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Identification and purification of a DNA-binding protein interacting with the promoter of *5′-nucleotidase* in *Dictyostelium discoideum*

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Abstract

The developmental management of *5′-nucleotidase* (*5nt*) expression in *Dictyostelium discoideum* has provided a focal point for studies of gene regulation at the level of transcription. To identify DNA–protein interactions involved in the *5nt* regulation, EMSAs were performed using short oligonucleotides, designed to span a 357 bp promoter region. A binding activity ($R_f = 0.33$) was identified and shown to be specific to the nucleotide sequence between −338 and −309 bp relative to *5nt* ATG. Characterization of the binding activity, including the effects of salt and temperature, provided insight into the nature and stability of the protein. The protein was purified in a series of chromatographic stages, including DEAE–Sephacel, heparin–Sepharose, DNA affinity, and gel filtration. SDS–PAGE analysis identified a polypeptide with a molecular weight of 70 kDa. Mass spectrometry revealed that the purified protein was a putative formyltetrahydrofolate synthase.

Keywords: 5′-Nucleotidase; *Dictyostelium*; Gene regulation; Electromobility shift assay; Protein purification; Column chromatography; Formyltetrahydrofolate synthase

5′NT[2] is an enzyme that is ubiquitous to numerous species and resides in a variety of cellular locations, including the cell membrane, the mitochondria, and the cytosol. The functions of the enzyme as it resides on the extracellular surface of the membrane include the conversion of nucleotides to nucleosides to enable transmembrane trafficking, cell adhesion activities, cellular recognition events, and cell/cell interactions [1]. 5NT is also involved in the cyclic AMP (cAMP) degradation pathway. 5NT removes 5′AMP by breaking it down into adenosine and inorganic phosphate. This activity precludes the accumulation of 5′AMP, which could otherwise prevent the additional synthesis of cAMP by feedback inhibition. The role of cAMP as a chemoattractant and morphogen during the growth and development of many organisms has been established, and extends to the model organism utilized in this study.

Initiation of the multicellular stages of the *Dictyostelium* life cycle is dependent upon the chemoattractant cAMP, which is emitted as pulses by individual amoebae when nutrients become diminished [2]. As a result of neighboring cells responding to the chemoattractant by migrating towards its most concentrated source, a multicellular aggregate structure is formed. Throughout the remainder of development, the differentiation of two major cell types occurs, a process that is dependent upon two morphogens: cAMP and differentiation inducing factor-1 (DIF-1) [3].

It has been established that the concentrations of cAMP and DIF-1 are different in prestalk and prespore cells [4]. Before the culmination stage of the life cycle is reached, cAMP induces cell-type specific gene expression in both cell types. After the culmination stage, however, prestalk cell

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[2] *Abbreviations used: 5nt*, *5′-Nucleotidase*; EMSA, electrophoretic mobility shift assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FTHFS, formyl tetrahydrofolate synthase; cAMP, cyclic Adenosine MonoPhosphate; DIF-1, differentiation inducing factor-1; HPLC, high performance liquid chromatography.

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differentiation is inhibited by cAMP, while prespore cell differentiation is induced [5]. In prestalk cells, the expression of a cAMP-phosphosdiesterase causes a reduction in cAMP concentration, thereby resulting in the inhibitory effects observed [6,7]. Another morphogen involved in differentiation, DIF-1, is present in high concentrations in the prespore region, where it is thought to be synthesized [8]. The presence of an enzyme in prestalk cells that degrades DIF-1 results in low levels of the morphogen in this zone. These low concentrations stimulate prestalk-specific gene expression and repress prespore-specific gene expression.

In *Dictyostelium*, *5nt* is expressed only during multicellular stages of development [9]. Expression of the gene is first apparent approximately 5 h after the initiation of development when nutrients are depleted. At this stage, individual amoebae have created a loose aggregate formation. The expression of the gene increases as the organism enters the first finger and slug stages of development, and high levels of expression are maintained throughout the remainder of the life cycle [9].

