Cloning and transcriptional regulation of the gene encoding the vacuolar/H^+ ATPase B subunit of *Dictyostelium discoideum*

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Abstract The main function of vacuolar H^+ ATPases in eukaryotic cells is to generate proton and electrochemical gradients across the membrane of inner compartments. We have isolated the gene encoding the B subunit of *Dictyostelium discoideum* vacuolar H^+ ATPase (*vatB*) and analyzed its transcriptional regulation. The deduced protein comprises 493 amino acids with a calculated molecular mass of 54 874 Da. The predicted protein sequence is highly homologous to previously determined V/H^+ ATPase B subunit sequences. The protein is encoded by a single gene in the *Dictyostelium* genome. The gene is maximally expressed during growth and it decreases during the first hours of development. Gene expression is rapidly enhanced by phagocytosis, but not by fluid-phase endocytosis. Acidic and alkaline conditions affect *vatB* gene expression differently.

Key words: V/H^+ ATPase; Transcriptional regulation; Phagocytosis; Endocytosis; *Dictyostelium*

1. Introduction

Vacuolar H^+ ATPases are ATP-dependent proton pumps responsible for acidification of the intracellular compartments of eukaryotic cells utilizing energy from the hydrolysis of ATP to generate H^+ gradients. They are multimeric enzymes composed of two distinct sectors each containing several subunits [1,2]. The catalytic part, V_1, is hydrophilic, contains the ATP-binding site and is responsible for the ATPase activity of the enzyme, whereas the transmembrane part, V_0, is highly hydrophobic and functions as a proton carrier across the membrane driving the protons into the lumen of the vacuole system. The V_1 sector is composed of different polypeptides designated A–F; the V_0 domain consists of at least three different subunits and two additional ones called a and c [2]. In *Dictyostelium* cells, unlike yeast, V/H^+ ATPases are associated with a variety of organelles including lysosomes, phagosomes, acidosomes and the contractile vacuole complex [3]. The vacuolar complex is an osmoregulatory organelle which has a special kidney-like function during water accumulation. It consists of a network of continuous membrane channels that are always present but change shape and organization during water accumulation [4]. V/H^+ pumps seem to play a crucial role in cellular life. Disruption of genes encoding the c subunit of the membrane sector and the B subunit of the catalytic sector result in conditional lethal mutants in yeast because the V/H^+ pump is strongly involved in acidification of endocytic compartments [5]. These mutants overcome the defect only if a low external pH allows for acidification by fluid phase endocytosis. In *Dictyostelium* two attempts have been made to obtain knockout mutants of two subunits of the V_0 sector, but both were not successful [6,7].

Here we report the primary structure and transcriptional regulation of the gene termed *vatB* encoding the B subunit, of the V_1 sector of the *Dictyostelium discoideum* V/H^+ ATPase. The gene was identified during analysis of mutants obtained by the REMI (restriction enzyme mediated integration) method, a method which is comparable to transposon tagging [8,9].

2. Materials and methods

2.1. Cell cultures

Dictyostelium discoideum strain AX2-214 (referred to as wild type strain) and the REMI mutant VIII-6 were grown at 21°C in axenic medium, pH 6.7, with shaking at 160 rpm [10].

2.2. Gene cloning

Genomic DNA from REMI mutant VIII-6 was extracted and purified by CsCl gradient centrifugation as described [11]. By digesting the genomic DNA with *Eco*RV we have recovered the inserted plasmid pUC-BsrΔBam [8] used to mutagenize the cells together with approximately 3.1 kbp of genomic flanking sequences carrying 1.1 kbp of A-T rich non-coding sequences and 1.69 kbp of sequences encoding the ATPase B subunit downstream of the insertion point of pUC-BsrΔBam. To clone the missing 3′ end of the gene a λgt11 cDNA library from growing cells was screened [12] using as probe a fragment of the ATPase gene belonging to a highly conserved region (aa 251–443). The clones obtained were sequenced with gene-specific primers using an automated sequencer (ABI 377 PRISM, Perkin Elmer, Norwalk, CT).

For sequence analysis the GCG package software was used (University of Wisconsin, Madison, WI) [13].

2.3. Development of D. discoideum

For analysis of development, cells grown to a density of $2–3\times10^6$ cells/ml were washed twice in 0.017 M Soerensen phosphate buffer, pH 6.0, resuspended at 1×10^7 cells/ml and allowed to develop on nitrocellulose filters (Millipore type HA, Molsheim, France) at 21°C as described [14]. For development in shaking suspension cells were washed as described above, resuspended in Soerensen phosphate buffer at a final concentration of 1×10^7 cells/ml and shaken at 160 rpm at 21°C. cAMP stimulation was done by pulsing cells in suspension with cAMP (2×10^{-8} M final concentration) using a syringe attached to a perfusion pump.

