recombinant VP4 protein was recovered from AcBTV10VP4-infected Sf9 cultures and the soluble form of the protein purified as described previously (Ramadevi *et al*., 1998). Recombinant baculovirus-expressed CLPs were recovered from Sf9 cells infected with AcBTV17VP3–BTV10VP7 and AcBTV10VP4 (LeBlois *et al*., 1992). The infected cells were grown in suspension culture at 28 °C for 72 h, the cells harvested and CLPs recovered from cell extracts following sucrose gradient (66–30%, w/v) centrifugation as described previously (French & Roy, 1990). The presence of VP3, VP7 and VP4 proteins in the particles was confirmed by SDS–PAGE and Western blot analyses using anti-BTV-10 polyclonal antibodies.

■ **NTPase reactions and colorimetric assay for phosphate ions (PO_4^{3-}).** The NTPase reactions were performed as described by Noble & Nibert (1997) in 1·5 ml microfuge tubes for ease of manipulation and incubation, and then transferred onto a 96-well microplate for development and measurement of PO_4^{3-} (Chen *et al*., 1956). Standard NTPase reaction mixtures contained 30 mM Tris–HCl pH 7·5, 4 mM $MgCl_2$, 0·1 mM DTT, 0·15 mg/ml purified VP4 protein and 1 mM ATP in a total volume of 50 µl. Reaction components were mixed on ice, incubated at 37 °C for 30 min and then returned to ice. Termination of each reaction was ensured by the addition of an equal volume of 10% (w/v) trichloroacetic acid (TCA). To measure the amount of PO_4^{3-} in each sample, 100 µl of each reaction mixture was transferred to a 96-well plate and mixed with an equal volume of freshly prepared colorimetric reagent (3 vols 0·8% ammonium molybdate, 1 vol. 3 M sulphuric acid, 1 vol. 10%, w/v, ascorbic acid) then incubated at 37 °C for 30 min. During development, a blue coloured, reduced phosphomolybdate complex was formed, which was assayed by absorbance at 655 nm (A_{655}) in a microplate reader (Bio-Rad). In each experiment, samples containing ATP but no protein were included as controls to estimate background PO_4^{3-} release due to non-enzymatic hydrolysis of ATP. A standardization curve for K_2HPO_4 (0, 2, 5, 10, 20, 40 and 100 nmol, in 100 µl samples and assayed as described above) was prepared. A_{655} values were converted to amounts of PO_4^{3-} released per reaction using these data (mean value of $0{\cdot}025 \pm 0{\cdot}003$ A_{655} units per nmol PO_4^{3-}).

■ **Thin layer chromatography (TLC) assay for NTPase products.** For an initial demonstration of NTPase activity, and for the determination of K_m and V_{max} values, TLC was used. Reaction mixtures were similar to those described by Warrener *et al*. (1993) with slight modifications. Briefly, reaction mixtures contained 30 mM Tris–HCl pH 7·5, 4 mM $MgCl_2$, 0·1 mM DTT, 3·3 pmol [α-^{32}P]ATP (2·5 Ci/mmol), or 12·4 pmol [α-^{32}P]GTP (12 Ci/mmol) and 1·5 µg recombinant VP4 proteins with or without 0·8 µg of the indicated polynucleotides in a total volume of 10 µl. The mixtures were incubated at 37 °C for 30 min and each reaction stopped by the addition of EDTA to a final concentration of 20 mM. One µl of each reaction product was spotted onto plastic-backed polyethyleneamine–cellulose TLC sheet (Merck) and developed by ascending chromatography in 0·75 M potassium phosphate buffer pH 3·5. After drying, the sheets were exposed to X-ray film. For quantitative evaluation, the radiolabelled spots identified in the X-ray film were excised and the radioactivity was measured using a scintillation counter.

Results

Purified BTV VP4 and CLPs containing VP4 exhibit nucleoside triphosphate hydrolase activities

To demonstrate the NTPase activity of VP4, purified VP4, CLPs and CLPs containing VP4 were incubated with either [α-^{32}P]ATP or [α-^{32}P]GTP and assayed for the release of di- and monophosphates using TLC. Triphosphates in the samples containing either purified VP4 or the CLPs containing VP4 were hydrolysed to their respective diphosphates (Fig. 1, lanes 3, 4, 7 and 8). This was not observed for the reactions of CLPs lacking VP4 (Fig. 1, lanes 2 and 6). When [α-^{32}P]ATP was incubated with either CLPs containing VP4 or purified VP4 only, ATP was converted to ADP and there was no detectable conversion to AMP (Fig. 1, lanes 3 and 4). When [α-^{32}P]GTP was incubated with CLPs containing VP4, or purified VP4, GTP was converted to GDP, and only very low quantities of GMP were detected (Fig. 1, lanes 7 and 8). Other nucleotide substrates like UTP and CTP were also hydrolysed to their respective diphosphates and no monophosphates were detected (data not shown). The data indicated that purified recombinant VP4 exhibits NTPase activity whether in the form of purified protein or when associated with CLPs.

