Calcium regulates the expression of a *Dictyostelium discoideum* asparaginyl tRNA synthetase gene

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In a screen for calcium-regulated gene expression during growth and development of *Dictyostelium discoideum* we have identified an asparaginyl tRNA synthetase (*ddAsnRS*) gene, the second tRNA synthetase gene identified in this organism. The *ddAsnRS* gene shows many unique features. One, it is repressed by lowering cellular calcium, making it the first known calcium-regulated tRNA synthetase. Two, despite the calcium-dependence, its expression is unaltered during the cell cycle, making this the first *D. discoideum* gene to show a calcium-dependent but cell cycle phase-independent expression. Finally, the N-terminal domain of the predicted ddAsnRS protein shows higher sequence similarity to Glutaminyl tRNA synthetases than to other Asn tRNA synthetases. These unique features of the *AsnRS* from this primitive eukaryote not only point to a novel mechanism regulating the components of translation machinery and gene expression by calcium, but also hint at a link between the evolution of *GlnRS* and *AsnRS* in eukaryotes.

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1. Introduction

Freshly starved cells of *Dictyostelium discoideum* are heterogeneous with regard to their levels of both free and sequestered calcium (Saran *et al* 1994; Azhar *et al* 1996). This pre-aggregative heterogeneity is correlated with a cell's post-aggregative fate; cells with relatively high calcium (HC cells) develop along the presumptive stalk (pst) pathway whereas cells with relatively low calcium (LC cells) develop along the presumptive spore (psp) pathway (Azhar *et al* 1996). The link between cellular calcium and cell fate is reinforced by the observation that artificially lowering the level of cellular calcium by a combination of the calcium ionophore A23187 and EGTA leads to an increase in the proportion of spore cells, while raising the cellular calcium by a combination of A23187 and high extracellular calcium leads to an increase in the proportion of stalk cells (Baskar *et al* 2000). Besides calcium, the cell cycle phase at starvation is another factor that affects cell fate in *D. discoideum* (McDonald and Durston 1984; Weijer *et al* 1984b; Huang and Pears 1999; Gomer and Firtel 1987; Azhar *et al* 2001). Cells that are in S or early G2 phase when starved tend to follow the pst pathway while those in mid to late G2 phase tend to adopt the psp pathway. As one might expect from this, the cell cycle status is correlated with the level of cellular calcium (Azhar *et al* 1998; Saran 1999). Treatment with cell cycle inhibitors leads to an increase or decrease in cellular calcium depending on the phase at which the inhibitors block the cell cycle (Azhar *et al* 2001).

Keywords. Calcium; cell cycle; *Dictyostelium*; RAP-PCR; tRNA synthetase

Abbreviations used: AARS, Amino acyl tRNA synthetase; AsnRS, asparaginyl tRNA synthetase; HC, high calcium; LC, low calcium; RAP-PCR, RNA arbitrary primed-PCR.

Calcium has also been shown to play a role during growth (Yuan *et al* 2001) and in the growth-to-development transition (Malchow *et al* 1996; Itoh *et al* 1998; Tanaka *et al* 1998) in *D. discoideum*. It is likely that calcium influences cell fate choice in *Dictyostelium* by regulating gene expression. A number of Ca^{2+} regulated genes have been identified in mammalian cells (Hardingham and Bading 1999), but little is known about such genes in *Dictyostelium*. The present study is aimed at identifying genes regulated by calcium in *D. discoideum* in the hope that one might thereby obtain insight into the functioning of metabolic and signalling pathways. Towards this goal we have employed the RNA Arbitrary Primed-PCR (RAP–PCR) technique.

RAP–PCR and a related technique, differential display-PCR (dd-PCR), have been used extensively for identifying differentially expressed genes in various organisms (Welsh *et al* 1992; Liang and Pardee 1995). Recently, dd-PCR has been used to identify *Dictyostelium* genes that are expressed in conjunction with the growth-to-differentiation transition (Inazu *et al* 1999; Hirose *et al* 2000). By using this approach we have found that expression of the Asparaginyl tRNA synthetase (*ddAsnRS*) gene in *Dictyostelium* is repressed by lowering of cellular calcium. Despite the strong link between cellular calcium and cell cycle phase, we find that expression of *ddAsnRS* gene is not influenced by the cell cycle phase. A feature of the predicted ddAsnRS protein is that its sequence is more similar to those of eukaryotic AsnRSs than to the prokaryotic and archaeal AsnRS proteins. Interestingly, it contains an N-terminal domain that shares little sequence overlap with eukaryotic AsnRS and is missing from the prokaryotic AsnRS proteins. However, the N-terminal domain of ddAsnRS is very similar to Glutaminyl tRNA synthetases (GlnRSs) from a number of organisms. In brief, we report the identification of an unusual tRNA synthetase gene that is also regulated in a novel fashion.

