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Isolation and Characterization of *mas1*$^+$ of *Schizosaccharomyces pombe*, a Homologue of Human *CIP29/Hcc-1* Involved in the Regulation of Cell Division

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The regulation of gene expression plays an important role in cell cycle controls. In this study, a novel gene, the *mas1*$^+$(mitosis associated protein) gene, a homolog of human *CIP29/Hcc1,* was isolated and characterized from fission yeast *Schizosaccharomyces pombe* (*S. pombe*) using a gene-specific polymerase chain reaction. The isolated gene contained a complete open reading frame capable of encoding 245 amino acid residues with a typical promoter, as judged by nucleotide sequence analysis. It was also found that a PCB (pombe cell cycle box) is located in the promoter region, which controls M-G_1 specific transcription in *S. pombe*. The quantitative analysis of the *mas1*$^+$ transcript against *adh1*$^+$ showed that the pattern of expression is similar to that of the septation index. Cytokinesis of *mas1* null mutant was greatly delayed at 25℃ and 36℃, and a large number of multi-septate cells were produced. The *mas1* null mutant had 2C, 4C and 6C DNA contents, as determined by FACS analysis. In addition, the number of multi-septate cells significantly increased. When cells were cultured in nitrogen starvation medium to increase proliferation, the abnormal phenotypes of *mas1* null mutant dramatically increased. These phenotypes could be rescued by an overexpression of the *mas1*$^+$ gene. The *mas1* protein localized in the nuclei of *S. pombe* and human HeLa cells, as evidenced by Mas1-EGFP signals. The abnormal growth pattern and the morphology of *mas1* null mutant were complemented by a plasmid carrying human *CIP29/Hcc-1*cDNA. In addition, *CIP29* / *Hcc-1* transcript level increased in active cell proliferation stages in the developing mouse embryos. These results indicate that the *mas1*$^+$ ishomologous to the human *CIP29/Hcc1* gene and is involved in cytokinesis and cell shape control.

Key words : Mas1, Hcc-1, cell cycle, *S. pombe*, cytokinesis

Introduction

The organization of eukaryotic chromatin into specific conformations that are associated with transcription, replication, repair and other nuclear processes are achieved via a series of DNA protein interactions. These interactions are mediated by a range of DNA binding domains or modules including the high mobility group 1 (HMG1) domain [7,21,23,25], the AT hook [4], the plant homeodomain (PHD) finger, the Bright domain, the Myb/SANT domain and SAP (after SAF A/B, Acinus and PIAS) domain [8,22,26,30].

A PSI-BLAST search of the non-redundant (NR) database at the NCBI seeded with the N-terminal DNA binding domain of SAF-A resulted in the detection of statistically significant similarity to several other chromatin associated proteins, such as the N-terminal region of plant poly (ADP-ribose) polymerase (PARP) and human Acinus [2]. Transitive searches with the sequences of these proteins resulted in the identification of new homologies, namely the DNA repair protein KU70, STAT inhibitors (e.g. PIAS), the yeast protein Tho1p, the yeast protein mlo1p as well as several uncharacterized animal, plant and fungal proteins.

It was previously reported that overexpression of the partial mlo1p containing the SAP domain of *S. pombe* causes a complete failure of chromosome segregation [22]. The mlo1p had homology with tho1 protein of *S. cerevisiae* and *CIP29* protein of human. The function of these proteins has not been yet characterized.

A bioinformatical prediction showed that the first 42 amino acids of the protein is a SAP domain with sequence matches to hnRNP from various vertebrate species. The *Hcc-1* protein was localized to the cell nucleus while the gene was localized to the chromosome 7q22.1. *Hcc-1* cDNA level increased in pancreatic adenocarcinoma. The level also increased in a well-differentiated hepatocellular carcinoma but decreased as carcinoma progressed to a poorly differentiated stage [9].

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To identify cytokine-induced proteins in hematopoietic cells, lysates from Epo-dependent UT-7/Epo leukemic cells cultured with or without Epo were analyzed by 2D-gel electrophoresis [18]. A full-lenth cDNA of a novel erythropoietin (Epo)-induced protein (*CIP29*) was cloned. The *CIP29* cDNA encodes a protein of 210 amino acids with a predicted molecular mass of 24kDa, with contains an N-terminal SAP DNA-binding motif. *CIP29* expression was higher in cancer and fetal tissues than in normal adult tissues [14]. Overexpression of CIP29-GFP in HEK293 cells enhances the cell cycle progression. *CIP29* appears to be a new cytokine-regulated protein involved in normal and cancer cell proliferation. The *Hcc-1* and *CIP29* genes were identified to encode the same protein [9,11].

On the other hand, *Hcc-1/CIP* appears to inhibit cell proliferation by inducing G_2/M arrest in the presence of growth factors, but inhibits cell proliferation by inducing apoptosis without arresting cells in G_2/M following deprivation of growth factors. The inhibitory effect on cell proliferation and induction of apoptosis or G_2/M arrest by *Hcc-1/CIP* is somewhat puzzling since *CIP29* is up-regulated by hematopoietic growth factors, which normally enhance cell survival, the cell cycle and proliferation, and is also up-regulated in several cancer cells, which possess enhanced survival and proliferation characteristics. Induction of *Hcc-1/CIP* by growth factors may, therefore, be part of a negative feedback loop regulating cell proliferation [15].

In the present study, the structure and function of the full sequence of *mlo1*, named here as *mas1*$^+$, was investigated in *S. pombe* and the functional relationship of *mas1*$^+$ with human *CIP29* was also studied.

Materials and Methods

Cell culture and general techniques

General molecular procedures were performed as described by Sambrook *et al.* [35], while the media used for the propagation of *S. pombe* were as described by Moreno *et al.* [28]. The standard genetic procedures of Gutz *et al.* [17] and Kohli *et al.* [24] were followed. For physiological experiments, cells were grown routinely in Edinbergh minimal medium (EMM, Gibco, USA) at 25 or 32℃ with shaking. Temperature-sensitive mutants were incubated at the restrictive temperature of 36℃ in order to display their mutant phenotype. Synchronization of cells by transient temperature shifts for the *cdc25-22* mutant was achieved by growing cells to mid-exponential growth at 25℃ before shifting to 36℃: cells were then back to 25℃ after 4 hr to enter the mitotic cell cycle in synchrony. Samples were removed for RNA extraction, and measured septation indices by microscopic examination. To overexpress Mas1 protein using the pREP1 vector [27], cells were grown in EMM with 5mg/ml thiamine (nmt1+ promoter 'off') to the early exponential stage of growth. Cells were washed three times in thiamine-free EMM, and then grown for 15 hr in thiamine-free EMM (nmt1+ promoter 'on'), at the same temperature. Cell number per ml of liquid culture was determined from a sample fixed in a 0.1% formaldehyde/0.1% sodium chloride solution.