2.4. Incubation with bacteria and pH treatment

For studying effects of bacterial uptake on gene expression cells were allowed to develop for 2 h in suspension as described above, centrifuged and resuspended at a final concentration of 3×10^6/ml either in axenic medium or on *Escherichia coli* B/r at 1×10^{10}/ml in Soerensen phosphate buffer. At various time points samples were harvested and RNA was isolated after removal of bacteria.

For pH treatment cells at a concentration of 3×10^6 cells/ml were incubated in axenic medium of different pH values (pH 5.5, 6.7, 9.0,

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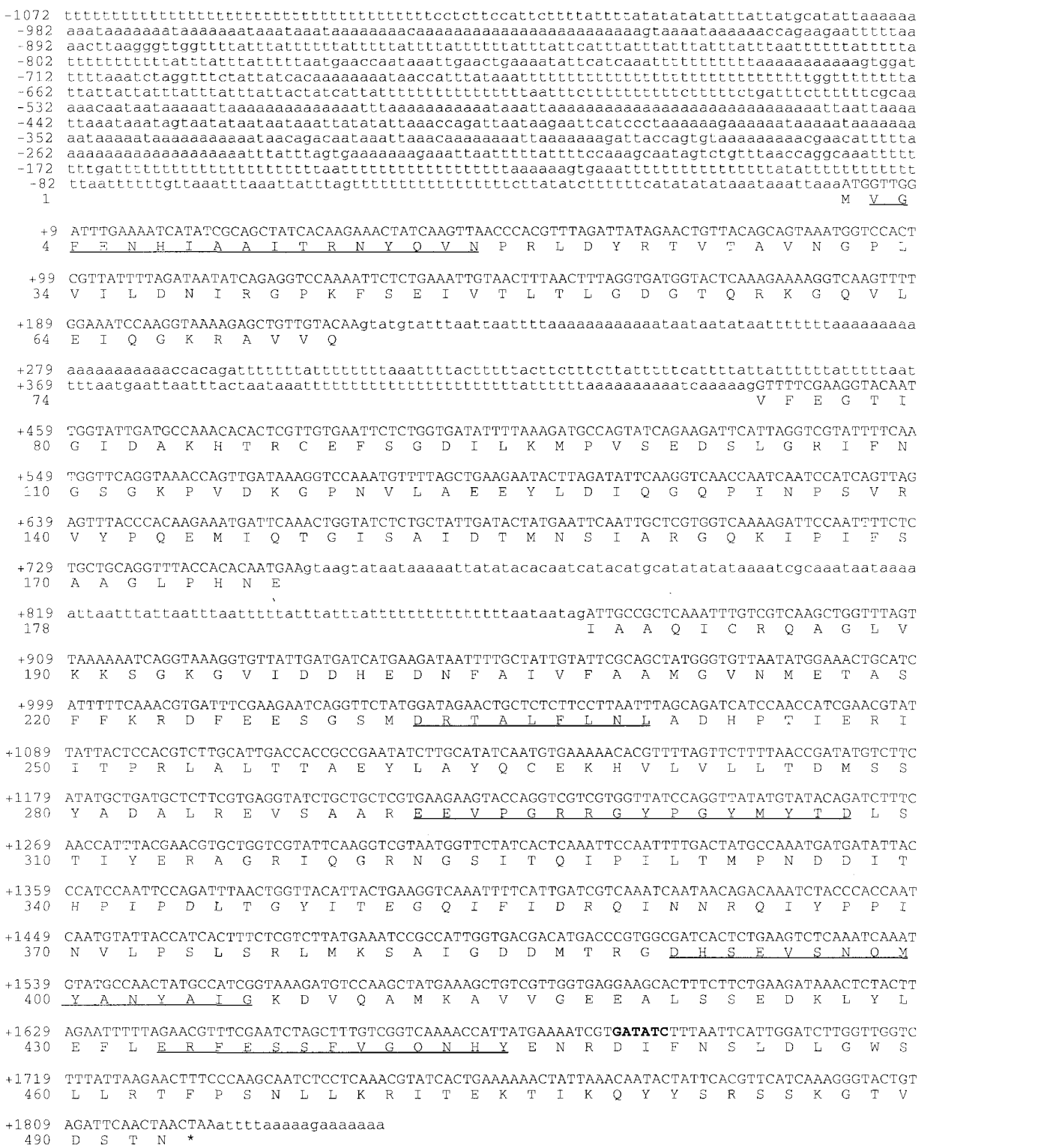

Fig. 1. Nucleotide and deduced primary sequence of *vatB*. Nucleotide residues are numbered starting at the first residue of the ATG encoding the putative initiation methionine. The coding sequence is shown in capital letters. The deduced amino acid sequence is displayed below the nucleotide sequence in the single letter code. Sequences upstream of the *Eco*RV site (bold characters) are derived from a genomic clone and downstream from a cDNA clone. An overlapping region of about 300 nucleotides was sequenced to confirm that both concern the same gene. Peptides previously determined by Adessi et al. [20] are underlined.

see below), and shaken at 21°C for 2 h. The axenic medium contained peptone, yeast extract and maltose in a 20 mM sodium potassium phosphate buffer [10]. The desired pH was established by altering the relative proportion of the buffering components (Na_2HPO_4 or KH_2PO_4). Aliquots of cells were harvested at various time points and used for RNA isolation.

