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Original Article

Analysis of a homologue of the adducin head gene which is a potential target for the *Dictyostelium* STAT protein Dd-STATa

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ABSTRACT A *Dd-STATa*-null mutant, which is defective in expression of a *Dictyostelium* homologue of the metazoan STAT (signal transducers and activators of transcription) proteins, fails to culminate and this phenotype correlates with the loss of expression of various prestalk (pst) genes. An EST clone, *SSK395*, encodes a close homologue of the adducin amino-terminal head domain and harbors a putative actin-binding domain. We fused promoter fragments of the cognate gene, *ahhA* (adducin head homologue A), to a *lacZ* reporter and determined their expression pattern. The proximal promoter region is necessary for the expression of *ahhA* at an early (pre-aggregative) stage of development and this expression is Dd-STATa independent. The distal promoter region is necessary for expression at later stages of development in pstA cells, of the slug and in upper cup and pstAB cells during culmination. The distal region is partly Dd-STATa-dependent. The *ahhA*-null mutant develops almost normally until culmination, but it forms slanting culminants that tend to collapse on to the substratum. The mutant also occasionally forms'fruiting bodies with swollen papillae and with constrictions in the prestalk region. The AhhA protein localizes to the stalk tube entrance and also to the upper cup cells and in cells at or near to the constricted region where an F-actin ring is localized. These findings suggest that Dd-STATa regulates culmination and may be necessary for straight downward elongation of the stalk, via the putative actin-binding protein AhhA.

KEY WORDS: *transcription factor, adducin, actin, cell differentiation, Dictyostelium*

Introduction

Organized movement of various types of cells is necessary for the development of multicellular organisms (Hou *et al.*, 2002; Baum, 2004). Such cell movements involve modulations of the cytoskeleton, such as the formation of actin stress fibers (Xia and Karin, 2004; Leptin, 2005). In order to understand the mechanism underlying such organized cell movement, the cellular slime mould *Dictyostelium* serves as an excellent model. *Dictyostelium* cells aggregate by a chemotactic movement in response to cAMP to form a multicellular structure and the structure requires morphogenetic cell movement to complete development (Kimmel and Firtel, 2004).

The transcription factor Dd-STATa, a functional homologue of metazoan STAT (Signal Transducers and Activators of Transcription) proteins (Kawata *et al.*, 1997), is necessary for efficient chemotactic cell movement at the aggregation stage and morphogenesis after the slug stage (Mohanty *et al.*, 1999); suggesting that Dd-STATa may play a role in aggregation and multicellular development by regulating cell movement. The anterior prestalk region of the slug is composed of at least three subtypes of prestalk (pst) cells, pstA, pstO and pstAB cells. This is based on promoter analyses of two genes encoding extracellular matrix proteins, *ecmA* and *ecmB* (Jermyn *et al.*, 1989; Gaskell *et al.*, 1992). PstAB cells express both the *ecmA* and *ecmB* genes (Gaskell *et al.*, 1992) and are localized in a funnel-shaped core of cells. The pstA cells express *ecmA* but not *ecmB* and *ecmB* expression is actively repressed in the pstA cells (Ceccarelli *et al.*, 1991; Harwood *et al.*, 1993). The repression of *ecmB* expression requires Dd-STATa; disruption of the *Dd-STATa* gene causes the pstA and pstO cells to express *ecmB* (Mohanty *et al.*, 1999). There is also a failure to culminate in the

Abbreviations used in this paper: ahh, adducin head homologue; Dd, Dictyostelium discoideum; EST, expressed sequence tag; pst, prestalk; STAT, signal transducers and activators of transcription.

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Dd-STATa null strain.

To further understand the molecular mechanism whereby culmination is regulated by Dd-STATa, we previously searched for Dd-STATa target genes that are expressed in the prestalk cells. These were analysed by microarrays and *in situ* hybridization (Maeda *et al.*, 2003) and we identified 13 candidate genes (Shimada *et al.*, 2004a). We previously analysed one of those genes, the *SSK395(ahhA)* gene. Here we show that *ahhA* encodes a protein similar to adducin, an actin-binding protein. We identify promoter regions that regulate its expression, investigate protein localization in the culminant and discuss its function.

Results

Prestalk cell-specific expression of the SSK395 *gene*

In a previous study, we identified 13 prestalk-specific genes whose expression is down regulated in the *Dd-STATa*-null mutant (Shimada *et al.*, 2004a). One of those is an EST clone *SSK395*, which is detectable at the tipped finger stage by *in situ* hybridization (Shimada *et al.*,2004a). We focused on the function of the gene encoding *SSK395*, because the expression pattern dramatically changes as development proceeds. At the first finger stage, expression in prespore cells became almost undetectable although weak expression in prestalk cells is still seen. The expression level becomes elevated again at the slug stage and it is observed throughout the entire prestalk region. Once culmination begins, expression in pstA cells disappears and localisation of the transcript then gradually shifts to the upper cup cells and the stalk tube entrance in mid culminants (Shimada *et al.*, 2004a).

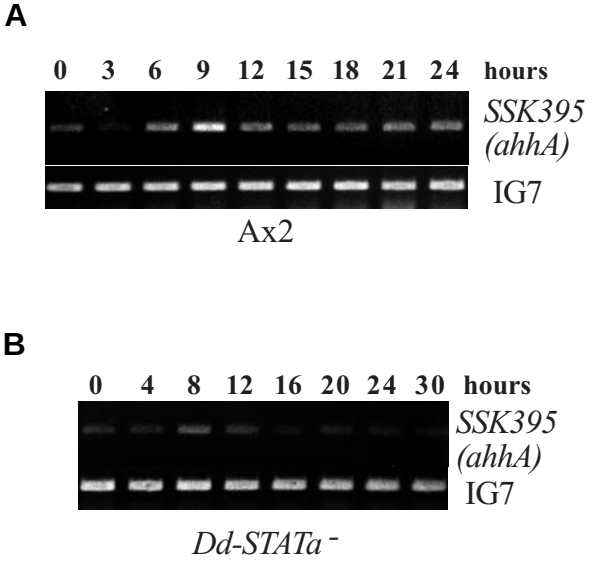

Fig. 1. Comparison of *ahhA(SSK395)* gene expression in Ax2 and *Dd-STATa*-null cells. (A) *Developmental time course of* ahhA(SSK395) *gene expression in Ax2 detected by semi-quantitative RT-PCR. Total RNAs were extracted from Ax2 cells at stages of growth phase (0h), aggregates (3h, 6h), mound (9h), tipped aggregate (12h), slug (15h), Mexican hat (18h), culminant (21h) and fruiting body (24h) were used to amplify the specific* ahhA(SSK395) *DNA fragment as described in the Materials and Methods.* **(B)** *Developmental time course of* ahhA(SSK395) *gene expression in* Dd-STATa*-null mutant detected by semi-quantitative RT-PCR. Cells were at stages of growth phase (0h), aggregates (4h, 8h), mound (12h), tipped aggregate (16h) and slugs (20, 24, 30h).* IG7 *was detected in the same reaction as a normalisation control. DNA was detectable after 19 cycles of amplification from 0.25 μg total RNA before PCR reaction saturated. Experiments (A) and (B) were performed at the same time with the same batch of reverse transcriptase to compare the amount of transcript. The experiments were repeated in triplicate and the representative data are shown.*

Semi-quantitative RT-PCR was used to monitor the expression time course of the *SSK395* gene in Ax2 cells (Fig. 1A). In accordance with previous microarray observations (Iranfar *et al.*, 2003), there are peaks of expression at 0 hour and 6~9 hours of development. *SSK395* gene expression is maintained at later stages of development (Fig. 1A).