Preparation of synchronous culture from *S. pombe*

Preparation of synchronous culture was carried out as described previously [36]. Strain Q356 cells carrying *cdc25-22* were grown to mid-log phase in rich medium (YES) at 25℃ and were collected by centrifugation and used to reinoculate into fresh medium which was cultured at 36℃ for 4 hr to block the progress of cell cycle. The culture was transferred back to 25℃, and 20 ml aliquots of the cells were harvested at 20 min intervals. These samples were stored at -70℃ until used.

Northern blot analysis

Total RNA (20 μg) extracted from JY1 (972 *h*$^+$ wild type) strain was denatured, electrophoresed through 1% agarose gel containing 2.2 M formaldehyde, and transferred onto membrane (Amersham, USA). The membrane was hybridized under 50% formamide at 42℃ for 20 hr with appropriate DNA probe. The probes were prepared by labeling with [α^{-32}P]dCTP (3000 Ci/mmole, NEN Corp.) according to oligo-labelling method [13].

Gene disruption/replacement

The 735 bp PCR fragment of the *mas1*$^+$ gene was deleted and replaced with a 1.7 kb fragment containing the *S. pombe ura4*$^+$ gene. The *Pvu* I fragment was isolated and used to transform the diploid *S. pombe* strain ED665/668. Two *ura*$^+$ transformants were identified and *h90/h*$^+$ derivative isolated and sporulated. The gene disruption was confirmed from the diploid and the haploid derivatives by Southern blotting.

Cytometry and cell staining

For DAPI and Calcofluor (Sigma, USA) staining, cells

were harvested, washed once in sterile water and re-suspended in 90% methanol. Samples were spotted onto microscope slides, stained with DAPI and Calcofluor, and photographed. For cytometry, cells were harvested, washed with sterile water and ethanol was added (while agitating the tube) to a final concentration of 70%. Cells were stored at -70°C. Before analysis a sample of the cells was removed, washed once with 50 mM sodium citrate (pH 7.0) and re-suspended in 0.5 ml of the same solution. After addition of RNase A to a concentration of 1 mg/ml, the cells were incubated for 3 hr at 37°C and stored on ice. The sample was diluted into PBS, and after addition of propidium iodide the cells were analyzed using a Coulter Elite cell sorter.

Staining with 4, 6-diamidino-2-phenylindole (DAPI) and microscopy

Aliquots of 900 µl of cultures in YES liquid media at 30℃ were collected and fixed in 100 µl of 37% formaldehyde. After washing the samples twice in water, cells were mounted on a microscope slide in DAPI mounting solution [28]. Samples were examined and photographed under a fluorescence microscope (Zeiss Axioskop 2 Plus Microscope, U.S.A) .

Results

Molecular cloning of a fission yeast homologue (*mas1*$^{+}$) of human *CIP29/Hcc1* gene

A fission yeast homologue of human *CIP29/Hcc1* gene was identified and characterized based on a homology search of the fission yeast database with the human *CIP29/Hcc1* gene sequence as a query sequence (Fig. 1A). The overall amino acid sequence of the identified fission yeast full-length Mas1 (Conserve domain database

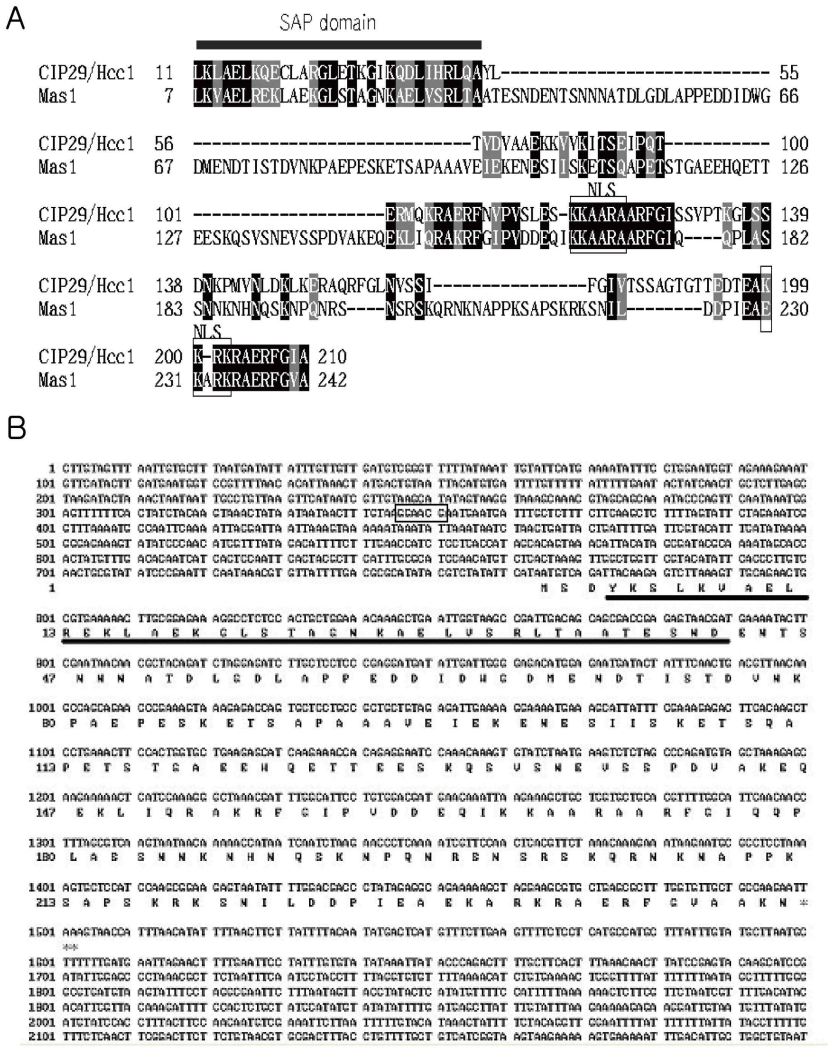

Fig. 1. Sequence of *S. pombe mas1*$^{+}$ gene and alignment of homologous gene. (A) This gene encodes a protein containing 245 amino acids. Numbers of nucleotides and amino acid residues are indicated on the left. The open reading frame starts at nt 735 and ends at the termination codon, corresponding to nt 1502. Underline indicates the SAP domain, a DNA binding domain. The PCB sequence (position -459) is shaded, which is GNAACg/c as a putative consensus sequence for the fission yeast M-G1 *cis*-acting promoter element. The positions of in-frame stop codons flanking the coding sequence are indicated by asterisks. (B) A SAP domain is underlined. The nuclear localization signals (NLS) are boxed. The putative active domains were shaded.

ID:KOG4259) was 35% homologous to human *CIP29/Hcc1.* The SAP domain and nuclear localization signal were highly conserved.