2. Materials and methods

2.1 *Reagents and growth media*

Dictyostelium discoideum strain AX2 was grown in HL5 (Watts and Ashworth 1970) supplemented with 1% penicillin-streptomycin (Sigma Chemical Company, USA). Peptone and yeast extract for HL5 was obtained from Oxoid (UK), and all other media components were obtained from Difco Laboratories (USA) and Sigma Chemical Company (USA). The RAP–PCR kit was purchased from Stratagene (La Jolla, CA, USA). Restriction enzymes were from New England Biolabs (UK); Amersham Pharmacia Biotech (UK); Bangalore Genei (India); and Roche Molecular Biochemicals (Germany). The oligonucleotides were synthesized by Bangalore Genei (India).

2.2 *Cell cycle synchronization and calcium treatments*

Cold synchronization was performed as described earlier (MacWilliams *et al* 2001). Cells were maintained for at least 48 h in continuous exponential growth, culminating at a density between $0{\cdot}5 \times 10^6$/ml and 1×10^6/ml. One hundred ml of cells in a 300 ml flask were then placed on a shaker in a cold chamber adjusted to give a temperature, in the medium, of 9·5°C, with shaking at 75 rpm. After 14–16 h, a sample was taken for the zero-time (T0) observation and the cells were then warmed to 22°C in 30–60 s by immersing the flask in a warm water bath. Cells for RNA extraction and BrdU incorporation measurements were taken from the removed material. For raising the cellular calcium level, cells growing in MES-HL5 (HL5 buffered at pH 6·4 with 10 mM MES) were treated with a combination of 7 μM A23187 and 1 mM CaCl2 (HC) or 7 μM A23187 and 1 mM EGTA (LC). In all the experiments involving calcium alterations, the growth media and all other relevant solutions were buffered using 10 mM MES.

2.3 *RNA preparation and Northern blot analysis*

RNA was prepared from 10 ml of cell suspension at a density of 5×10^6 using the guanidine hydrochloride method (Chomczynski and Sacchi 1987). For RAP–PCR, RNA was treated with RNase-free DNAse (Startegene, USA, Amersham Pharmacia Biotech., Sweden), extracted with acid phenol and precipitated with ethanol. The RNA was suspended in DEPC-treated water and quantified by spectrophotometry. For Northern blot analysis, 10–20 μg of total RNA was resolved on a 1·2% agarose-formaldehyde gel in 1X MOPS/EDTA buffer as described in Sambrook *et al* (1989). Before loading, RNA was denatured by heating at 68ºC for 15 min with three volumes of RNA loading buffer (67% formamide and 9% formaldehyde in 1X SSC with 0·2% bromophenol blue). RNA was then transferred by capillarity onto a Hybond N (Amersham Pharmacia Biotech., Sweden) or Nytran membrane (Schleicher and Schuell, USA) in 10X SSC. After transfer, RNA was cross-linked using Stratalinker 1800 (Stratagene, USA). Northern blots were carried out according to the "Neverfail" Northern blot protocol of McCaughern-Carucci (http://www.nwfsc.noaa.gov/protocols/northernblot.html). Blots were exposed to a Fuji phosphoimager screen and quantified with the Fuji Science(tm) software (Fuji Laboratories, Japan). To control for loading differences, mRNA

levels were normalized to the level of the IG7 (Hopper *et al* 1993) message in the same blots.

2.4 *RAP–PCR*

RAP–PCR involved the following two steps:

(i) cDNA synthesis was carried out using 1 μg of total RNA and a randomly selected primer; in the present study the primer used was called A2G and had the sequence AATCTAGAGCTCCAGCAGG. The reaction was carried out for 1 h at 37°C. As a control, another tube that contained all the components for cDNA synthesis except for the reverse transcriptase enzyme was included to check for the presence of residual DNA that did not get completely degraded by the DNase treatment. RAP–PCR products from those reactions whose controls (meaning parallel samples that lacked reverse transcriptase) yielded more than 4–5 bands were not examined any further.
(ii) PCR was carried out for 30 cycles using 10 μl of 1 : 10 diluted cDNA from the above in the presence of 5 μCi-P^{32}dATP and using the following cycling parameters: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and 10 min at 72°C. Following PCR the sample was resolved on a 6% denaturing urea polyacrylamide gel, the gel was dried and put for autoradiography. The bands excised from the gel were re-amplified using the cycling conditions used above for PCR, but this time without the radiolabel.

2.5 *DNA sequencing and analysis*

BLASTP searches and amino acid sequence similarities were calculated using the BLAST software at the NCBI Blast server (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al* 1990). The sequences of all the clones were determined by an ABI-PRISM automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequence data were processed using the Clone Manager software (Scientific and Educational Software, Durham, NC, USA). *AsnRS* sequences from different organisms were obtained from Genbank database and aligned using the CLUSTAL W program (Higgins and Sharp 1988; Thompson *et al* 1994). Amino acid sequence similarities were calculated using BLAST. Sequence alignments were carried out using the ClustalW program using default settings at GeneBee molecular biology server at the Russian EMBnet node (http://www.genebee.msu.su/). The software 'Boxshade 321' was used to highlight the regions of similarities in the multiple sequence alignment files generated using ClustalW.