Reduced SSK395 *gene expression in* Dd-STATa*-null mutant at the late slug stage but not at the early stages*

Interestingly, although expression at the tipped finger stage is detectable in the *Dd-STATa* null mutant, the transcript is hardly detectable in the mutant at the late slug stage (Shimada *et al.*, 2004a). Therefore, only later expression appears to be positively regulated by Dd-STATa. To confirm whether expression of the *SSK395* gene is Dd-STATa-dependent, we performed semi-quantitative RT-PCR in a *Dd-STATa*-null strain and compared expression to that in the parental strain, Ax2. *SSK395* transcripts were detectable in both strains at the tipped finger stage (9 hours of development for Ax2 and 12 hours for the *Dd-STATa*-null strain) (Fig. 1B). Interestingly, the transcript level at the mound stage in the mutant (8 hours of development) was rather stronger than that at the tipped finger stage. The expression level dropped during late first finger and early slug stages in both strains (12~15 hours of development for Ax2 and 16 hours for *Dd-STATa*-null strain). Then, the expression increased slightly as development proceeded to the migratory slug stage in the *Dd-STATa*-null strain (20 hours of development).

The SSK395 *gene encodes a protein related to adducin*

The *SSK395* gene contains an open reading frame (ORF), which is capable of encoding a protein of 268 amino acids with a predicted molecular mass of 30.1 kDa. A database search using BLAST showed that the *SSK395* gene encodes a protein with high homology to mammalian adducin (Fig. 2A). However, the gene does not encode the whole of adducin, it only encodes a domain corresponding to the head domain (Fig. 2B). Therefore, we named the *SSK395* gene *ahhA* (adducin head-homologue A).

Promoter fragment of the ahhA *gene confers prestalk-specific expression*

Comparison of *ahhA* gene expression between Ax2 and *Dd-STATa* - null strains by *in situ* hybridisation (Shimada *et al.*, 2004a) and semi-quantitative RT-PCR (Fig. 1) imply the presence of at least two regulatory promoter elements; one is for the early stages and is independent of Dd-STATa and the other is for the later stages and is partly Dd-STATa-dependent. To clarify whether this is true for *ahhA* gene expression, we amplified a promoter fragment of the *ahhA* gene and subcloned it into pDd-gal17(H+) to make a *lacZ*-fusion reporter construct