Determination of nucleotide substrate specificity for the VP4 NTPase activity

To determine whether the NTPase activity of VP4 exhibited substrate specificity, a variety of nucleotides were used as substrates. For these experiments, a colorimetric assay was utilized to detect the release of PO_4^{3-} (Chen *et al*., 1956; Noble & Nibert, 1997). This allowed us to measure the amounts of PO_4^{3-} released due to nucleotide hydrolysis. When GDP or GMP were used as substrates, very low levels (0·5% for GDP, 0·2% for GMP) of hydrolysis were observed with VP4 (data not shown). In contrast, all four ribonucleoside triphosphates were hydrolysed completely, although at different efficiencies. The relative rates of hydrolysis were in the

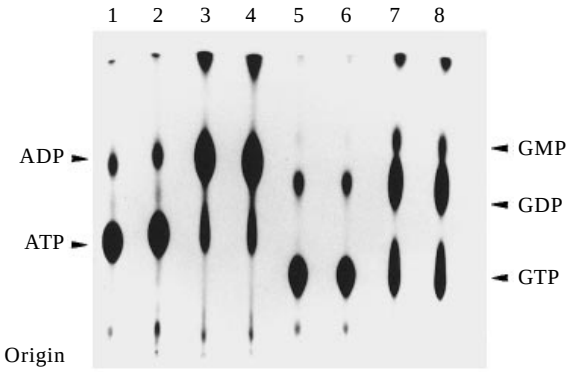

Fig. 1. Nucleotide products of ATP and GTP hydrolysis by VP4 protein. Standard reaction mixtures included 30 mM Tris–HCl pH 7·5, 4 mM $MgCl_2$, 0·1 mM DTT and either 3·3 pmol [α-^{32}P]ATP (2·5 Ci/mmol) (lanes 1–4) or 12·4 pmol [α-^{32}P]GTP (12 Ci/mmol) (lanes 5–8). Reactions were incubated at 37 °C for 30 min. After the reactions were stopped with EDTA, the reaction products were spotted on TLC plates and developed by ascending chromatography in 0·75 M KH_2PO_4 pH 3·5. The positions of origin, ATP, ADP, GTP, GDP, GMP were first determined by UV absorption of cold markers, and by autoradiography of labelled products. The [α-^{32}P]ATP reaction mixtures were incubated with buffer only (lane 1), CLPs consisting of VP3 and VP7 only (lane 2), purified VP4 protein (lane 3) or CLPs encapsidating VP4 (lane 4). The [α-^{32}P]GTP reaction mixtures were incubated with buffer only (lane 5), CLPs consisting of VP3 and VP7 (lane 6), purified VP4 protein (lane 7) or CLPs containing VP4 (lane 8).

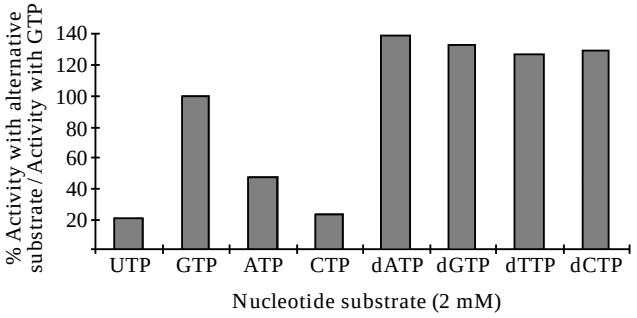

Fig. 2. Determination of the substrate specificity for the NTPase activity of VP4. Reactions contained the non-radioactive substrate at 2 mM concentration. Reactions were stopped by adding an equal volume of 10% TCA. To measure the amount of PO_4^{3-} released, 100 µl of the substrate reaction mixtures were mixed with an equal volume of freshly prepared colorimetric reagent, then incubated at 37 °C for 30 min. The blue coloured, reduced phosphomolybdate complex was quantified by measuring at A_{655}. The relative activity of other substrates was calculated by taking the activity observed in the presence of GTP as 100%.

order GTP ATP UTP CTP (Fig. 2). Interestingly, each mRNA species of BTV has a guanosine at the 5′ end which is capped and methylated (Roy *et al.*, 1990). ATP was hydrolysed to ADP at about 40% of the efficiency of GTP, whereas UTP and CTP were hydrolysed at 20% efficiency. Similarly, dNTPs were hydrolysed to dNDPs by VP4, although in contrast to the ribonucleotides dATP was hydrolysed more efficiently than dGTP, and each of the dNTPs was hydrolysed more efficiently than its counterpart ribonucleotide (Fig. 2). The data indicate that VP4 has non-specific NTPase activity.