3. Results

3.1 *Calcium regulates gene expression in vegetative amoebae*

The level of cellular calcium and the cell cycle phase at starvation are both known to regulate post-starvation cell fate choice. In order to identify calcium-regulated genes during growth and early starvation, we carried out RAP–PCR on total RNA extracted from cells grown to high densities (5×10^6 cells/ml) in HL5 medium. The medium was supplemented with 7 μM calcium ionophore A23187 and 1 mM $CaCl_2$ (high calcium; HC) or 1 mM EGTA (low calcium; LC). We have previously shown that these approaches are effective in altering the level of the Ca^{2+} in *D. discoideum* amoebae (Baskar *et al* 2000). Both environments reduced the growth rate of cells but did not affect their ability to initiate and undergo development (Azhar 1997; Baskar *et al* 2000). Treatment of cells with 7 μM A23187 alone did not affect the overall pattern of gene expression [compare lanes untreated (U) and ionophore treated (I) in figure 1]. Even in response to HC or LC treatment, gene expression was not drastically altered, as of the total of 4 primers that we tested for RAP–PCR only one (primer A2G) showed altered expression of a few genes (compare lanes HC, LC and U in figure 1). These genes can be classified into the following categories: repressed by LC (figure 1, band A); induced by LC (figure 1, band D); induced by HC (figure 1, band B); repressed by HC (figure 1, band C); and repressed both by LC and HC (figure 1, band E). These cDNAs were isolated from the gel, amplified, cloned and sequenced. The cDNA encoded by band B (figure 2a) is identical to the *D. discoideum* gene that encodes the cAMP binding protein CABP1/P34 (Grant and Tsang 1990; Bain *et al* 1991), while that corresponding to band E (figure 2b) shows highest similarity to the sequence of the gene that encodes *D. discoideum* alpha-mannosidase (Bush *et al* 1994). Bands C and D remain to be explored further. An additional cDNA, not visible in figure 1, was repressed by HC and was found to encode the 26s ribosomal RNA gene (figure 2c and Jaiswal 2001). Band A encoded a cDNA with maximal sequence similarity to the Asn tRNA synthetase gene (*AsnRS*) (Glöckner *et al* 2002). Amino acyl tRNA synthetases (AARS) have been shown to be regulated by a variety of signals including cell differentiation and the cell cycle; correspondingly, mutations in AARS have been shown to affect these processes (Sen *et al* 1997; Pelchat and Lapointe 1999; Zhou *et al* 1999). So far glutaminyl tRNA synthetase (*GlnRS*) is the only tRNA synthetase that has been identified in *D. discoideum*, a fact which motivated further analysis of the organization and expression of the *AsnRS* gene.

3.2 *Organization and sequence analysis of* D. discoideum *AsnRS*

The cDNA corresponding to band A was cloned and sequenced. BlastN analysis carried out for this sequence using the *Dictyostelium* genomic DNA sequence database at the Sanger Center identified a contig (Contig 17359) that contained a sequence identical (> 99% nucleotide sequence similarity) to this cDNA (figure 3a). Analysis of contig 17359 for a coding region using the GrailEXP Gene finder software (http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html) indicated the presence of a single ORF with 3 exons (470 bp, 1455 bp, and 173 bp respectively) and 2 small introns (121 and 131 bp) (figure 3a). Analysis of predicted protein encoded by this ORF using the 'pfam' protein domain database (Sonnhammer *et al* 1997) indicated the presence of the OB-fold nucleic acid binding domain (OB domain) and a class II tRNA synthetase domain (figure 3b). Both domains are characteristic of AsnRS (Commans *et al* 1998). A blast search of the putative protein encoded by this ORF identified the DNA sequence to be that of the *D. discoideum AsnRS* (*ddAsnRS* Genbank Accession# AAO52345) that is encoded by a gene on chromosome 2 (Glöckner *et al* 2002).

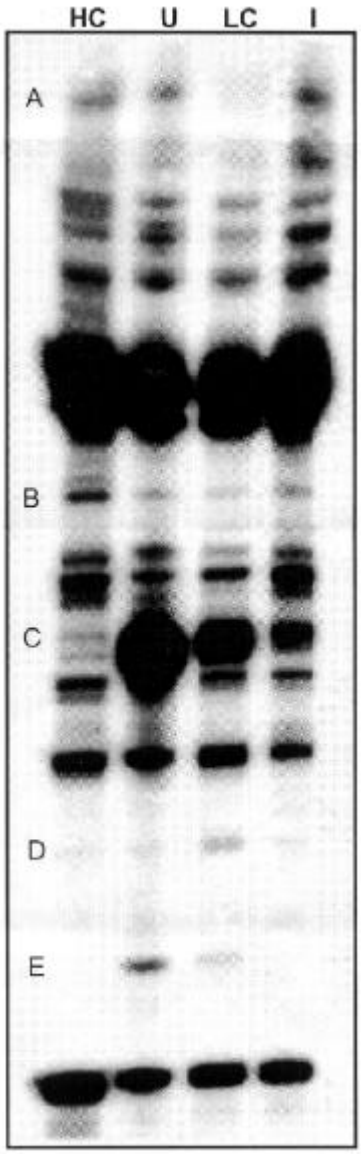

Figure 1. Effect of calcium alterations on gene expression. RNA extracted from cells growing in normal growth media with no additions (U), with the addition of 10 μM A23187 (I), with A23187 and 1 mM $CaCl_2$ (HC), or with A23187 and 1 mM EGTA (LC) were subject to RAP–PCR analysis. The different classes of calcium regulated cDNAs identified are represented by the cDNA clones A (repressed by LC), B (induced by HC), C (repressed by HC), D (induced by LC), and E (repressed both by LC and HC).