The entire coding sequence of 735 bp *mas1*$^+$ ORF was amplified by gene specific PCR from the *S. pombe* cDNA. This *mas1*$^+$ cDNA was then cloned into the *S. pombe* expression vector pREP1 and fully sequenced. The nucleotide sequence with the deduced amino acid sequences are shown in Fig. 1. Comparison of the genomic and cDNA nucleotide sequences indicated that the *mas1*$^+$ gene is 735 base pairs in length. Intron was absent in agreement with the predicted structure in the *S. pombe* sequence data bank. The predicted Mas1 protein might be a 24 kDa hydrophilic protein consisting of 245 amino acids and containing a SAP domain which a putative DNA-binding motif involved in a chromosomal or N-terminal leader sequence (Fig. 1).

Identification of PCB sequence in the *mas1*$^+$ promoter

The consensus sequence GNAACg/c has been defined as PCB (pombe cell cycle box), which is a *cis*-acting element found in *S. pombe* promoter regulating M-G_1 transition in the cell cycle [3]. The typical PCB sequence GGAACG was found at position -495 of *mas1*$^+$ promoter (Fig. 1B). The transcription profile of the *mas1*$^+$was analyzed and compared to that of *cdc15*$^+$ in the present study. For the above purpose, *cdc25-22* mutant cells cultured at 25℃ were synchronized by transient arrest at the G_2-M boundary by reversible temperature shifts to 36℃ for 4 hr and then a shift back to 25℃ (Fig. 2A). Northern blot analysis was performed on RNA from cells taken at 20 min intervals following release from the restrictive temperature. The blot was hybridized consecutively with *cdc15*$^+$, *mas1*$^+$ and *adh1*$^+$ probes, the latter as a loading control, and the quantification of each transcript against *adh1*$^+$ is shown (Figs. 2B and 2C). When compared with the septation index (Fig. 2A), the *cdc15*$^+$ mRNA as a positive control was transcribed in M-G_1, peaking before septum formation as expected. The *mas1*$^+$ mRNA level was low in G_2 phase, but high in M-G_1 phase. Especially when the percentage of septated cells was maximal, *mas1*$^+$ mRNA level was highest (Fig. 2) suggesting that the *mas1*$^+$ is associated with septum formation or deposition during cytokinesis.

Disruption of *mas1*$^+$ gene

To identify the function of *mas1*$^+$, disruption of the *mas1*$^+$ gene was performed using the 1.8 kb *S. pombe ura4* gene, which was inserted into the ORF of *mas1*$^+$ (Fig. 3A). A linearized DNA fragment containing the deleted *mas1*$^+$ gene was integrated into the chromosome of a diploid by homologous recombination. The resulting cells were analyzed by

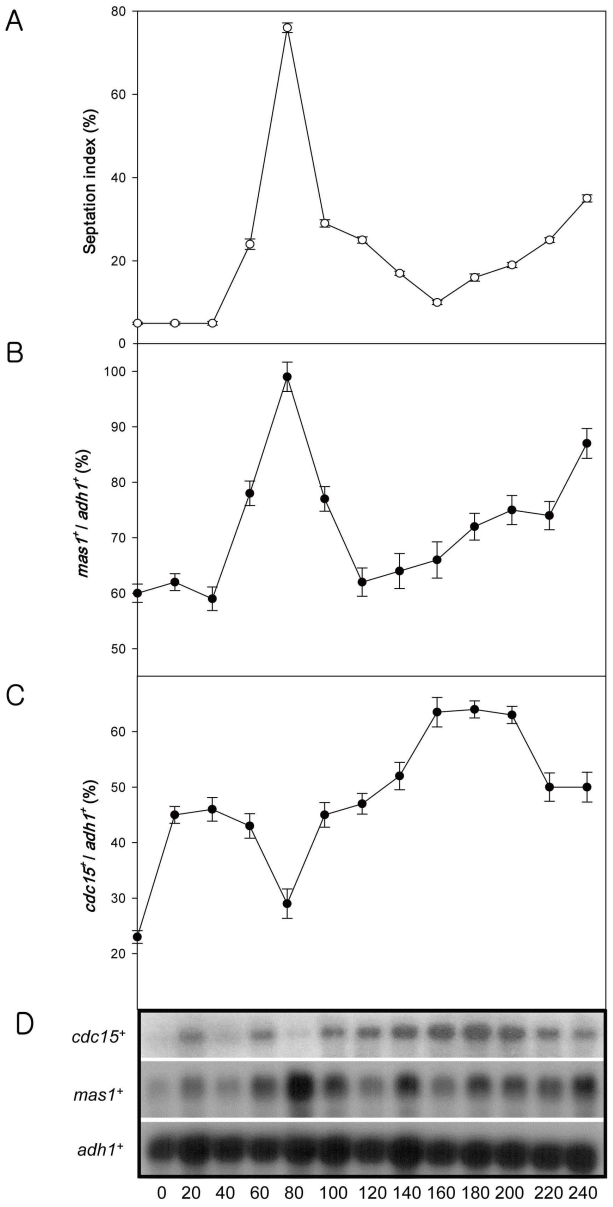

Fig. 2. *mas1*$^+$ mRNA level is maximal when the percentage of septated cells is maximal. Septation index (A) and mRNA levels of *mas1*$^+$ (B) or *cdc15*$^+$ (C) during mitosis in *S. pombe*. *cdc25-22* mutant cells cultured at 25℃ were synchronized by transient temperature shift to 36℃ for 4 hr and then shifted back to 25℃for various time periods. Quantification of *mas1*$^+$ (B) and *cdc15*$^+$ (C) transcript against *adh1*$^+$ from the autoradiogram of northern blot (D). Each data represents the mean±S. D. obtained from three independent experiments.

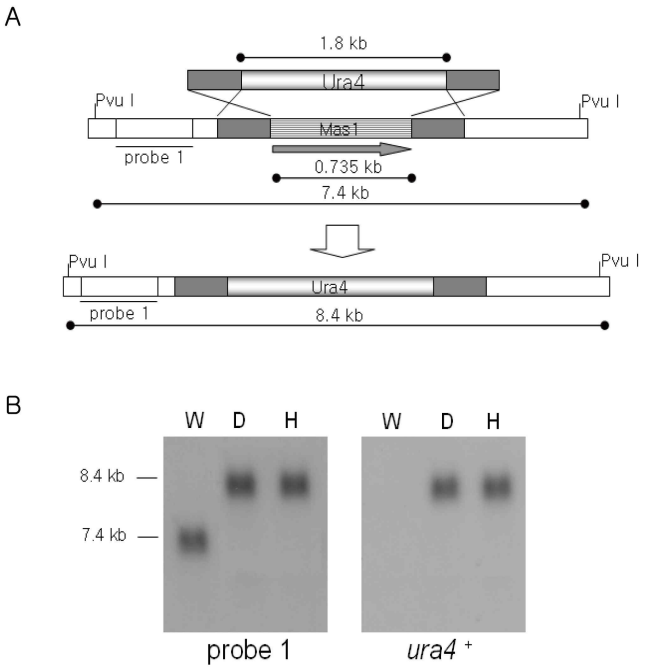

Fig. 3. Gene disruption of *mas1*$^+$ gene using *ura4*$^+$. (A) The diagram of *mas1*$^+$ gene (the coding region indicated by the shaded arrow) disrupted by PCR based gene targeting method using *S. pombe ura4*$^+$ gene. (B) Southern blot analysis. Chromosomal DNA cleaved with *Pvu* I was hybridized with probe 1 (left panel) or *ura4*$^+$ gene (right panel). W, wild type; D, disruption diploid; H, disruption haploid.