A

```
AhhA            ------------------------------------------------------------
α-adducin     1 MNGDSRAAVVTSPPPTTAPHKERYFDRVDENNPEYLRERNMAPDLRQDFNMMEQKKRVSM  60

AhhA          1 ------------------------------------------------------------MIS   3
α-adducin    61 ILQSPAFCEELESMIQEQFKKGKNPTGLLALQQIADFMTTNVPNVYPAAPQGGMAALNMS 120
                                                                            :*

AhhA          4 NNKFTSSDDLSLVNKTKYSDEEYLVRVKLAATYRMVAHLGWDELIYNHLTARVPG-TDHM  62
α-adducin   121 LGMVTPVNDLRGSDSIAYDKGEKLLRCKLAAFYRLADLFGWSQLIYNHITTRVNSEQEHF 180
                  . .*. :**   :.  *.. * *:* **** **:.  :**.:*****:*:** .  :*:

AhhA         63 LLNAFGMRFDEVTATSLVTIDMDGKIIDAGSTDLGINKTGYVIHGAIHKARPDILATMHV 122
α-adducin   181 LIVPFGLLYSEVTASSLVKINLQGDIVDRGSTNLGVNQAGFTLHSAIYAARPDVKCVVHI 240
                  *: .**: :.****:***.*:::*.*:* ***:**:*::*:.:*.**: ****: ..:*:

AhhA        123 HQEDTVAVACYKEGLLPISQNSFIIGDISYHNYEGISINAQEQERIVKSLGPVNKNLILR 182
α-adducin   241 HTPAGAAVSAMKCGLLPISPEALSLGEVAYHDYHGILVDEEEKVLIQKNLGPKSKVLILR 300
                  *    .**:. * ****** ::: :*:::**:*.** :: :*:  * *.*** .* ****

AhhA        183 NHGIVSCGNSIEEAFFFLYQLVKTCEIQVKMLSMVGGDISKLDIPDKQIRDFSTRTAASF 242
α-adducin   301 NHGLVSVGESVEEAFYYIHNLVVACEIQVRTLASAGGPDNLVLLNPEKYKAKSRSPGSPV 360
                  ***:** *:*:****:::::** :*****: *: .**  . : :  :: :  *  ..:..

AhhA        243 TKQGNGKKEWKSYYRIVEKLDDSFKN---------------------------------- 268
α-adducin   361 GEGTGSPPKWQIGEQEFEALMRMLDNLGYRTGYPYRYPALREKSKKYSDVEVPASVTGYS 420
                  : ..  :*:   : .* *   :.*
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B

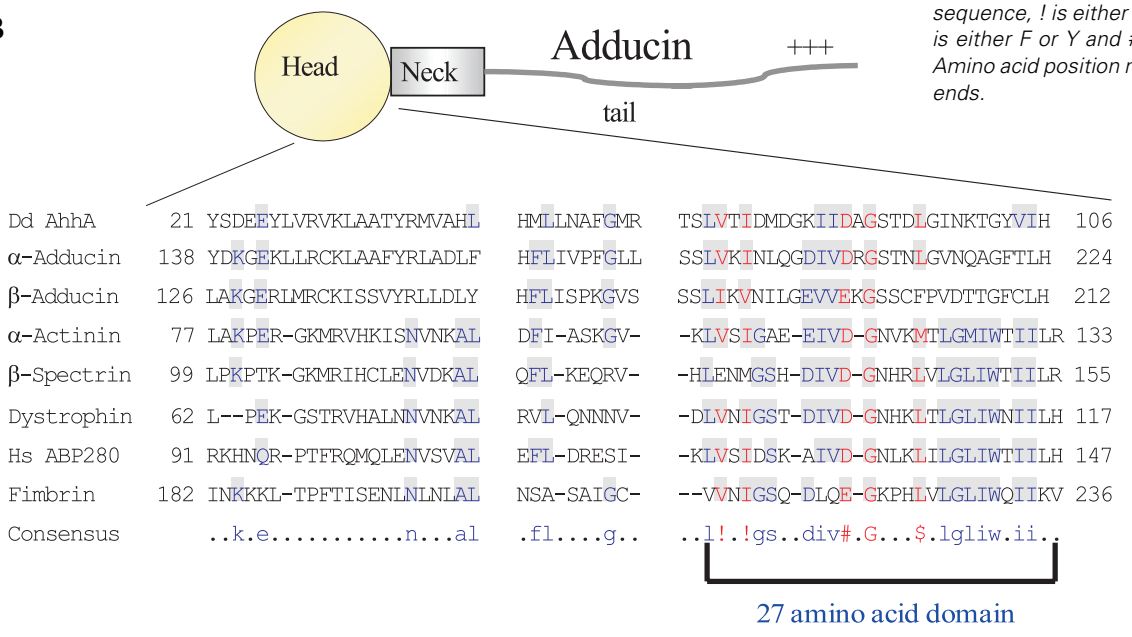
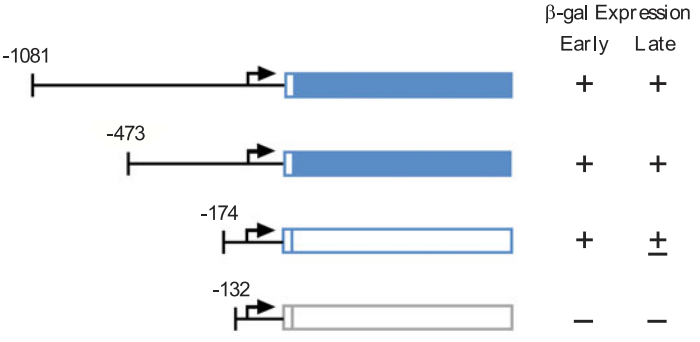


Fig. 2. Homology of the *ahhA* gene product with α-adducin, together with a scheme of the adducin protein and alignment with a putative actin-binding domain. (A) *Alignment of the AhhA amino acid sequence with human α-adducin 1 (accession number L29296.1; Lin* et al., *1995). Amino acid sequences of AhhA and human α-adducin 1 were aligned by using ClustalW program. Asterisks indicate identical amino acid residues between human α-adducin 1 and AhhA. Similarly, colons denote the amino acid residues with conservative change; dots indicate the residues with semi-conservative change. Amino acid position numbers are shown at both ends. Only the N-terminal portion corresponding to 420 residues is shown for human α-adducin 1.* **(B)** *Schematic representation of adducin protein and an alignment of a putative actin-binding domain. The schematic structure of the α- or β-adducin subunit is shown at the top (adapted from Hughes and Bennett, 1995; Li* et al., *1998). Adducin consists of head, neck and C-terminal tail regions. AhhA spans a region corresponding to head region of adducin. A sequence alignment of a putative actin-binding domain with conserved 27 amino acid-domain of already known actin-binding proteins and AhhA is shown at the bottom. Sequences of human α-adducin, human α-actinin (accession number P12814), human β-adducin (accession number P35612),* Dictyostelium *AhhA (accession number C91531), human β-spectrin (accession number P11277), human Dystrophin (accession number P11532), human endothelial ABP-280 (accession number P21333) and chicken fimbrin (accession number P19179) were aligned according to MultAlin program (Corpet 1998; http://prodes.toulouse.inra.fr/multalin/cgi-bin/multalin.pl). Conserved amino acids are shaded. Hyphens indicate gaps of sequence alignment. For symbols of consensus sequence, ! is either I or V, $ is either L or M, % is either F or Y and # is either N,D,Q,E,B or Z. Amino acid position numbers are shown at both ends.*

A

Fig. 3. Analyses of the *ahhA* gene promoter. (A) *Nucleotide sequence of the* ahhA *gene promoter region. Positions of nucleotide numbers are shown to the left. The position of a putative translational start site is referred to as +1 and is boxed. The positions of each deletion points at -1081, -473, -174 and -132 are indicated by arrows. The region with shaded letters is necessary for the prestalk-specific expression at multicellular stages of development (see Fig. 6).* **(B)** *Schematic representation and summarized expression of each 5' deletion construct. Expression at both early stages (until tip formation) and late stages are shown schematically as + (expressed), ± (barely detectable) and – (not expressed).*

B



and the promoter activity was assayed by its β-galactosidase activity.

A DNA fragment, which has a 5' end-point 1081 bp upstream of the putative translational start codon of the *ahhA* gene (*ahhA-5'Δ-1081::lacZ*, see Fig. 3), gave a similar staining pattern to that observed in the *in situ* hybridisation experiment (Shimada *et al.*, 2004a). The signal was detected during aggregation (Fig. 4Aa). Later, a signal became detectable in the pstA region at the tipped mound and slug stages (Fig. 4A b,c,d). At the Mexican hat stage, cells expressing *lacZ* are found in the tip region (Fig. 4A e). The stained cells eventually locate as a thin ring-shaped structure in the prestalk region, stalk tube entrance and disk cells (Fig. 4A f,g).