Blast analysis of the protein encoded by the putative *D. discoideum AsnRS* indicated a > 50% amino acid similarity with other eukaryotic and prokaryotic Asn tRNA synthetases (figure 4a). At the N-terminus, the predicted ddAsnRS contains a ~ 130 amino acid stretch (encoded by exon 1) which is absent from prokaryotic AsnRSs and shares very little similarity with eukaryotic AsnRSs (figure 4a). On the other hand, this extended N-terminal domain shares a significant degree of amino acid sequence similarity (55–60%) with the eukaryotic glutaminyl tRNA synthetase (GlnRS) (figure 4b). It has been suggested that the *AsnRS* and *GlnRS* genes has evolved separately along independent paths (Shiba *et al* 1998). Thus it is interesting to note the presence of a GlnRS homology domain in the *Dictyostelium* AsnRS.

3.3 *Expression of* D. discoideum *Asn tRNA synthetase is repressed by calcium*

As a first step towards analysing the expression of the *ddAsnRS*, we carried out a Northern blot analysis using a band A-specific probe. This indicated the presence of a ~ 2 kb mRNA whose expression was reduced by the lowering of cellular calcium (figure 2d). In order to further confirm the calcium regulated expression of this *AsnRS*, we obtained a shotgun clone (JC2a186g08) from the *Dictyostelium* Genome sequencing centre at IMB Jena (Germany) that spans most of the *AsnRS* gene. This clone was used to perform a Northern blot analysis (figure 5). Similar to the result obtained with the smaller cDNA clone obtained by RAP–PCR (figure 2d), this probe also identified a single ~ 2 kb mRNA that was repressed in response to a decrease in cellular calcium (figure 4); the IG7 transcript was used as an internal control for RNA loading (Hopper *et al* 1993).

One of the consequences of altering cellular Ca^{2+} in *D. discoideum* is that post-aggregation cell fate choice is affected. The cell cycle phase at starvation too influences cell fate choice (McDonald and Durston 1984; Weijer *et al* 1984a; Gomer and Firtel 1987; Huang and Pears 1999). Besides, the manner in which it does so indicates that calcium levels are influenced by the cell cycle phase (Saran 1999; Azhar *et al* 2001). We therefore looked