Southern hybridization with the upstream region of *mas1*$^+$ or *mas1*$^+$ full gene as probes: *Pvu* I bands with the expected sizes (8.4 and 7.4 kb) were obtained (Fig. 3B). The transformed diploids were then sporulated, and the resulting tetrads were dissected. Haploid cells containing the disrupted *mas1*$^+$ gene were viable, indicating that the *mas1*$^+$ gene is nonessential for viability.

The phenotype of *mas1* null mutant

By DAPI staining, a small fraction of abnormal, multiply septate and branched cells were observed at 36˚C and 25˚C (Fig. 4). At these temperatures, *mas1* null mutant cells were elongated with multi-septa and contained multiple nuclei. Each compartment formed by the septa usually contained a single nucleus, but not always. The size of the compartments was often smaller than that of single wild type cells and multiple septa were found adjacent. Thus the progression of cytokinesis was delayed, and the positioning of septation became abnormal in the disruption mutant. In contrast to *mas1* null mutant, no branching or delay of cytokinesis was found in wild type cells grown at the restriction temperatures 25˚C and 36˚C (Fig. 4). Interestingly, the increase in the turbidity of cultures was similar between the wild type and *mas1* null mutant at 25˚C after the shift to 36˚C for 4 hr, supporting the view that the frequency of cytokinesis, rather than increased cell mass, was severely affected in *mas1* null mutant at the temperature (data not shown).

The abnormal phenotype of cultured *mas1* null mutant in the rich YES medium was found only in 10% of cells (Fig. 4C). However, this population constituted 30% of the cells when cultured for 72 hr at the restrictive temperature in minimal medium, after which multi-septate cells began to appear (data now shown). These experiments showed that the Mas1 is associated with cytokinesis at M-G_1 in *S. pombe*.

Flow cytometry analysis

DNA content of wild type or *mas1* null mutant cells grown at 25℃, shifted to 36℃ for 4 hr, and then shifted back to 25℃ for various time periods were analyzed by flow cytometry. Figure 11 shows that a considerable proportion of the *mas1* null mutant had 2C, 4C and 6C DNA content (Fig. 5).

Septum staining of *mas1* null mutant indicated that these cells had some interesting features. After culturing the *mas1* null mutant at the restrictive temperature of 25℃, the population of the multi-septate cells dramatically increased. This population constituted 30% of the cells when cultured for 10 hr on minimal medium at the restrictive temperature, after which multi-septate cells began to appear. In contrast, only 1% of wild type cells showed such features (data not shown). FACS analysis revealed that the majority of the *mas1* null mutant at the restrictive temperature contained a 2C, 4C and 6C DNA contents. In this result, *mas1* null mutant growing at the restriction temperature showed abnormal cytokinesis. It is suggested that *mas1*$^+$ is involved in septum deposition during cytokinesis.

The abnormal phenotype of *mas1* null mutant cells increased in nitrogen starvation medium

As described above, the multi-septate pattern of the *mas1* null mutant appears when cultured for 10 hr on the minimal medium and for 72 hr on rich medium (Fig. 4). To determine whether the abnormal phenotype of *mas1* null mutant is associated with cell proliferation or not, the mutant cells were grown in the nitrogen starved medium to increased cell proliferation and analyzed by FACS and Calcofluor staining (Fig. 5, 6A and C). As expected, when the *mas1* null mutant