Unlike the *in situ* hybridisation results (Shimada *et al.*, 2004a), the signal was also detected in the *Dd-STATa*-null mutant transformed with *ahhA-5'Δ-1081::lacZ* construct throughout entire development (Fig. 4B). This may reflect traces of β-galactosidase activity during the early stages of development because of its stable character. Therefore, we replaced the *lacZ* gene with ile::*lacZ*, a fusion gene that produces unstable β-galactosidase (Detterbeck *et al.*, 1994) and compared the staining pattern between in Ax2 and *Dd-STATa*-null strains (Fig. 5).

The construct showed weakened staining, particularly in pstA cells, at late stages of development in the *Dd-STATa*-null strain compared to the staining of Ax2 strain (Fig. 5B), implying the involvement of Dd-STATa in the expression of the *ahhA* gene at late stages of development but not at early stages as already seen in the *in situ* and RT-PCR experiments (Shimada *et al.*, 2004a, Fig. 1). There is still a discrepancy between RT-PCR data and unstable *lacZ* data because of the absence of expression of the unstable β-galactosidase before tipped aggregates (Figs. 1 and 5). If a half-life of unstable β-galactosidase is the same, it could be possible that the stability of *ahhA* mRNA may change depend on developmental time course, although the precise reason is unknown.

Mapping of the ahhA *promoter regions necessary for early and late stage-specific expression*

To identify the regions necessary for expression at early and late stages, a series of 5'-3' deletion constructs were created. Deletion to 712 bp upstream of the putative translational start codon (termed ATG hereafter) weakened the staining in all pst cells at the slug and culminant stages in Ax2 and at the slug stage in the *Dd-STATa*-null mutant (data not shown). Further deletion to 473 bp upstream of the ATG caused ectopic expression of the *lacZ* gene in the late prespore/spore region of the culminats in addition to the prestalk staining of slugs (Fig. 6A). This indicates that a late prespore/spore-specific repressor region is located between nt (nucleotide) -712 and nt -474.

Cells transformed with construct *ahhA-5'Δ-174::lacZ* showed no pst- nor late staining. The observed diffuse staining (not a specific pst-staining) may due to scattered cells expressing stable β-galactosidase from beginning of development or pre-aggregation stage (Fig. 6B). Cells transformed with construct *ahhA-5'Δ-132::lacZ* lacked staining entirely at all stages of development (Fig. 6C). These results demonstrate that the region necessary for expression at the later stages in pstA cells is between nt -473 and -175, while expression at the early stages requires sequences between nt -174 and -132.

Disruption of the ahhA *gene leads to a tendency to form slanting culminants and aberrant papillae*

To determine the function of the AhhA protein, mutants lacking

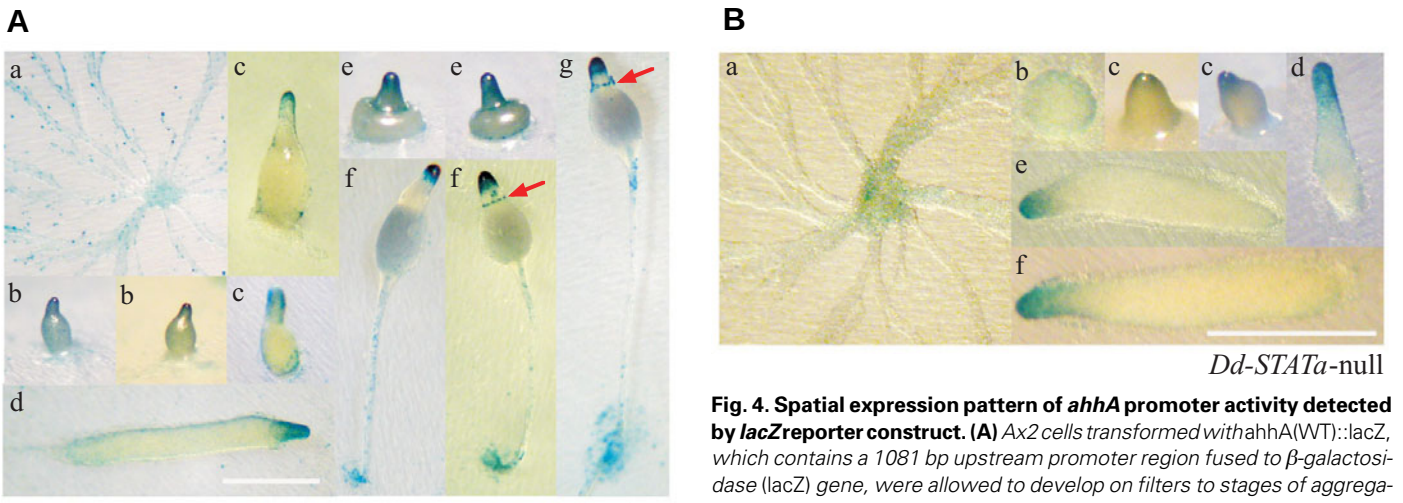


Fig. 4. Spatial expression pattern of *ahhA* promoter activity detected by *lacZ* reporter construct. (A) *Ax2 cells transformed with* ahhA(WT)::lacZ, *which contains a 1081 bp upstream promoter region fused to β-galactosidase* (lacZ) *gene, were allowed to develop on filters to stages of aggregation stream (a), tipped finger (b,c), slug (d), Mexican hat (e), mid culminant (f) and late culminant (g). Bar indicates 500 μm. Arrows indicate the ring shaped staining specific to late stages of development (see text for details). Staining was performed at 37°C for 30 min to detect* ahhA*-expressing cells.* **(B)** Dd-STATa*-null cells transformed with* ahhA(WT)::lacZ *were allowed to develop on filters to stages of aggregation stream (a), mound (b), tipped finger (c) and slug (d-f). Bar indicates 500 μm. Staining was performed at 37°C for 45 min to detect* ahhA*-expressing cells.*

the *ahhA* gene were created by homologous recombination (Fig. 7A). Two independent targeted clones were isolated by PCR screening using genomic DNA as a template and a semi-quantitative RT-PCR analysis showed that the *ahhA* transcript is almost undetectable (Fig. 7A). When the *ahhA*$^-$ cells were allowed to develop on either phosphate-buffered filters or on water agar plates, the *ahhA*$^-$ cells developed more rapidly, by approximately one hour (data not shown), but morphologically normally until early culmination (data not shown). At the mid stage of culmination, ~5% of the mutants formed swollen papillae with a constriction at the prespore-prestalk boundary (Fig. 7B c,d). The terminal structure of most mutant entities was indistinguishable from that of the parental strain, Ax2, except that the mutant formed a slanting fruiting body and easily collapsed on to the substratum (Fig. 7 Be). This latter, weaker phenotype is more prevalent, as more than 50% of the culminants show such behaviour (data not shown). All these phenotypic defects were rescued by expressing AhhA protein under the control of its own promoter (Fig. 7Bf). Therefore, all defects of the mutant are attributed to inactivation of the *ahhA* gene.