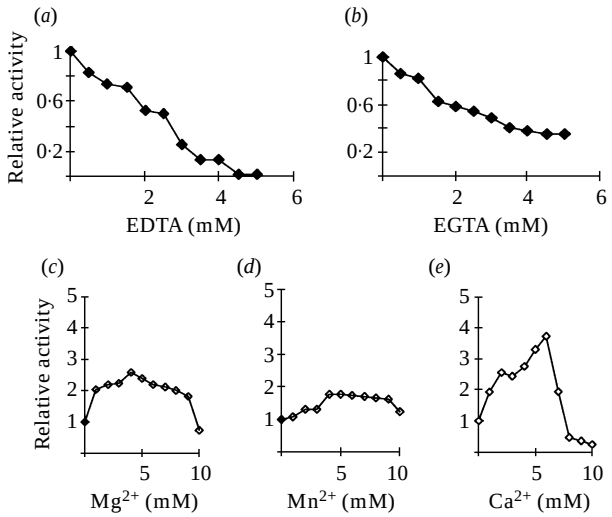

Fig. 3. Effect of divalent cations and chelating agents on VP4 ATPase activity. Reactions were performed as described in Fig. 2, except that no divalent cations were added to the reaction mixtures. The effect of EDTA (*a*) or EGTA (*b*) was studied by adding the indicated amounts of either EDTA or EGTA. The relative effect of EDTA and EGTA was calculated relative to the value obtained with no added EDTA or EGTA. The effects of $MgCl_2$ (*c*), $MnCl_2$ (*d*) or $CaCl_2$ (*e*) were studied by including the respective divalent cations as indicated. The relative activity was calculated by taking the value obtained with no added divalent cations in the reaction as 1·0. Relative activity was plotted against the amount of added divalent cation.

The NTPase activity of VP4 requires divalent cations

Most viral proteins that possess NTPase activity require divalent cations to activate the enzyme efficiently. We therefore examined the divalent cation requirement of the VP4 NTPase activity by the addition of Mg^{2+}, Mn^{2+} or Ca^{2+} ions, and in the absence and presence of chelating agents such as EDTA and EGTA (Fig. 3). EDTA reduced the activity drastically (see Fig. 3*a*) and only 1% residual activity was observed in the presence of 4 mM EDTA. The addition of EGTA, on the other hand, reduced the NTPase activity by 30% (5 mM was the highest concentration tested; Fig. 3*b*). The effect of addition of Mg^{2+} ions is shown in Fig. 3(*c*). The NTPase activity increased rapidly with increasing Mg^{2+} concentration, reaching a maximum in the presence of 4 mM Mg^{2+}. However, higher Mg^{2+} concentrations had an inhibitory effect. While Mg^{2+} ion addition stimulated the reaction up to 2·5-fold, addition of Mn^{2+} stimulated it some 1·7-fold (Fig. 3*d*). For both Mg^{2+} and Mn^{2+} ions, the NTPase activity for reactions containing 1 mM ATP reached its maximum when the added cation concentration was 4 mM, slightly lower than for Ca^{2+} addition, for which the reaction reached its maximum at 6 mM, with a stimulatory effect of some fourfold (Fig. 3*e*). The profiles of ATP hydrolysis for VP4 were very similar in the presence of either $MgCl_2$ or $MnCl_2$. However, with 5 mM ATP as substrate, the profiles of ATPase activity in the presence of Mg^{2+} or Mn^{2+} were different and the maximum activity was observed when Mg^{2+} and Mn^{2+} concentrations