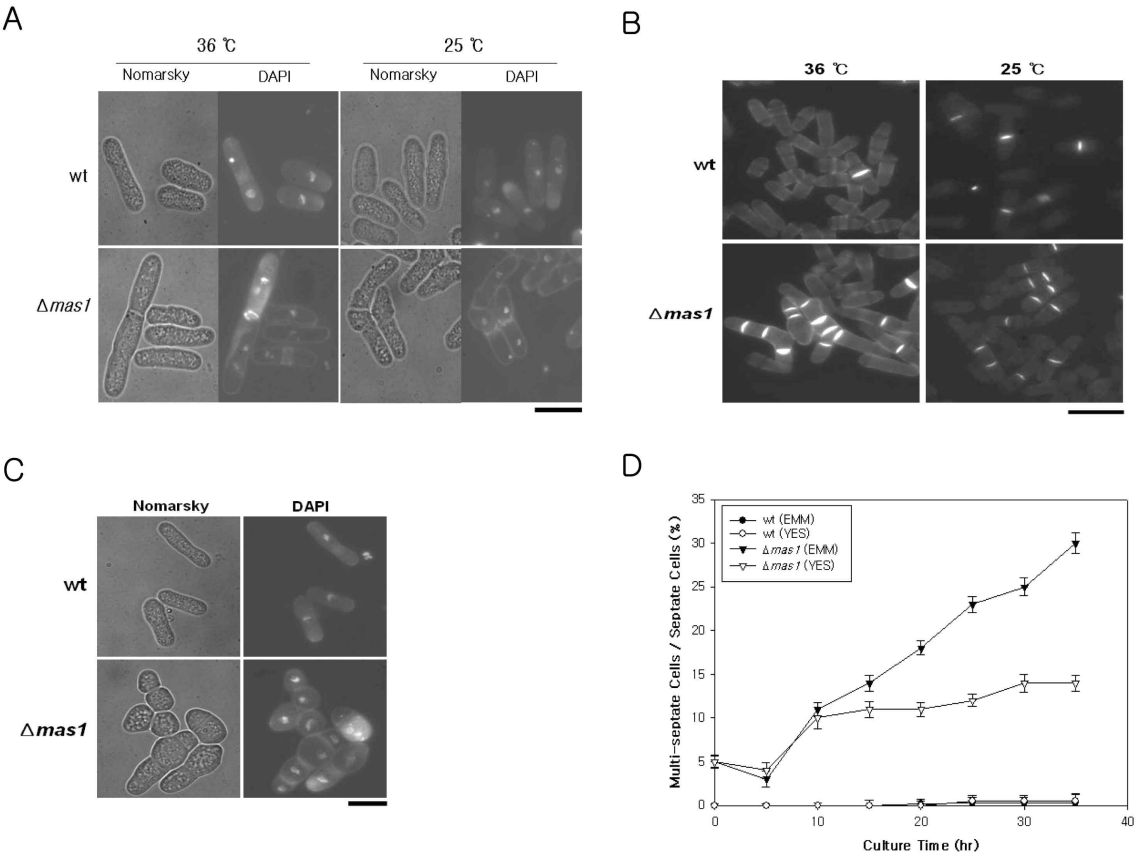

Fig. 4. Formation of multi-septate cells under various medium conditions. Wild type (wt) and *mas1* null mutant (Δ*mas1*) cells grown at 25°C in the minimal EMM medium were transferred to 36℃ in the rich YES or EMM medium and grown for various time periods. Wild type (wt) and *mas1* null mutant (Δ*mas1*) cells grown in EMM medium at 36°C or 25°C were stained by DAPI (A) and Calcofluor (B). Bar, 10 μm. (C) Wild-type (wt) and *mas1* null mutant (Δ*mas1*) cells grown for 72 hr at 36°C on rich YES medium and were stained by DAPI. Bar, 10 μm (D) Frequency of multi-septate cells from septate cells are shown. Each data represents the mean±S.D. of at least seven independent experiments.

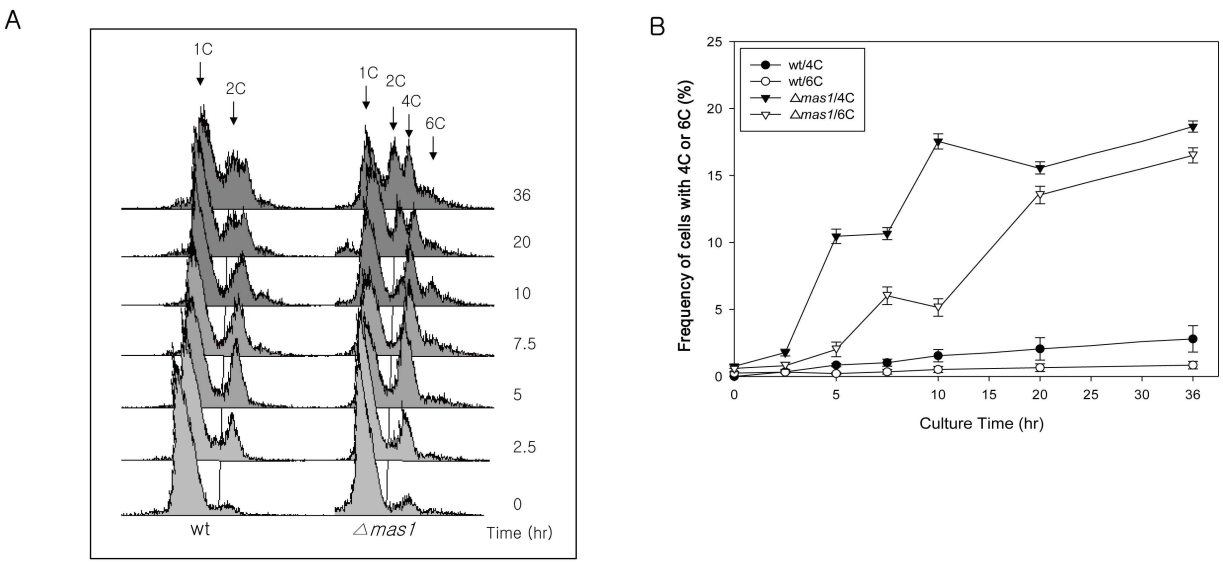

Fig. 5. DNA content (A) and quantification (B) of muli-septate of a *mas1* null mutant cells cultured in EMM medium. Wild type (wt) or *mas1* null mutant (Δ*mas1*) cells grown at 25℃ were shifted to 36℃ for 4 hr, shifted back to 25℃ for indicated time periods and then subjected to FACS analysis. Each data of panel B represents the mean±S.D. determined from three independent experiments.