Since several null mutants of genes encoding actin-binding protein show a more severe phenotype when they are allowed to develop on soil (Ponte *et al.*, 2000), we tested the phenotype *ahhA*$^-$ cells on soil plates. However, no phenotypic change was observed in such experiments (data not shown).

Localization of the AhhA protein at the tip, pstO cells and cells near the constricted region

In order to elucidate AhhA function, we made a construct, which expresses AhhA protein with a human c-myc epitope tag, under the control of the *ahhA* promoter (Fig. 8A). The construct was transformed into the *ahhA*-null mutant and AhhA protein

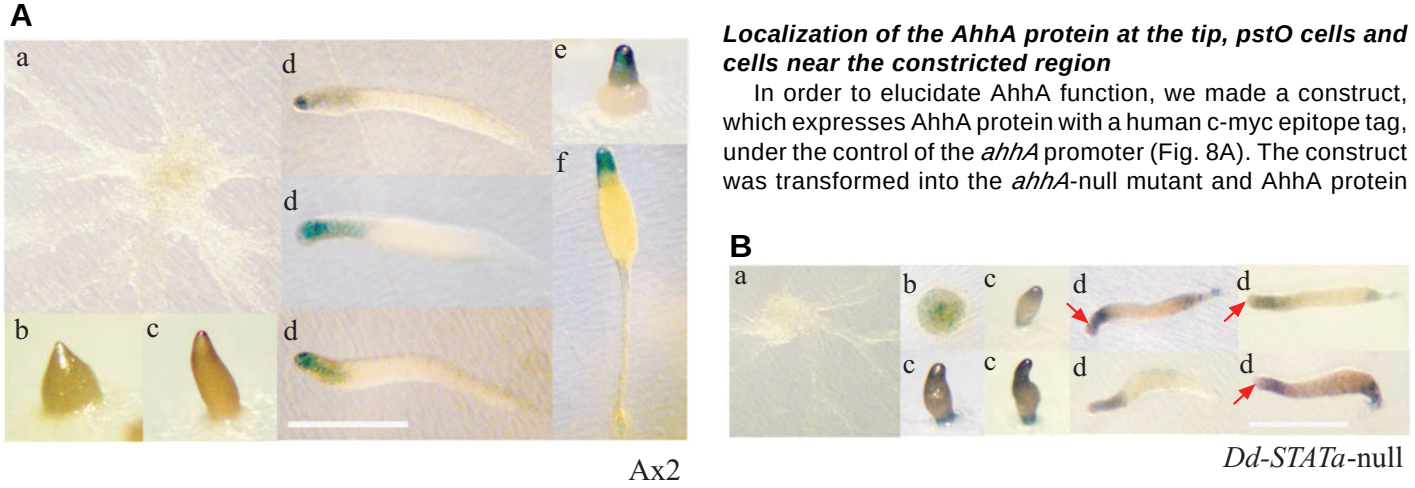


Fig. 5. Spatial expression pattern of *ahhA* promoter activity detected by ile::*lacZ* reporter construct. (A) *Ax2 cells transformed with* ahhA(WT):: *ile* ::lacZ, *which contains an1081 bp upstream promoter region fused to unstable β-galactosidase* (*ile* ::lacZ) *gene, were allowed to develop on filters. Stages are the same as described in the legend of Fig. 4A. Bar indicates 500 μm. Staining was performed at 37°C for 3 hours to detect* ahhA*-expressing cells.* **(B)** Dd-STATa*-null cells transformed with* ahhA(WT)::*ile* ::lacZ *were allowed to develop on filters. Stages are the same as described in the legend of Fig. 4B. Bar indicates 500 μm. Staining was performed at 37°C for 5 hours to detect* ahhA*-expressing cells. Arrows indicate the weakened staining of pstA cells in the* Dd-STATa*-null mutant (see text for details). After prolonged incubation, the staining before tipped aggregates becomes visible, thus to be expressed at a very low level.*

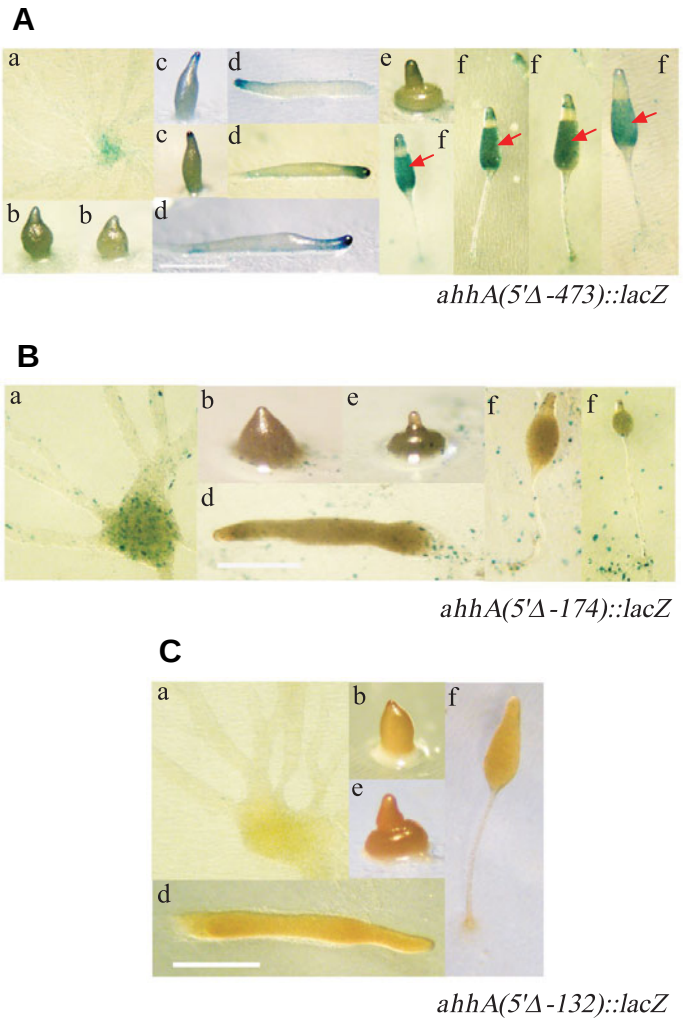


Fig. 6. Deletion analysis of the *ahhA* promoter in Ax2 cells. *Expression patterns are shown for the deletion constructs* **(A)** Δ-473::lacZ, **(B)** Δ-174::lacZ *and* **(C)** Δ-132::lacZ. *Transformed cells with each* lacZ *construct were allowed to develop on filters. Stages are the same as described in the legend of Fig. 4A. Bar indicates 500 μm. Staining was performed at 37°C for 1 hour for (A) and overnight for (B) and (C) to detect* ahhA*-expressing cells. Arrows indicate the ectopic staining in the psp cells for the* Δ-473::lacZ *construct.*

expression was monitored. Western blotting of total cell proteins from three independent clones identified a protein with an approximate molecular weight of 30 kDa, which matched well to the size predicted from the AhhA amino acid sequence (Fig. 8B).

Next we examined AhhA protein localization by immunohistochemical staining (Fig. 8C). The protein localized at the stalk tube entrance and pstO cells (arrowhead). The protein also localized in cells near the constricted region, where the *ahhA* transcript localized (Shimada *et al.*, 2004a; Fig. 4A). Since the AhhA has a putative actin-binding domain and the F-actin ring localizes at the constricted region, we tried co-staining with fluorescent phalloidin. As shown in Fig. 8C, AhhA protein co-localized with F-actin at the cells near the constriction (coloured arrows).

Discussion

A new Dd-STATa-dependent marker expressed in prestalk cells

In this study we identify a new marker gene, *ahhA*, which is expressed in all cells at the early stages and comes to be expressed in prestalk cells at later developmental stages, after the tipped finger stage (Fig. 4A). Expression in pstA cells at stages later than the slug stage is down-regulated in the *Dd-STATa*-null mutant, while expression up to the tipped finger stages is not (Shimada *et al.*, 2004a; Figs. 1 and 5). The spatial and temporal patterns of *ahhA* expression look almost the same in Ax2 and *Dd-STATa*-null cells but longer staining is required to visualize it (Figs. 1 and 4). However, *lacZ* staining in pstA cells is down-regulated in the *Dd-STATa*-null mutant compared to the staining in pstO cells (see Fig. 5d), The prestalk marker gene *ecmA* shows a similar expression pattern at the slug stage but there is a critical difference in the expression patterns of these two genes during culmination. During culmination, the *ecmA* gene is expressed in all prestalk cells. However, expression of the *ahhA* gene in the pstA cells disappears and then reappears in the upper cup cells, pstAB cells, stalk tube entrance and disk cells. There is no detectable expression in the bottom part of the stalk (Fig. 4A). This expression pattern is rather similar to the *ecmB* gene expression pattern. However, there is a difference in the expression pattern in the late culminant. Unlike the *ahhA* gene, the *ecmB* gene is expressed in the entire stalk. Most notably, the *ecmB* gene is also expressed in the lower cup cells. Therefore, the *ahhA* gene is a novel type of prestalk marker gene whose expression is partly dependent on Dd-STATa. It provides a tool to investigate the function of Dd-STATa.