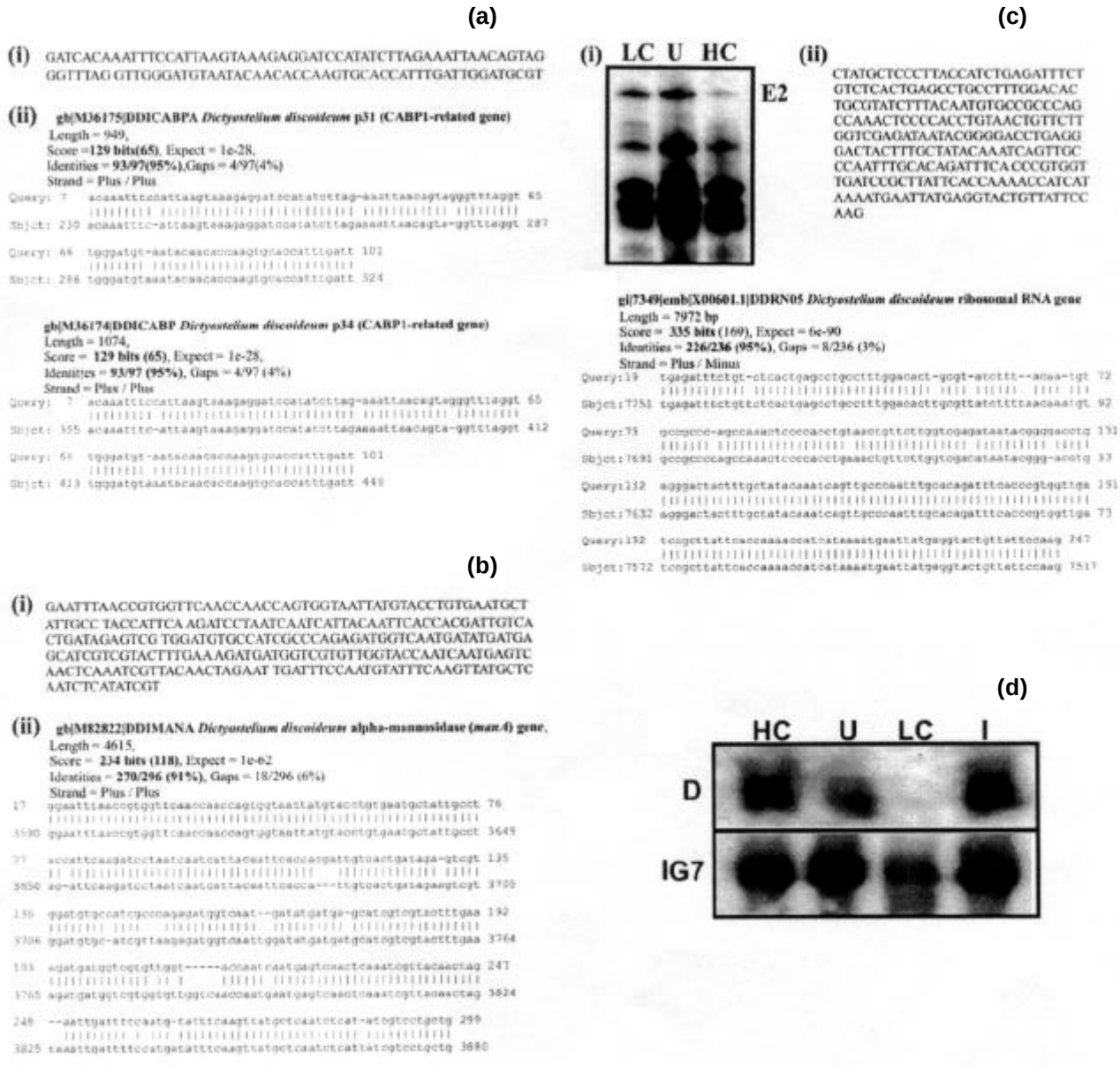

Figure 2. BLAST analysis of cDNA corresponding to various bands shown in figure 1. (**a**) Band B. (**i**) cDNA corresponding to band B was cloned and partially sequenced. (**ii**) The partial sequence was used to carry out a BLAST homology search using the Genbank nucleotide database. The sequence showed maximal (~ 97%) similarity with two CABP1 related *Dictyostelium* genes, *p34* and *p31*. (**b**) Sequence and BLAST analysis of cDNA corresponding to band E. (**i**) cDNA corresponding to band E was cloned and sequenced. (**ii**) BLAST homology search using the band cDNA identified this cDNA to correspond to the *Dictyostelium* alpha mannosidase gene (*manA*) gene. (**c**) RAP–PCR analysis and BLAST analysis of cDNA corresponding to band E2. (**i**) RAP–PCR was carried out as described in text and the sample was resolved on Urea PAGE. (**ii**) The cDNA corresponding to band E2 was excised out of the gel, cloned and sequenced. BLAST analysis of E2 cDNA carried out using Genbank database, indicated high sequence similarity with the *D. discoideum* 26S rRNA. (**d**) Northern blot analysis using total RNA and the cDNA corresponding to band A as probe. Note almost undetectable expression of mRNA corresponding to this cDNA in cell treated with LC. The blot was subsequently probed using IG7 to control for loading differences.

at the effect of cell cycle phase on the expression of *ddAsnRS*. To assay the expression of *ddAsnRS* over the cell cycle, AX2 cells were synchronized by the cold release method (Maeda *et al* 1989); synchrony was checked by the increase in cell numbers and by nuclear BrdU incorporation (figure 6a). Although there is a strong correlation between cell cycle and cell calcium levels, we found that *ddAsnRS* expression remained unchanged all through the cell cycle, in particular when calcium levels are expected to be relatively low (mid-late G2 phase, or between 4–8 h in figure 6b).

4. Discussion

The AARSs are highly conserved proteins and their evolution has been found to be representative of the evolutionary history of the organisms themselves (Woese *et al* 2000). The 20 cellular AARS enzymes, besides carrying out esterification of specific tRNAs with their cognate amino acids, are also essential for processes that go beyond protein synthesis (Martinis *et al* 1999). Expression of these proteins is regulated by a variety of signals including starvation, cell cycle and cellular differentiation (Sen *et al* 1997; Pelchat and Lapointe 1999; Zhou *et al* 1999). Mutations in tRNA synthetases cause cell cycle arrest both in prokaryotic (Holland *et al* 1999) and eukaryotic (Motomura *et al* 1996) cells. Modified versions of the catalytic domains of tRNA synthetases are reported to serve a variety of roles such as kinases and as enzymes involved in histidine and asparagine biosynthesis (reviewed in Weiner 1999). In short, the term 'tRNA synthetase' does not do justice to the range of capabilities exhibited by these versatile proteins. Our observation of calcium-regulated expression of *ddAsnRs* adds another feature to their properties. To our knowledge this is the first report of a calcium-dependent expression of a tRNA synthetase. Given that during the developmental phase of *Dictyostelium* calcium and the cell cycle phase act in concert to regulate cell fate (Azhar *et al* 2001), it is significant that the expression of the *ddAsnRS* gene is sensitive to calcium but not to the cell cycle phase (compare figure 5 with figure 6). This indicates that calcium can affect gene expression independently of the cell cycle phase. The cell cycle dependent and independent regulation of gene expression could be mediated by different calcium-binding proteins that in turn regulate different signalling pathways leading to differential effects on gene expression.