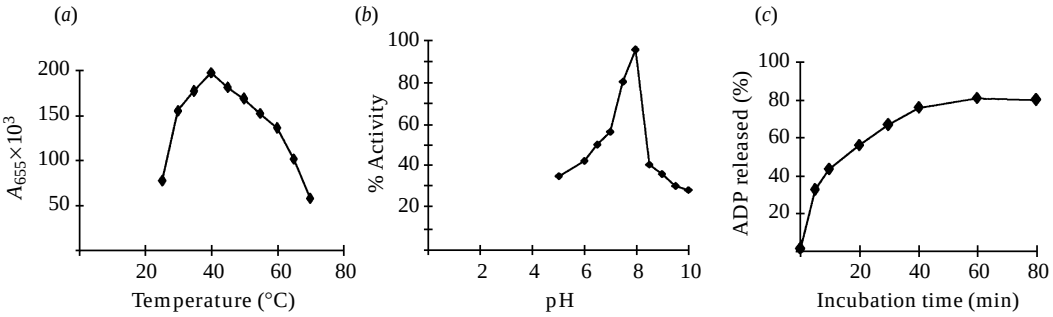

Fig. 4. (*a*) Temperature dependence of VP4 ATPase activity. Reactions were incubated at different temperatures as indicated. The amount of PO_4^{3-} released was measured at A_{655}. (*b*) The pH dependence of the VP4 ATPase activity was determined using [α-^{32}P]ATP as substrate. The reaction mixtures were buffered at the indicated pH. The percentage activity was calculated from the amount of ATP hydrolysed to ADP. (*c*) The kinetics of conversion of [α-^{32}P]ATP to [α-^{32}P]ADP were determined.

were nearly 6 mM (data not shown). This suggests that the formation of complexes between ATP and divalent cations is important for the level of activity. These results suggest that the NTPase activity is dependent on the presence of divalent cations, and that the purified protein most likely has some bound divalent cations. This issue was not investigated further.

The *in vitro* NTPase activity of VP4 is influenced by the reaction pH and temperature

The rate and duration of the activity of an enzyme is commonly based on enzyme concentration at defined conditions of pH and temperature. To determine the optimum temperature for the VP4 NTPase activity, we performed assays at temperatures from 25 to 70 °C. ATP hydrolysis increased with temperature until 40 °C, above which the activity declined rapidly. When the NTPase activity of VP4 was monitored using reaction mixtures buffered at different pH (Fig. 4 *b*), the optimum activity was achieved between pH 7·5 and 8·0.

At pH 8·0 and 37 °C, some 3·3 pmol [α-^{32}P]ATP hydrolysis was achieved by 1·5 µg purified VP4 in 40 min (Fig. 4 *c*), by which time 78% of the ATP was hydrolysed.

Does nucleic acid stimulate the NTPase activity of VP4?

For many viral NTPases, the activity is stimulated (to different degrees) by the presence of nucleic acids (DNA/RNA or homopolymers). To investigate whether the BTV VP4 NTPase activity exhibited similar properties, the effect of the presence of a variety of polynucleotides, poly(U), poly(A), poly(C) and poly(G), as well as ss- and dsDNA, was tested. No enhancement in activity was detected when ssDNA (M13 DNA) or dsDNA (pUC19) was present, although a slight stimulation (1·2–1·4-fold) was detected when poly(U), poly(C) or poly(A) was used. Interestingly, poly(G) exhibited no effect on the NTPase activity (data not shown). With poly(A), the maximum activity was observed when the concentration was 0·8 µg. The data indicate that the activity was not dependent on the presence of nucleic acids, in contrast to the effect of nucleic acids on the BTV VP6 helicase (Staueber *et al.*, 1997).

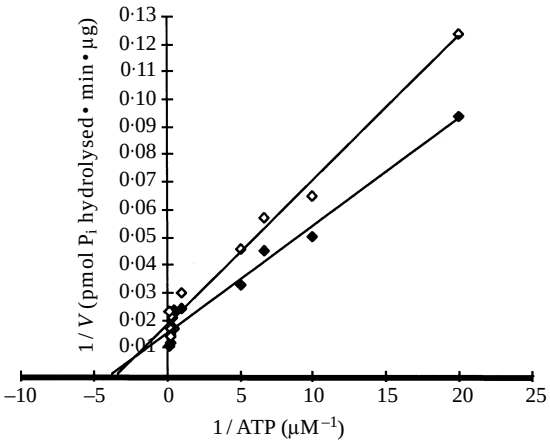

Fig. 5. Lineweaver–Burk plot of VP4 ATPase activity in the presence and absence of poly(A). The ATPase reactions contained 2 µCi [α-^{32}P]ATP plus non-radioactive ATP (0·2, 0·4, 0·6, 0·8, 1, 2, 3, 4 or 5 µM). The K_m and V_{max} values were determined by the least square estimates, with standard errors of the estimates. ◆, Reactions with added poly(A); ◇, reactions without any added poly(A).