Subregions of the ahhA *promoter directing early and late stage expression*

Although the *ahhA* gene is expressed throughout development, the transcript amount and spatial expression pattern of *ahhA* show very complicated profiles (Shimada *et al.*, 2004a; Figs. 1A, 4A). Deletion analysis of the *ahhA* promoter mapped a region necessary for expression in prestalk cells (particularly in pstA cells) at “later stages” to between -473 and -175, a region necessary for expression at early stages to between -174 and -133 (Figs. 3~6). A region between -712 and -474 must have a negative regulatory element(s) repressing *ahhA* expression in late prespore/spore cells and a region between -473 and -175 may contain a region required for the expression in those cells (Fig. 6A). The expression pattern of the *ahhA-5'Δ-473::lacZ* deletion construct in those cells is similar to that observed for *spiA* gene, a marker gene of late prespore/spore cells (Richardson *et al.,* 1994).

Since 5’-deletion constructs for further fine mapping are all unstable in bacterial cells, we have been unable to identify sequences necessary for prestalk cell expression at later stages in the region between -473 and -175 (data not shown). There are several GC sequences in this region (Fig. 3). Placing such GC sequences upstream of the *ahhA-5'Δ-174::lacZ* deletion construct gave no staining (data not shown). Therefore, it is likely that at least two GC sequences in this region are necessary for prestalk specific expression and the sequence element

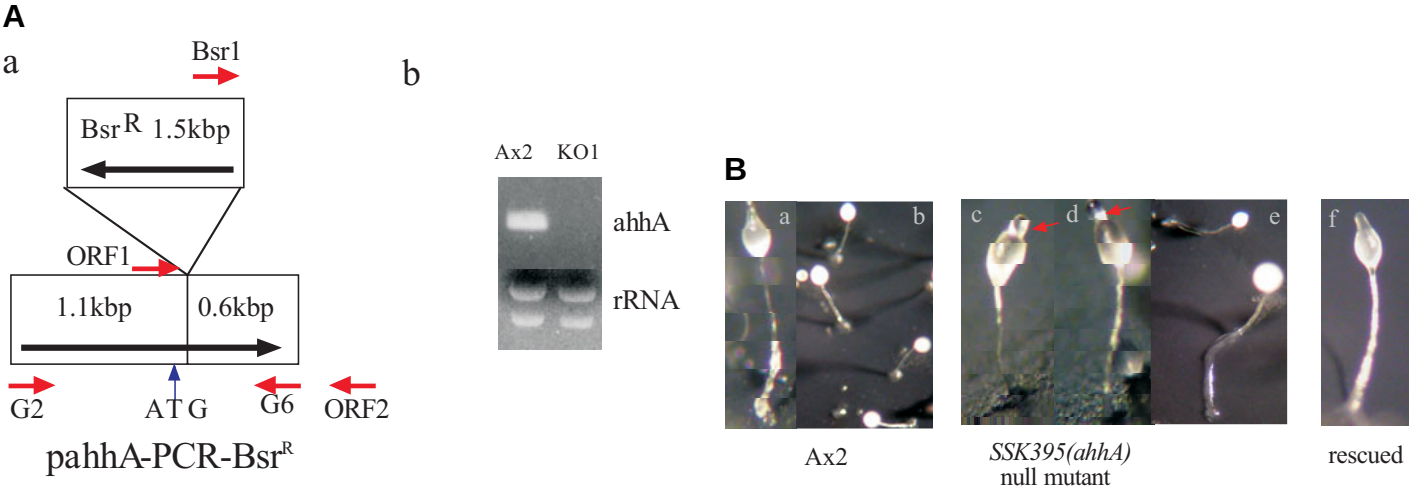


Fig. 7. Knock out mutants of *ahhA* gene. (A) *Creation of* ahhA *knock out construct and screening of* ahhA$^-$ *strains. Knock out construct of* ahhA *gene. Bsr*^{R} *cassette was inserted as described in Materials and Methods at 81 bp down stream from the* ahhA *gene translational start site (panel a). Targeted gene disruption was screened by genomic PCR with primer pair, Bsr-1 and 395-ORF-2 (data not shown). The disruption was further confirmed by both genomic PCR (data not shown) and RT-PCR (panel b). Genomic PCR was performed with primer pair 395-G2 and 395-G6 for two independently isolated* ahhA*-null mutants (KO1 and KO2). One microgram of total RNAs isolated from wild-type (Ax2) and the* ahhA*-null mutant (KO1) at vegetavely growing stage were used for RT-PCR. Ribosomal RNAs (1 μg total RNA/lane) were electrophoresed and stained with EtBr for loading standard.* **(B)** *Developmental phenotype of* ahhA*-null mutant. Cells of wild-type (Ax2, panels a and b), the* ahhA*-null mutant (panels c-e) and the rescued strain (panel f) were allowed to develop on phosphate-buffered filters to mid-culmination stage (panels a, c, d and f). Panels b and e show the overhead views of the late-culminants of the wild-type (Ax2) and the* ahhA*-null cells, respectively. Arrows indicate the swollen papillae of the mutant (see text for details).*

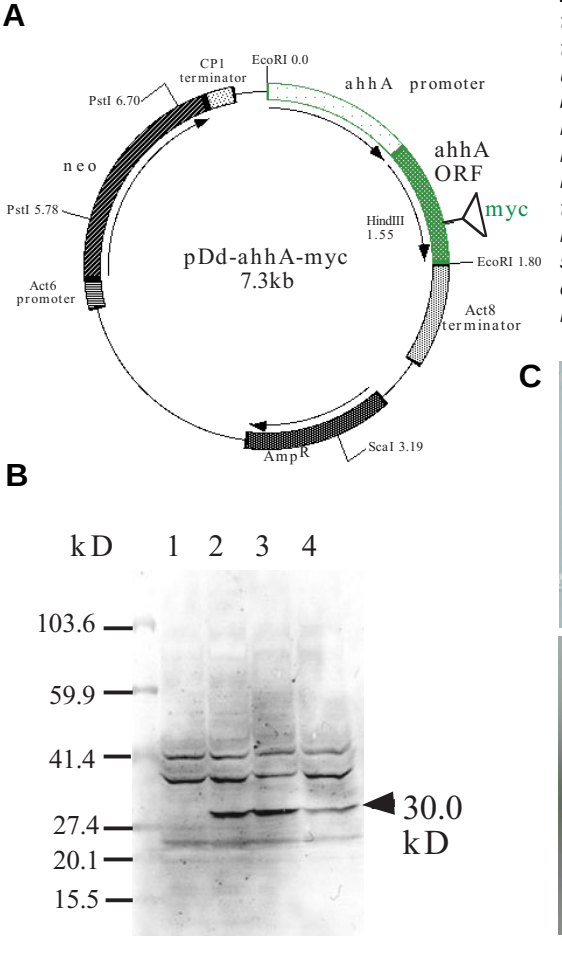
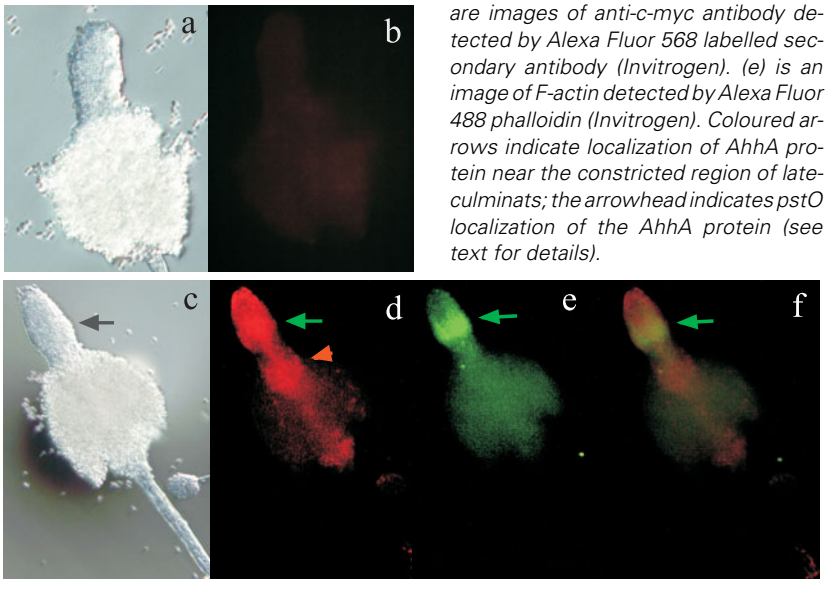


Fig. 8. Detection of the AhhA protein in the culminants. (A) *Expression construct of the AhhA protein, pDd-ahhA-myc. The ORF region of the* ahhA *gene was placed under the control of its own promoter. Human c-myc epitope tag was introduced into the unique* Hind*III site in the coding region.* **(B)** *Expression of the AhhA protein as detected by Western blotting. pDd-ahhA-myc was transformed into* ahhA*-null cells and three independent transformants (r1, r2, r3) were analysed. For Western analysis, approximately 20 μg of total cell protein was loaded per each lane and analysed as described in Materials and Methods. Lane 1 indicates Ax2 proteins, lanes 2-4 correspond to the transformants r1-r3, respectively. A protein band with a molecular weight of approximately 30 kDa (arrowhead) was visible in lanes of transformants but not of Ax2, thus, supposed to be specific AhhA band.* **(C)** *Spatial expression of the AhhA protein as detected by immunohistochemical staining. Photographs (a) and (c) are relief contrast images, (b,d,e,f) are fluorescent images of the mid-culaminants of Ax2 strain (a,b) and AhhA::c-myc expressing strain (c-f). (b,d) are images of anti-c-myc antibody detected by Alexa Fluor 568 labelled secondary antibody (Invitrogen). (e) is an image of F-actin detected by Alexa Fluor 488 phalloidin (Invitrogen). Coloured arrows indicate localization of AhhA protein near the constricted region of late-culminats; the arrowhead indicates pstO localization of the AhhA protein (see text for details).*