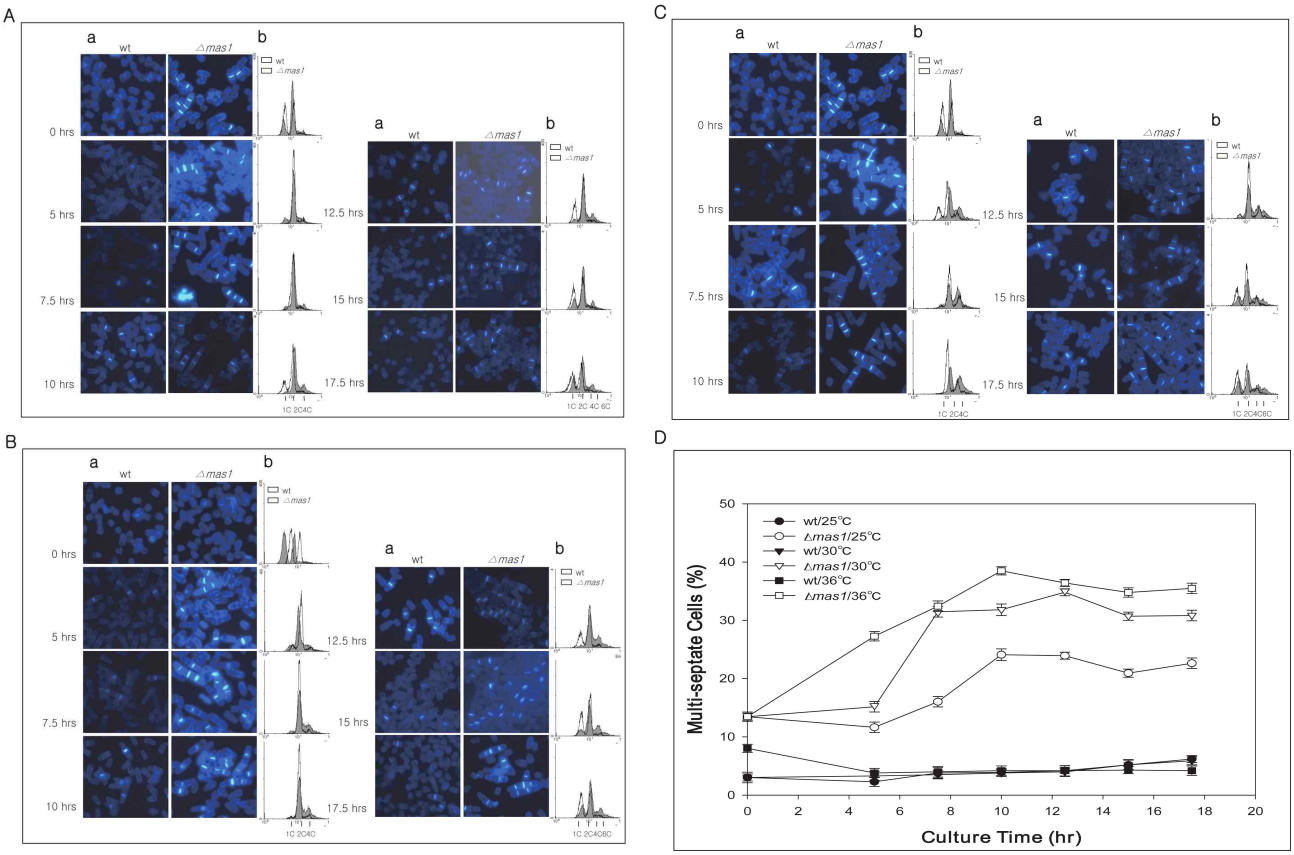

Fig. 6. Morphology (a) and DNA content (b) of wild type (wt) or *mas1* null mutant (Δ*mas1*) cells grown in nitrogen starved medium at 25℃. The cells grown at 30℃ were transferred to 25℃ (A), 30℃ (B) or 36℃ (C) in the nitrogen starved medium, cultured for various time periods and then analyzed by Calcofluor staining or FACS. (D) Fraction of multi-septate cells formed under various temperature condition in nitrogen starved medium. Each data represents the typical results that were reproduced in at least three independent experiments.

was grown in the nitrogen starved medium, the frequency of multi-septate cells and abnormal phenotype dramatically increased (Fig. 6). The frequency of multi septate cells reached maximum level when cells were incubated for 17.5 hr at 25 (Fig. 6A), 30 (Fig. 6B) or 36℃ (Fig. 6C). In addition, cells incubated at 36℃ showed increased abnormality as compared to those at 25℃ or 30℃. All these results suggest that the abnormal phenotype of *mas1* null mutant might be accelerated or potentiated by the depletion of nitrogen energy source.

Phenotype of the *mas1*$^+$ overexpressed cell

To overexpress the *mas1*$^+$ gene, the *S. pombe* inducible promoter *nmt1* was employed, which is repressed in the presence of thiamine (+T) and induced in the absence of thiamine (-T). The restriction sites, *Nde* I and *Sma* I were introduced at the putative initiation codon of *mas1*$^+$, and the resulting restriction fragment was cloned into the plasmid pREP1. Plasmid nmt1-*mas1* thus made was introduced into wild type or *mas1* null mutant, and transformants were selected in +T. Cells were first grown in +T, and then transferred to -T in order to derepress the nmt1 promoter. The nmt1 promoter-directed synthesis of *mas1*$^+$ tanscript was observed at 10 hr after the removal of thiamine.

By northern hybridization analysis, it was shown that this resulted in an increase in the transcript level by a 2.1 or 1.8 fold in wild type or the *mas1* null mutant, respectively, as compared to wild type cells transformed with pREP1 only (Fig. 7A). The induction of *mas1*$^+$ by the removal of thiamine, significantly decreased the abnormal phenotype of *mas1* null mutant (Fig. 7B), suggesting a sufficient complementation of disrupted *mas1*$^+$ gene. Cells carrying plasmid *nmt1-mas1* were fixed by 70% ethanol and analyzed by FACS at various time periods after the transfer to -T (Fig. 7C). After induction

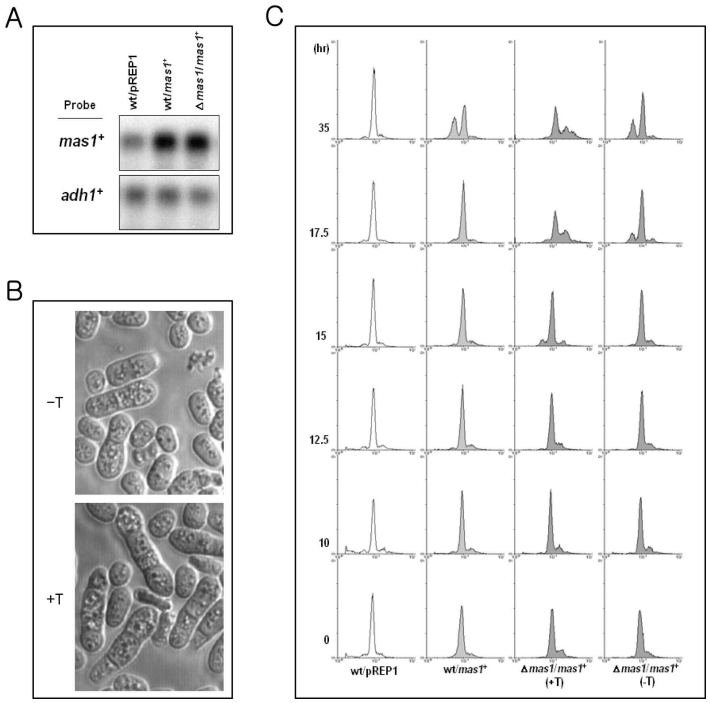

Fig. 7. Overexpression of Mas1 in fission yeast. (A) Northern blot analysis of overexpressed *mas1*$^+$ gene. Wild type cells transformed with pREP1 vector only (wt/pREP1) or with pREP1 harboring *mas1*$^+$ gene (wt/*mas1*$^+$) or *mas1* null mutant transformed with pREP1 harboring *mas1*$^+$ gene (Δ*mas1*/*mas1*$^+$) were grown for 16 hr at 30℃ in the absence of thiamine and then subjected to northern hybridization with *mas1*$^+$ and *adh1*$^+$ probes, the latter as a loading control. (B) Morphology of *mas1* null mutant transformed with pREP1-*mas1*$^+$ cells were cultured in the presence (+T) or absence (-T) of thiamine to repress or induce *mas1* gene, respectively. (C) FACS analysis of overexpression of Mas1 in *S. pombe*.