The regulation of a tRNA synthetase gene by calcium hints that changes in Ca^{2+} levels might not only be regulating cell differentiation but also general protein synthesis. This possibility is supported by the observed effect of calcium changes on normal cellular metabolism. For example, an increase in Ca^{2+} induced by the calcium-specific ionophores, A23187 and ionomycin affects the nucleotide content and rate of protein synthesis in several types of mammalian cells (Gmitter *et al* 1996; Xu *et al* 1999). Further, calmodulin antagonists suppress translation, both in mammalian (Kumar *et al* 1991) and *Dictyostelium* (Sonnemann *et al* 1993) cells. Also, the interaction of calmodulin with the ribosomal protein L19 has been shown to be important for *in vitro* translation of *Dictyostelium* genes. Our observation that the expression of the

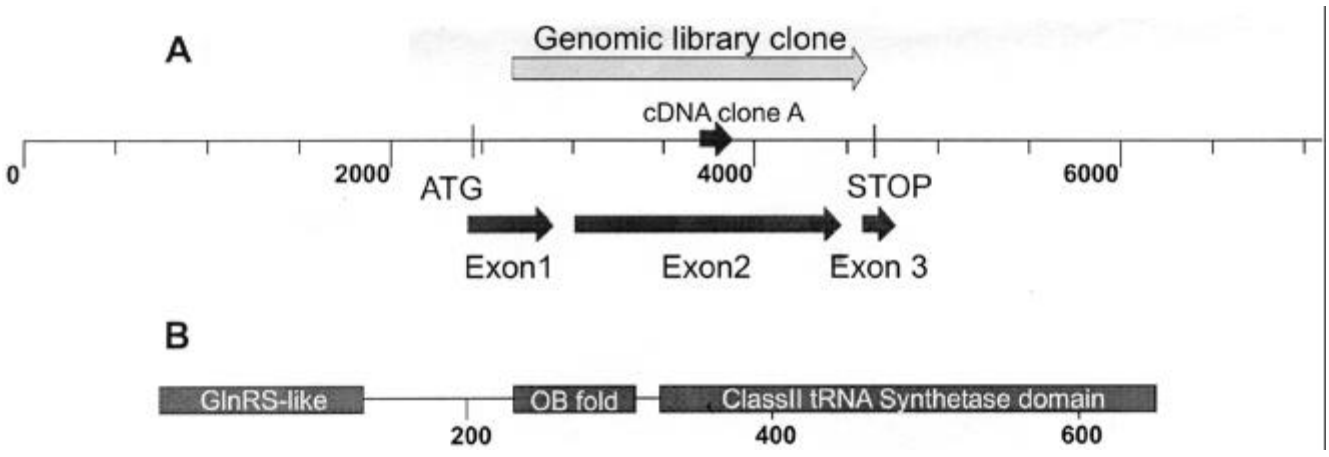

Figure 3. Gene and protein structure of Contig 17359. (**A**) Contig contains the full-length AsnRS protein encoding region and also the regions upstream and downstream of it. The ORF encoding *AsnRS* consists of three exons and 2 small introns. The position of cDNA clone A, and the genomic library clone (JC2a186g08) are indicated by shaded arrows. (**B**) The N-terminus sequence of the AsnRS protein shows homology to the GlnRS proteins, while the C-terminus contains the OB-fold nucleic acid binding domain and the class II tRNA synthetase domain.