responsible for expression in prestalk cells remains to be identified.

Putative function of the AhhA protein

AhhA is a protein with a putative actin-binding domain and it is highly homologous to mammalian adducin (Fig. 2). Data base search shows that there is no other gene encoding an adducin-like protein in the *Dictyostelium* genome (Eichinger *et al.*, 2005). Adducin is a heterodimeric cytoskeleton protein involved with actin and spectrin in forming specialized contact sites in various multicellular organisms (Yue and Spradling, 1992; Robinson *et al.*, 1994). Interestingly, the AhhA protein lacks domains corresponding to neck and protease-sensitive carboxy-terminal tail but harbors a globular head domain (Hughes and Bennett, 1995). The C-terminal tail is a target of protein kinase C and Rho kinase and has an important role in forming a complex with fast growing ends of actin filaments that recruit spectrin (Hughes and Bennet, 1995; Li *et al.*, 1998). Therefore, the AhhA protein may not be an authentic adducin.

Despite lacking the C-terminal domain of adducin, localization of AhhA protein is in cells where F-actin is enriched and where there is a constriction in the stalk tube (Grimson *et al.*, 2000; Fig. 8C). Although there is no evidence to show a direct interaction of AhhA protein with F-actin, the above result and the presence of putative actin-binding domain imply that the AhhA protein may interact with F-actin.

Inactivation of the *ahhA* gene causes almost no phenotypic change but *ahhA*-null cells form aberrant culminants: slanting culminants, frequently than is observed for wild-type cells (Fig. 7C). The mutants easily collapse on to the substratum (Fig. 7Be). Under microscopic observation the mutant stalk is indistinguishable from that of wild-type stalk (data not shown), therefore, the collapse may be caused by the slanting of culminants but not by the weakness of the stalk. In addition, the AhhA protein co-localizes at a region near or where the stalk tube is constricted. This may indicate that AhhA is necessary for the correct downward elongation of the stalk. It may control the shape of the culminant through interaction with other molecules, possibly F-actin or adherens junction molecules (Grimson *et al.*, 2000; Coates and Harwood, 2001; Coates, 2003; Williams and Harwood, 2003). A protein supposed to be adducin has been identified in *Physarum polycephalum*. It forms large aggregates of actin filaments and modifies actin-myosin interaction by blocking it (Ogihara and Tonomura, 1982). The AhhA protein may have similar function, although it remains to be elucidated.

A link between Dd-STATa signaling and the AhhA protein and the cytoskeleton?

Our finding that the *ahhA* is down-regulated in the *Dd-STATa*-null mutant at later stages of development (Figs. 1 and 5), suggests that Dd-STATa has a role(s) during culmination of regulating this adducin-like protein. As the protein harbors a putative actin-binding domain, this provides a tool to investigate Dd-STATa function on morphogenesis, through the cytoskeleton.

Materials and Methods

Cells and growth conditions

D. discoideum Ax2 and *Dd-STATa*-null cells were cultured axenically in HL5 medium at 22°C. Cells of *Dd-STATa*-null and *SSK395(ahhA)*-null strains were grown in HL5 supplemented 10 μg/ml blasticidin S (Kaken Pharmaceutical, Tokyo, Japan). Transformants containing the Neo^R cassette were selected in HL5 supplemented with 10 μg/ml G418 (geneticin; ICN Biochemicals Inc., Ohio, USA) and then the concentration of G418 was increased to 20~80 μg/ml.

Genomic DNA cloning

The genomic sequence of the *SSK395(ahhA)* gene was obtained via a BLAST search (http://dictybase.org/db/cgi-bin/blast.pl) using the SSK395 cDNA sequence from the Dicty-cDNA database (http://www.csm.bio.tsukuba.ac.jp/cDNAproject.html) as a search term. The genomic fragment was amplified by PCR using the oligonucleotides 395-G2 (5'-TGC TCT AGA GCA CAT TAG TTT TAA CTC ATT TAG CTC-3') and 395-G6 (5'-CCG CTC GAG CGG CTT GTT TGG TGA AAG AAG CAG CTG-3') as primers (see Fig. 7A) and genomic DNA from the Ax2 strain as the template. A PCR fragment of approximately 1.7 kbp, was amplified and subsequently subcloned into pCR4-TOPO (Invitrogen Life Technologies) to yield pTOPO-395.

Construction of targeting vector

An *SSK395(ahhA)* targeting vector was constructed by use of the inverse PCR. Both oligonucleotides 395-G1 (5'-CGC GGA TTC GCG ATT AAC TAA TGA AAG GTC ATC ACT ACT TG-3') and 395-G5 (5'-CGC GGA TTC GCG AGT TCG TGT CAA ATT GGC AGC AAC-3') contain *Bam*HI site (underlined) at their 5' end, which were used as primers and pTOPO-395 as the template. Amplified fragments were digested with *Bam*HI and ligated with a bsr^R cassette digested with *Bam*HI.