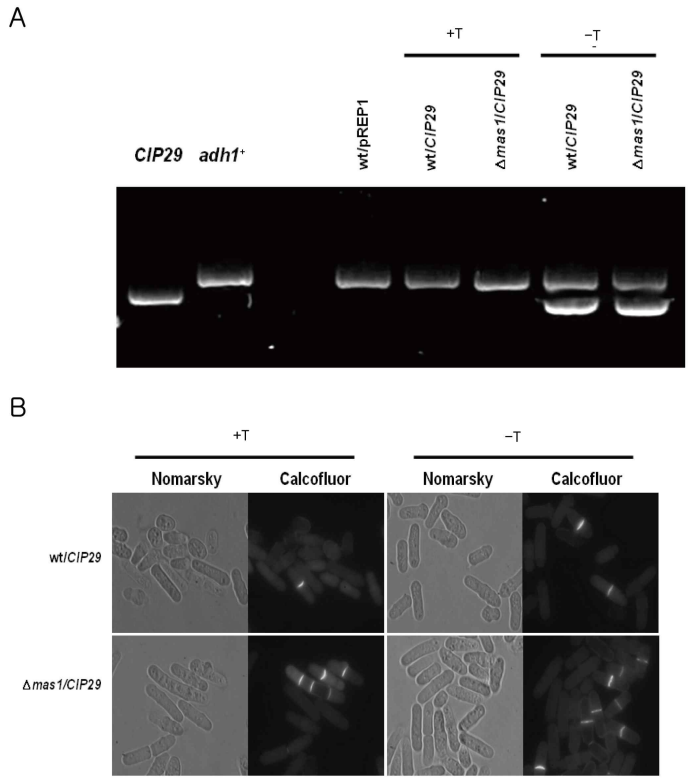

Fig. 8. Expression of human *CIP29* gene in the *mas1* null mutant or wild type *S. pombe* cells. (A) RT-PCR of overexpression of *CIP29* in *S. pombe*. wt/pREP1, wild type cells transformed with pREP1 vector only. wt/*CIP29*, wild type cells transformed with pREP1 harboring *CIP29*. Δ*mas1*/*CIP29*, *mas1* null mutant transformed with pREP1 harboring *CIP29*. Cells were grown at 36°C then cultured in the presence (+T) or absence (-T) of thiamine to repress or induce *CIP29* gene, respectively. *adh1*$^+$ was used as a loading control. (B) Morphological examination of the *mas1* null mutant or wild type cells transformed with *CIP29* by Nomarsky optics and Calcofluor staining. (C) FACS analysis of overexpression of Mas1 in *S. pombe*. Cells, denoted identically with Fig. 6(c), were first grown in +T at 30℃, and then transferred at 25℃ for various time periods in +T or -T in order to repress or derepress the nmt1 promoter, respectively. The data represent the typical results that were reproduced in three independent experiments. The data represent the typical results that were reproduced in three independent experiments.

of *mas1*$^+$ for 17.5 hr, both *mas1* null mutant and wild type cells (OD_{595}=0.5) showed considerable cell population with 1C DNA content which was not readily seen in control cells transformed with pREP only.

The function of *mas1*$^+$ is conserved in *S. pombe* and human cells.

To determine whether the function of *S. pombe mas1*$^+$ and human *CIP29* is identical, human *CIP29* cDNA was cloned

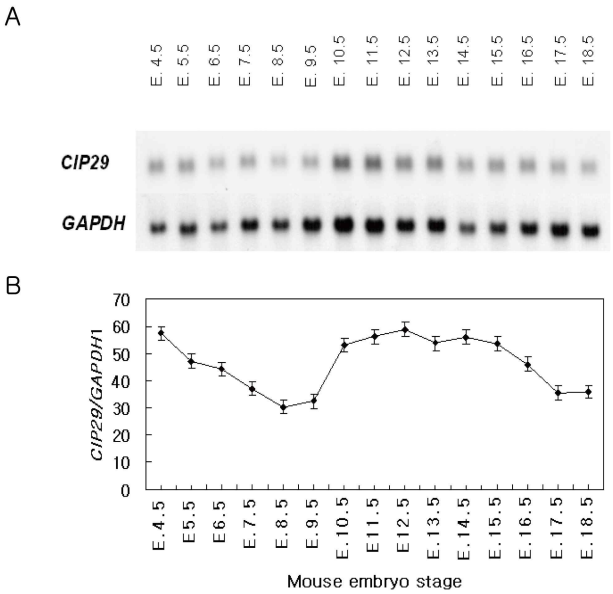

Fig. 9. Northern blot analysis of differentially expressed *CIP29/Hcc1* mRNA during mouse embryogenesis. (A) Northern blot result of RNAs from cell samples taken at mouse embryo stages E4.5-E18.5. The blot was hybridized consecutively with mouse *CIP29/Hcc1* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes, the latter as a loading control. (B) Quantification of *CIP29/Hcc1* transcript against GAPDH. Each data represents the mean±S.D. of three independent experiments.

into pREP vector. The resulting plasmid, pREP1-*CIP29*, was transformed into the wild type cells or the *mas1* null mutant and transformants were selected in a medium containing thiamine in absence of leucine. Cells were first grown in +T, and then transferred to -T in order to derepress the nmt1 promoter. Induction of *CIP29* in -T was clearly demonstrated in both wild type or *mas1* null mutant, as analyzed by RT-PCR (Fig. 8A). The induction of *CIP29* by the removal of thiamine, also decreased the frequency of abnormal phenotype of *mas1* null mutant (Fig. 8B), which is very similar to the case of *mas1*$^+$ induction (Fig. 7B and C). Under this expressing condition, the *mas1* null mutant carrying the pREP1-*CIP29* plasmid grew normally and showed wild-type morphology, suggesting that the function of Mas1 is conserved in *S. pombe* and human cells.

Northern blot analysis of *CIP29/Hcc1* mRNA in the embryonic mouse

In order to know one of the *in vivo* roles of *CIP29/Hcc1* gene, we examined the expression of *CIP29/Hcc1* mRNA during embryonic mouse development. As a control for RNA quality, a northern blot was also performed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The RNA of master blot (SeeGene) was isolated from 5.5- and 6.5-day-old embryos (E5.5and E6.5) that included the extraembryonic tissues and the maternal uterus, or from 7.5- to 9.5-day-old embryos (E7.5 to E9.5) that included the conceptuses along with embryos and extra-embryonic tissues, or from 10.5- to 18.5- day-old embryos (E10.5 to E18.5) that contained only the embryos. Expression of a single 705 bp mouse *CIP29/Hcc1* mRNA was detected in the mouse embryos examined, with higher levels of *CIP29/Hcc1* mRNA occurring in RNA extracted from 4.5-, and 12.5-day-old embryos (E4.5, E12.5) than from older embryos (Fig. 9). Quantification against GADPH shows that the *CIP29/Hcc1* mRNA level gradually decreased in E4.5-E8.5 and then increased in E9.5-13.5. Interestingly, the *CIP29/Hcc1* transcript level decreased in E17.5 and 18.5 when the proliferation rate decreased.

Discussion

We report the identification and cloning of a full length novel cDNA for a *S. pombe* mitosis associated protein, Mas1. Mas1 possesses a SAP domain, which is a putative DNA-binding motif predicted to be involved in chromosomal organization [5]. The SAP proteins regulate transcription, DNA repair, RNA processing, and apoptotic chromatin degradation.