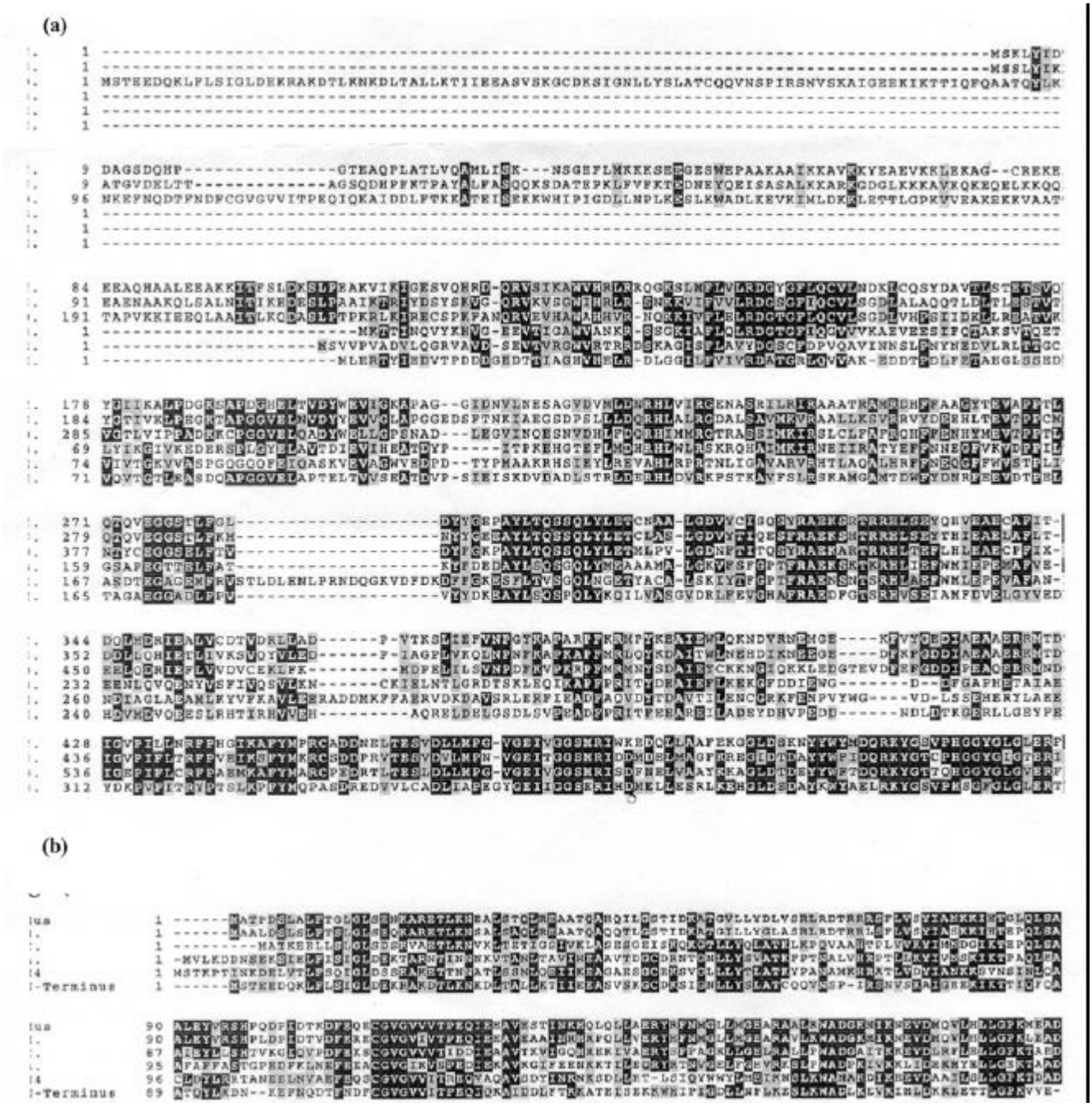

Figure 4. Multiple protein sequence alignment. (**a**) The predicted *AsnRS* of *Dictyostelium discoideum* (D.) was aligned with AsnRSs from other eukaryotes *C. elegans* (C.), *S. cerevisiae* (S.), *Halobacterium salinarum* (H.) and prokaryotes *Bacillus subtilis* (B.), and *E. coli* (E.), using the ClustalW sequence alignment software and displayed using the boxshade software. The region shaded black represent sequence identity, while the gray shaded region represent sequence similarity. (**b**) The N-terminus 90 amino acids of the *D. discoideum* AsnRS identified in this study (N-terminus) were aligned using ClustalW with the N-terminal GlnRS sequence of *Mus musculus* (M.), *Homo sapiens* (H.), *C. elegans* (C.), *Arabidopsis thaliana* (A.), and predicted GlnRS from *D. discoideum* (H4), displayed using the boxshade software.

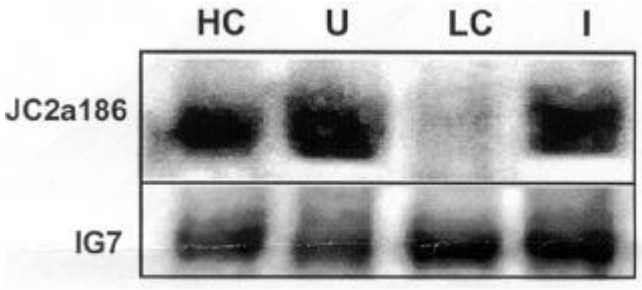

Figure 5. Northern blot analysis using the genomic DNA clone JC2a186 as probe. RNA prepared as in figure 1 was probed with a DNA probe prepared using the JC2a186 DNA. Subsequently the same blot was probed using IG7 to control for loading differences. Note that similar to the expression profile of the probe made from cDNA clone corresponding to band A in figure 1, low calcium (LC) treatment also inhibited the expression of JC2a186.

26S ribosomal gene (figure 2C) and the putative asparaginyl tRNA synthetase genes in the RAP–PCR screen is dependent on Ca^{2+} (figure 5) add to the growing evidence for the role of calcium in *Dictyostelium* protein synthesis, and hence general cellular metabolism. Growth in glucose-rich (G+) or glucose-deficient (G–) medium respectively raises or lowers the level of stored sugar reserves (Noce and Takeuchi 1985), and also presumably the metabolic activity of the cells. It is known that when combined, starved G+ and G– cells exhibit pre-spore and pre-stalk tendencies respectively (Leach *et al* 1973). It is conceivable that a raising or slowing down of cellular metabolism is the means by which pre-aggregation differences in the level of calcium alters post-aggregation cell fate choice.