Screening of the SSK395(ahhA)*-null strains*

The target vector was digested with *Not*I and *Spe*I. After phenol/chloroform extraction followed by ethanol precipitation of the vector DNA, Ax2 cells were transformed by electroporation. Transformants were selected in 96-wells plates containing HL5 medium supplemented with 10 μg/ml blasticidin S. To screen the clones for those in which the *SSK395(ahhA)* gene had been successfully targeted, genomic DNA from each clone was amplified by PCR using two pairs of primers 395-ORF-2 (5'-CCG CTC GAG TTA ATT TTT AAA AGA ATC ATC TAA TTT TTC AAC AAT TC-3') / Bsr-1 (5'-GCT CCC ACA TCA TGT TTA TTA TCC-3') and 395-G2 / 395-ORF-2 (see Fig. 7A).

Analysis of gene expression by semi-quantitative RT-PCR

RT-PCR was performed as described previously (Shimada *et al.*, 2004b) by RNA LA PCR Kit (AMV) version 1.1 (TaKaRa, Kyoto, Japan) using a pair of primers 395-ORF-1 (5'-CGG AAT TCA TGA TTT CAA ATA ATA AAT TTA CAA GTA GTG ATG ACC TTT C-3') and 395-ORF-2.

Construction of various lacZ *fusion construct and expression construct*

The promoter of the *SSK395* gene was amplified by PCR to add an *Xba*I site at its 5' end and a *Bgl*II site at its 3' end. After digestion with *Xba*I and *Bgl*II, the fragment was gel-purified and subcloned into *Xba*I/*Bgl*II cut pDd-gal17(H+) (Harwood and Drury, 1990) to make pSSK395::*lacZ*. For 5' deletions, primers corresponding to each deletion point with *Xba*I site at the 5' end were used to amplify the deleted fragment, then subcloned into pDd-gal17(H+) as above.

For construction of human c-myc-tagged SSK395(AhhA) expression vector, pDd-gal17H(+) was digested with *Bgl*II and *Xho*I to delete the *lacZ* gene to produce pDd-gal17H(+)Δgal. The genomic fragment was amplified by PCR with a pair of oligonucleotides 395-G2 and 395-ORF-2 as primers using genomic DNA from Ax2 cells as a template. A DNA fragment, which contains an entire *ahhA* ORF and its promoter was amplified and subcloned into pCR2.1-TOPO (Invitrogen). After digestion with *Eco*RI, the fragment containing SSK395(ahhA) ORF

and its promoter was gel purified and ligated into *Eco*RI site of pDd-gal17H(+)Δgal to yield pDd-ahhA. As a human c-myc epitope EQKLISEEDL, two oligonucleotides corresponding to sense (5'-AGCT GAA CAA AAA CTT ATT TCT GAA GAA GAT CTT GC-3') and antisense (5'-AGCT GC AAG ATC TTC TTC AGA AAT AAG TTT TTG TTC-3') strands were annealed then ligated into an unique *Hin*dIII site of pDd-ahhA to make pDd-ahhA-myc (see Fig. 8). The *Hin*dIII site was located 584 bp downstream from translational start codon of the SSK395(ahhA) ORF. Direction of the inserted double stranded oligonucleotides and the reading frame was confirmed by PCR and sequencing.

Promoter analysis by β-galactosidase staining

For the detection of promoter activity, cells transformed with various deleted promoter fragments fused to *lacZ* or ile::*lacZ* (Detterbeck *et al.*, 1994) reporter plasmids were grown and developed on nitrocellulose filters. Fixation and staining were performed as described (Dingermann *et al.*, 1989).

Western analysis and immunohistochemical staining

For Western analysis, cells were solubilized in SDS sample buffer containing EDTA-free Complete Mini (Roche, Penzberg, Germany) as proteinase inhibitors. The proteins were analyzed on 5~20% gradient SDS-polyacrylamide gels and blotted onto Hybond-ECL filters (Amersham Biosciences, Ltd, UK). The filters were blocked and detected by Promega Proto Blot II AP System with Stabilized Substrate according to the supplied protocol (Promega, WI, USA). Mouse anti-human c-myc monoclonal antibody 9E10 (1:2,000 dilution, Exalpha Biologicals, Inc., MA, USA) was used as a primary antibody and alkaline phosphatase-conjugated anti-mouse IgG(HL) antibody (1:10,000 dilution, Promega) was used as a secondary antibody.

For whole mount immunohistochemical staining, pre-culminant stage multicelllar structures were allowed to settle on poly-L-lysine coated slides and fixed with methanol for 10 min then dried. Samples were incubated with 9E10 antibody (1:200 dilution, Exalpha Biologicals, Inc.) for 20 hrs in blocking solution. After washing with PBS, they were incubated with Alexa Fluor 568 goat anti-mouse antibody (1:1000 dilution, Invitrogen life technologies, USA) for 20 hrs in PBS. For the detection of F-actin, 170 nM Alexa Fluor 488 phalloidin (Invitrogen life technologies) were used. The samples were mounted and visualized in OLYMPUS fluorescent relief-contrast microscope (model CKX41). The images were taken by Penguiun 150CL Digital Camera System (Pixera, Los Gatos, USA).

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