The effect of VP4 concentration on the rate of ATP hydrolysis

The NTPase activity of VP4 was further characterized by determining whether the initial velocity of the hydrolysis was proportional to the concentration of VP4. Experiments were performed using variable amounts (1–7·5 µg per 10 µl reactions) of protein. At higher concentrations (e.g. 7·5 µg) ATP hydrolysis was not linear with time. However, in the presence of 1 and 1·5 µg protein, the reaction proceeded in a linear fashion with time (data not shown).

To characterize the enzyme activity further, we determined the V_{max} and the Michaelis constant (K_m). The reactions were carried out with 1·5 µg VP4 protein in the presence of variable amounts of ATP and a constant amount of [α-^{32}P]ATP. The K_m

and V_{max} were determined in the presence and absence of poly(A) using least square estimates ($\pm$SE of the estimate) on Eadie–Hofstee direct plots, and the results are presented as a Lineweaver–Burk double-reciprocal plot (Fig. 5). As expected, the ATPase activity of the VP4 protein followed Michaelis–Menten kinetics, yielding a straight line on a Lineweaver–Burk plot. The K_m and V_{max} for the VP4 protein in the absence of added poly(A) was $0{\cdot}25 \pm 0{\cdot}05$ μM ATP and 55 ± 4 pmol ATP hydrolysed $min^{-1}\,\mu g^{-1}$, respectively. The avidity for ATP hydrolysis decreased 1·3-fold in the presence of poly(A), with an increasing of the K_m to $0{\cdot}26 \pm 0{\cdot}04$ μM ATP (Fig. 5). However, as expected in the presence of added poly(A), the V_{max} value increased significantly from 55 pmol ATP hydrolysed $min^{-1}\,\mu g^{-1}$ to 71·4 pmol ATP hydrolysed $min^{-1}\,\mu g^{-1}$. When calculated, the total increase was equivalent to some 1·3-fold over the V_{max} value obtained in the absence of poly(A).

Discussion

The BTV minor core protein VP4 has been shown to have NTPase activity in addition to the previously reported guanylyltransferase and RNA 5′-triphosphatase activities. Like the NTPase proteins of other dsRNA viruses, BTV VP4 prefers purine over pyrimidine nucleotides. Although NTP hydrolysis products were predominantly NDPs, in some reactions (e.g. when GTP was used as substrate) a low level of monophosphate was also produced. Most likely the BTV VP4 protein cleaves GTP into GMP prior to cap formation of the 5′ end of the diphosphate-ended mRNA made during transcription. Like other viral NTPases, the VP4 enzyme is stimulated by added divalent cations. The activity observed in the absence of added cations probably indicates the presence of endogenous cations, since the activity was essentially abrogated by addition of EDTA. In the presence of EGTA some activity was still detected, suggesting that bound Ca^{2+} ions may be present in the purified VP4 preparations. This issue has not been investigated further. Like reoviruses, the NTPase activity of BTV VP4 exhibited very distinct pH (7·0–8·0) and temperature (37–40 °C) optima.

We characterized the NTPase activity of BTV VP4 by determining both K_m and V_{max} values using ATP as a substrate. The K_m for ATP hydrolysis was in the range 0·25–0·26 μM ATP, which is similar to that of other viral NTPase enzymes such as those of tamarillo mosaic potyvirus (Eagles *et al.*, 1994), the large T-antigen of simian virus 40 (Scheffner *et al.*, 1989) and the NS3 protein of yellow fever virus (Warrener *et al.*, 1993). A slight decrease in ATP avidity upon poly(A) addition was compensated by an increase in the rate of ATP catalysis caused by an increase in V_{max}.

For many viruses, an RNA-dependent NTPase activity has been observed in association with viral helicase enzymes (Warrener *et al.*, 1993; Tamura *et al.*, 1993). For BTV, the VP6 protein has been shown to be an ATP-dependent helicase (Staueber *et al.*, 1997). Previous data showed that the BTV VP4 is a guanylyltransferase (LeBlois *et al.*, 1992), and more recent results indicate that VP4 also has 5′ RNA triphosphatase activity (Costas *et al.*, 1998). Since the 5′ terminus of each mRNA species of BTV starts with a guanosine residue, it is perhaps not surprising that GTP is the preferred substrate for the NTPase activity of VP4. This raises the question of whether the two activities are coupled and performed by the same domain of VP4. It is also possible that in virus cores the VP4 NTPase is involved in the process of extrusion of newly synthesized mRNA species out from virus cores into the cytoplasm. In this context, the NTPase activity of P4 protein of bacteriophage $\phi 6$ has been implicated in ssRNA packaging and assembly of procapsids (Gottlieb *et al.*, 1992).

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