2.5. Northern and Southern blotting

RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). For Northern blot analysis RNA was separated on 1.2% agarose gels in the presence of 6% formaldehyde [15] and blotted onto nylon membranes (Biodyne B, Pall Filtron, Dreieich, Germany). Genomic DNA was isolated as described [16]. For Southern blot analysis the DNA was separated in agarose gels, blotted onto nylon membrane and incubated with ^{32}P-labeled probes generated using a random prime labeling kit (Stratagene, La Jolla, CA). Hybridization was performed at 37°C for 15–16 h in hybridization buffer containing 50% formamide plus 2×SSC. Blots were washed twice for 5 min in 2×SSC and 0.01% SDS at room temperature followed by a 1 h wash in 50% formamide, 2×SSC at 37°C. The membranes were then exposed to X-ray films (Kodak, Rochester, NY) at −80°C with intensifying screens. For quantitative analysis the membranes were exposed to Phosphorimager screen (Fujix BAS 1000, Japan).

3. Results and discussion

3.1. Sequence of the Dictyostelium discoideum vacuolar ATPase B subunit

The *vatB* gene was found during the analysis of mutants obtained by insertional mutagenesis (REMI). By cutting the genome of mutant VIII-6 with *Eco*RV we recovered the inserted plasmid used to mutagenize the cells together with approximately 3.1 kbp of genomic DNA flanking region which contained an open reading frame encoding 450 amino acids of the vacuolar ATPase B subunit. The plasmid was inserted approximately 1.1 kbp upstream of the start codon of the *vatB* gene. The sequence of this non-coding region is extremely A+T rich with extensive homopolymeric regions as are present in 5′ non-coding regions of *D. discoideum* genes [17] and may contain the regulatory sequences of *vatB* (Fig. 1).

To clone the missing 3′ end of the gene a λgt 11 cDNA library was screened using as probe a fragment corresponding to amino acids 251–443. The isolated cDNA contained the missing 129 bp of coding sequences. The whole open reading frame codes for a polypeptide of 493 amino acids with a calculated molecular mass of 54 874 Da. The known genomic sequence is interrupted by two introns in positions 228–448 and 754–873 (Fig. 1). The amino acid sequence of peptides derived from a protein associated with endosomal membranes

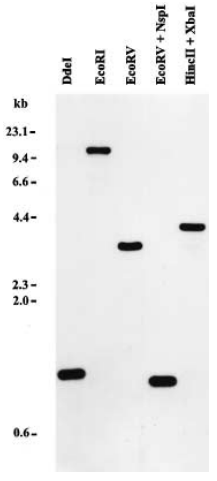

Fig. 2. Southern blot analysis. Genomic DNA was digested with the indicated restriction enzymes, blotted and probed with a highly conserved fragment of *vatB* encoding aa 251–443.

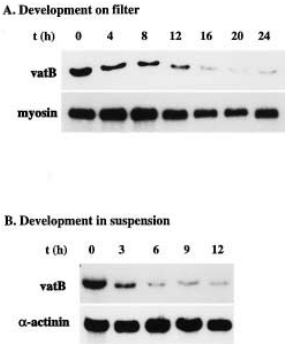

Fig. 3. Transcription of *vatB* during development. Cells were allowed to develop on nitrocellulose filters (A) or in suspension (B). Northern blots were loaded with 30 μg of total RNA per lane and probed with a *vatB*-specific probe. Myosin and α-actinin [21] gene-specific probes were used for control. The numbers above the panels indicate the time in hours.

matches the predicted sequence of the protein encoded by *vatB* [18].

Sequence comparison of the *Dictyostelium* protein with B subunits from different organisms showed an identity of 72–77% in eukaryotic cells, dropping to 55% when compared with the corresponding subunits of prokaryotic F-ATPases (data not shown). All B subunits known so far differ in size and show sequence variability at the N- and C-terminal regions whereas the core region containing the nucleotide binding site is highly conserved [2]. The hydropathy profile indicates a peripheral location of the subunit as was suggested for members of this protein family from other organisms. The hydropathy plot closely matches that of the yeast B subunit (not shown).