Fusion of the *5nt* promoter to the *lacZ* reporter gene enabled the cellular localization of enzyme activity to be determined within the organism at each developmental stage [10]. During the formation of the aggregate, activity was determined to be highest in cells residing in the aggregate center relative to cells still streaming. In the tight aggregate, activity resided mainly in cells from the upper portion of the structure. While in the first finger stage of development, activity was highest in cells at the base of the structure, activity was prevalent in anterior-like cells (ALCs) and slime sheath cells at the slug stage. Activity in early culminants was concentrated to the boundary between the prespore and prestalk cells, while in the more mature culminants activity was found in the lower section of the prespore mass. Upon fruiting body formation, activity was localized to the basal disc, lower stalk, and upper cup [10].

The changes of *5nt* expression levels and localization during the course of development in *Dictyostelium* require the employment of mechanisms to regulate activity in a temporal and spatial context. The transcriptional regulation of *5nt* was investigated in our laboratory through a thorough analysis of the gene's promoter (Wiles et al., unpublished results). A study of numerous internal and 5′ promoter deletions enabled the identification of a general region of the promoter in which regulatory elements may reside. Further evaluation of this promoter region by site-directed mutagenesis provided additional insight into potential sequences in the promoter critical to the achievement of normal levels of expression. In this study, EMSA was performed to identify DNA–protein binding activities involved in the temporal regulation of *5nt* expression. By designing and utilizing numerous short oligonucleotide probes that spanned a chosen region of the promoter, DNA–protein binding activities specific to particular nucleotide sequences in the *5nt* promoter could be identified.

We report here the identification of a DNA–protein interaction specific to a 30 bp sequence of the promoter. We describe the characterization of this binding activity with respect to parameters including temperature, salt, and the presence of different non-specific competitors. We explain the purification processes utilized to isolate the DNA-binding protein, describe the mass spectrometry analysis of the characterized protein, and discuss the potential role of the presumed FTHFS as a regulator of the *5nt* expression.

Materials and methods

Extraction of cytoplasmic and nuclear proteins from Dictyostelium amoebae and slugs

Cytoplasmic and nuclear protein extracts for EMSA were prepared from two sources: AX3K *Dictyostelium* amoebae induced with cAMP in shaking flasks, and AX3K *Dictyostelium* slugs grown on 2% water agar plates. Preparation of amoebae extracts were obtained by enabling cells to grow to a density of about 3×10^6 cells/ml. Cells were harvested by centrifugation at 2100*g* in a CRU-5000 centrifuge (International Equipment Company) for 3 min. The cell pellets were washed in a $1 \times$ MES-LPS buffer (pH 6.5) and resuspended in a volume of $1 \times$ MES-LPS required to obtain a final concentration of 1×10^7 cells/ml. Cells were grown in non-nutrient conditions overnight, after which cAMP was added to a final concentration of 1 mM. Cells were shaken in the presence or absence of cAMP for 8 h, and harvested by centrifugation at 10,000*g* in a Sorvall GSA for 10 min. The cell pellets were resuspended in a volume of lysis buffer A (50 mM Tris, pH 7.5, 10% glycerol, 2 mM $MgCl_2$, 1% NP40, 1 mM DTT, 20 μg/ml TLCK, and 100 μg/ml PMSF) five times the pellet weight, and harvested by centrifugation at 10,000*g* for 10 min. The supernatant was stored at −80 °C until EMSA was performed. The pellet was washed and resuspended in 4 ml lysis buffer A, centrifuged at 10,000*g* for 10 min and the supernatant was stored at −80 °C. The remaining pellet was suspended in $2 \times$ volume lysis buffer A. NaCl was added to a final concentration of 0.42 M, and the samples were incubated on ice for 1 h. After centrifugation at 11,000*g* for 10 min, the supernatant was stored at −80 °C.

For protein extraction from slugs, the cells were grown, harvested, and suspended in $1 \times$ MES-LPS buffer (pH 6.5) to obtain 1×10^7 cells/ml as described above. To induce multicellular development, the samples were spread on 2% water agar plates and incubated at room temperature until slugs formed, in approximately 18 h. Slugs were collected with 20 mM potassium phosphate buffer (pH 7.5), disaggregated by passing through a 21 G syringe, and centrifuged at 2600*g* for 5 min. The supernatant was removed and the pellets were resuspended in five times the pellet weight of lysis buffer A. Cytoplasmic and nuclear protein fractions were obtained as described for the amoebae samples.