With regard to the high sequence similarity of 56% at the N-terminus of the ddAsnRS with other eukaryotic

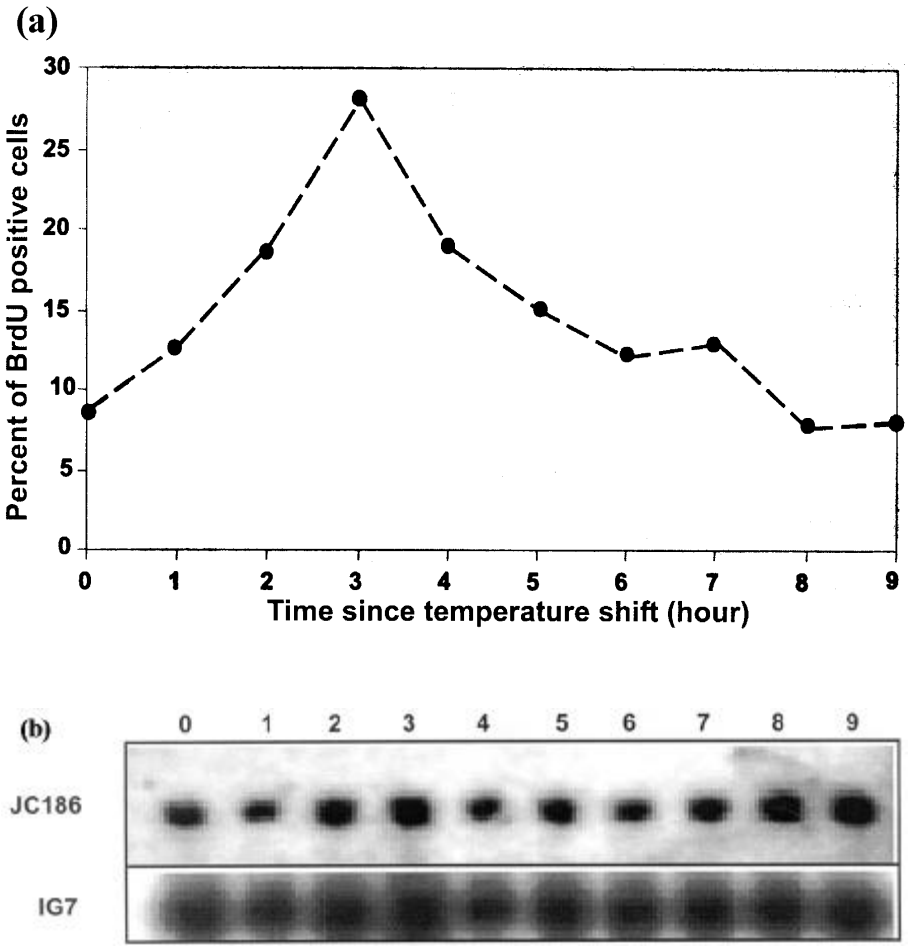

Figure 6. Cell cycle-dependent expression of JC2a186. (**a**) AX2 cells synchronized by release from cold were assessed for synchrony using nuclear Brdu incorporation. (**b**) RNA extracted from the synchronized AX2 cells were probed using JC2a186 DNA as probe. Compared to the internal control, IG7, no change in the expression of *AsnRS* can be observed during the various stages of the cell cycle.

GlnRS proteins, we wish to draw attention to two features: (i) It has been noted that a domain present in one aaRS is present in others belonging to the same class (Diaz-Lazcoz *et al* 1998; Woese *et al* 2000). However, to the best of our knowledge this is the first example of a domain seen in a class II (AsnRS) synthetase which is also present in a class I enzyme (GlnRS). (ii) Genome analysis reveals that a wide spectrum of bacteria rely on the tRNA-dependent transamidation pathway as the sole route for the synthesis of asparagine and glutamine tRNAs (Tumbula *et al* 2000). This initially made us wonder whether the open reading frame (ORF) identified by us might code for a protein with both AsnRS and GlnRS activities. On further consideration this seemed to be unlikely for two reasons. Firstly, the encoded protein lacks the tRNA synthetase class I domain characteristic of GlnRSs; and secondly, the H4 protein, which was previously identified as the putative *GlnRS* from *D. discoideum* (Singleton *et al* 1989) shares sequence identity with only 180 N-terminal amino acids encoded by the ORF, and even this is restricted to 38% of the residues. Also, the Sanger Center *Dictyostelium* genome database lists a contig (No. 15417) which appears to contain the full-length ORF corresponding to the GlnRS (data not shown). The GlnRS encoded by this contig also contains the class I tRNA synthetase domain. Taken together, these observations indicate that the *D. discoideum* GlnRS activity is separate from that of the AsnRS. The origin and implications of the high sequence similarity between the N-terminus of the ddAsnRS protein with the GlnRS protein remain to be investigated. The hypothesis that suggests itself is that these two tRNA synthetases have arisen by tandem gene duplication.

This being only the second putative tRNA synthetase predicted in *D. discoideum*, we are just beginning to explore the range of functions and regulation of tRNA synthetases in the cellular slime moulds. The peculiar regulation and genetic organization of *ddAsnRS* will not only provide us with an opportunity to better understand the role of amino acyl tRNA synthetases in growth and development of *D. discoideum*, but will also help address the question of origin and evolution of this fascinating class of proteins.

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