Southern blot analysis indicates that both under low (30%) and high stringency (50% formamide) only single fragments are labeled with *vatB* probe after digestion of genomic DNA with a variety of restriction enzymes (Fig. 2). We conclude that, as in yeast, this gene is present in a single copy in *Dictyostelium*. In Northern blots a single message of 1.75 kbp is detected (Fig. 3) during the whole developmental cycle.

Mutant VIII-6 is defective in phagocytosis. The mutant phenotype is probably independent of the *vatB* gene, as the *vatB* gene was not disrupted by the inserted plasmid, and its expression is only slightly altered in the mutant (data not shown).

3.2. Temporal regulation of vatB

One of the prominent features of the *Dictyostelium* life cycle is the transition from solitary amoebae to a multicellular fruiting body. This transition is triggered by starvation of the cells, and involves coordinated activation and/or repression of certain genes. We analyzed the expression of the *vatB* gene during development of *Dictyostelium* cells on nitrocellulose filter or in suspension. In both cases Northern blot analysis revealed that transcription of *vatB* is developmentally regulated. The mRNA is expressed at highest levels during growth and decreases in abundance during development. Low levels of *vatB* mRNA continue to be present throughout the whole life cycle (Fig. 3A). A comparable decrease in mRNA accumulation is observed in cells starved in suspension, which develop only up to tight aggregate stage (Fig. 3B). Treating cells with cAMP pulses, which are known to stimulate expres-

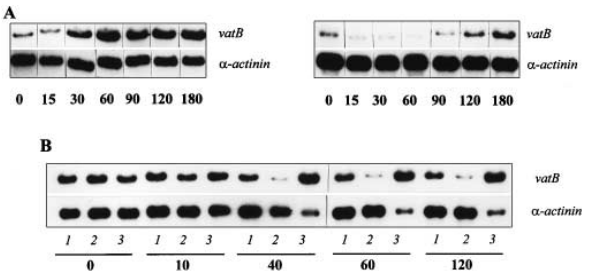

Fig. 4. Transcriptional regulation of *vatB* during bacterial phagocytosis. *vatB* transcript levels in cells incubated in shaken suspension of bacteria (A, left panel) or in axenic medium (A, right panel). The numbers below the panels indicate the incubation time in minutes. B: Incubation in growth medium at different pHs: 1, 6.7; 2, 5.5; 3, 9.0. For control blots were reprobed with an α-actinin-specific probe [21]. The blots contained 30 μg of total RNA per lane.

sion of some developmental genes and to repress a class of growth phase genes [18], did not affect the expression of *vatB* (data not shown).

3.3. Regulation of vatB by bacterial uptake

Dictyostelium is a natural phagocyte, and it has been shown previously [3] that phagosomes are among the compartments containing vacuolar ATPase proton pumps. Therefore we analyzed the behavior of *vatB* in response to bacteria as food source or in axenic medium. To allow for identical starting conditions, cells were first starved for 2 h by shaking in phosphate buffer and then transferred to either *E. coli* bacterial suspension or axenic medium.

In both conditions an increase in *vatB* mRNA amount was observed, but with clearly different patterns of transcriptional kinetics. Addition of bacteria reversed quite rapidly the expression of *vatB*: within 30 min the mRNA level markedly increased, reaching values comparable to the vegetative one at 60 min (Fig. 4A, left panel). In contrast, adding axenic medium did not affect gene expression until 120 min, when mRNA slowly re-accumulated (Fig. 4A, right panel). These data are consistent with the ATPase being indeed an essential component for acidification of phagosomes [19], and suggest that bacteria provide a stimulus to enhance the activity of the phagocytic machinery.

3.4. Transcriptional regulation of vatB by pH

We found that low pH values (5.5) down-regulated the transcript levels of *vatB* whereas a pH of 9.0 had the opposite effect (Fig. 4B). In both cases a significant effect was observed 40 min after the start of the experiment. The *vatB* amount decreased by a factor of 4 and increased by a factor of 2 when the cells were placed in, respectively, acidic or alkaline conditions suggesting that the V/H^+ ATPase has a role in survival at high pH. In contrast to the up-regulation of the B subunit at high pH, α-actinin, which was used as control in all experiments, was down-regulated at pH 9.0 (Fig. 4B). A similar pattern as for α-actinin was found when the blot was reprobed with actin (not shown). Since methylene blue staining of ribosomal genes did not show significant changes in the amount of loaded RNA, these results suggest that high pH has differential effects on gene expression. The results obtained for *vatB* are in agreement with those found in yeast where mutants lacking B and c subunits of ATPases can grow at acidic pH (pH 5.5). This might be due to their ability to acidify the vacuolar system by equilibration with external medium via fluid-phase endocytosis [5].

In conclusion, we have shown that *vatB* gene expression is down-regulated during development, up-regulated by bacterial uptake and sensitive to changes in pH. These results are consistent with the V/H^+ ATPase playing a major role during *Dictyostelium* cells growth, particularly on bacteria.

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