A novel protein Hcc-1 that has the same sequence as *CIP29* was identified in carcinoma cells but no biological function was described [9,14]. A fission yeast homologue of human *CIP29/Hcc1* gene was identified and characterized based on a homology search of the fission yeast database with the human *CIP29/Hcc1* gene sequence as query sequence. In the present study, both the genomic and cDNA copy of the *mas1*$^+$ gene were cloned and sequenced. Comparison of the genomic and cDNA nucleotide sequences indicated that the *mas1*$^+$ gene is 735 base pairs in length (Fig. 1). The Mas1 is a hydrophilic protein of 245 amino acids with a predicted weight of 24 kDa and contains a SAP motif: a putative DNA-binding motif involved in chromosomal or N-terminal leader sequence were evident.

The GNAACg/c as a putative consensus sequence for the fission yeast M-G1 *cis*-acting promoter element defined as the PCB (pombe cell cycle box) sequence [3]. Previous studies demonstrated that *spo12*$^+$, *cdc19*$^+$, *fin1*$^+$, *mid1*$^+$/*dmf1*$^+$, *sid2*$^+$, *ppb1*$^+$ and *plo1*$^+$ all showed transcription profiles similar to

that of *cdc15*$^+$. The same molecular processes may regulate transcription of all of these genes [16]. In the present study, a putative PCB sequence, GGAACG, was also found at position -495 in *mas1*$^+$ promoter (Fig. 4). In the northern blot analysis of Mas1 mRNA during the cell cycle, the pattern of graph was similar to that of septation index (Fig. 2). Thus, *mas1* seems to play a role in the control of septation possibly through regulating actin ring formation, the co-ordinated constriction of this ring, or septum deposition. The genes involved in the above stages include *cdc15*$^+$, *ppb1*$^+$, *sep1*$^+$ and *sid2*$^+$, which have important roles in different aspects of septation. *cdc15*$^+$ controls deposition of the actin ring [10,26]. The *S. pombe sep1* gene encodes a nuclear protein that is required for periodic expression of the *cdc15* gene [36].

Deletion of the *sep1*$^+$ forkhead transcription factor homologue is not lethal but causes hyphal growth in *S. pombe* [32]. *sep1*$^+$ inactivation by mutation or disruption, causes a failure of the daughter cells to separate. Since the entry into a new cell cycle is not dependent on a successful completion of cell separation, *sep1*$^+$ null mutant will continue growing with a hyphal morphology instead of producing unicellular daughter cells. In the present study, the inactivation of *mas1*$^+$ by disruption, also caused a failure of the daughter cells to separate (Fig. 4). FACS analysis revealed that the majority of the *mas1* null mutants at the restrictive temperature contained a 2C, 4C and 6C DNA content (Fig. 5). In this result, *mas1* null mutant grown at the restriction temperature showed abnormal cytokinesis, as incomplete cell separation. All these results suggested that *mas1*$^+$ may be involved in septum deposition in cytokinesis.

In *S. pombe*, nitrogen starvation is known to induce transient acceleration of cell division and reduction in cell size with a final arrest in G_1. The division size control appears to be impaired by mutations in *cdr1/nim1* and *cdr2*, genes that encode protein kinases mediating nutritional control over the mitotic cycle. cdr$^-$ cells arrest after fewer rounds of division and are larger than the wild type [34]. In the present study, when the *mas1* null mutant cells were grown in the nitrogen starved medium, the frequency of multi-septate and branched cells dramatically increased (Fig. 6). All these results suggest that the above abnormal phenotypes of *mas1* null mutant dramatically increased when the cell division was accelerated following depletion of nitrogen energy source.

If *mas1+* is assumed to be actively involved in septum separation then its overexpression might result in an accelerated entry into G_1 via the rapid processing of mitosis. The consequence of ectopic overexpression of *mas1*$^+$ confirmed the role of Mas1p in septum regulation, increased in the frequency of G_1-arrested cell population (Fig. 7). In order to clarify the exact role of Mas1, additional studies including the measurements of β-glucanase activity are necessary.

One of the possibilities for Mas1 protein as a regulator of mitosis is that the Mas1 is a nuclear protein controlling the expression of other gene(s). In the present study, it was shown that Mas1p has a SAP domain as DNA binding domain and a KKRKR amino acids sequence as nuclear localization signal (Fig. 1) and that Mas1p localizes in the nucleus of *S. pombe* cells (data not shown). The SpMas1p-EGFP signal was also found in the nucleus in HeLa cell (data not shown) like the case of *CIP29/Hcc1* in mammalian cells. Possibly acting analysis of the Mas1 as a transcription factor or as a component of mitotic machinery needs to be elucidated in further studies.

Mas1 protein is 35% homologous to human CIP29/Hcc-1. The functional homology of Mas1 to CIP29 was demonstrated by the complementation of *mas1* null mutant phenotype upon induction of human CIP29 gene (Fig. 8). It is therefore suggested that the function of Mas1 is highly conserved.

A novel protein Hcc-1 that has the same sequence as *CIP29* was identified in carcinoma cells but no biological function was described [9,14]. Hcc-1 is overexpressed in pancreatic adenocarcinoma and hepatocellular carcinoma [9]. CIP29 is known to be induced by a cytokine, EPO, and to accelerate the cell division cycle resulting in a rapid cell proliferation [14]. Consistent with the above observation, the present result indicate that the *CIP29/Hcc1* mRNA level in the embryo stages, decreased in E4.5-E8.5 and increased in E9.5-13.5. In the E17.5 and E18.5 stages, in which it is known that the proliferation is decreased and the *CIP29/Hcc1* transcript decreased (Fig. 9). E4.5-E6.5 stage is the stage for the differentiation of the egg cylinder into embryonic and extra embryonic regions and the formation of the proamniotic cavity [6]. Also, E6.5-E8.5 is the stage for the formation of streak and somites. In E9.5, cell division for formation of forelimb bud, forebrain vesicle and neuropore is increased [10]. *CIP29/Hcc1* dose not seem to be involved in differentiation but in the regulation of cell division. Detailed action mechanism of Mas1 in the cell should be clarified in future studies including the interaction of Mas1 with other proteins or the role of Mas1 in the transcription processes.

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초록 : 세포분열에 관여하는 인간의 CIP29/Hcc-1 유전자와 상동성을 가지는 분열형 효모의 새로운 유전자 mas1+의 특성분석

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세포주기조절에서 유전자 발현의 조절은 매우 중요한 부분이다. 본 연구에서는 인간의 유전자인 CIP29/Hcc-1과 상동성을 가지는 분열형 효모의 새로운 유전자 mas1+을 분리하였다. 중합효소연쇄반응을 수행하여 cDNA를 얻고 이 cDNA의 염기서열을 분석한 결과 *mas1+*의 전체 염기서열은 735 bp로서, 245개의 아미노산을 암호화하고 있다. *mas1+*의 프로모터에서는 M-G1에 특이적인 전사를 보이는 유전자들에 보존되어 있는 PCB 서열이 발견되었다. 세포주기별 *mas1+*의 전사 수준을 분석한 결과 격막이 형성된 세포수의 빈도를 나타내는 격막 세포지표의 양상과 유사하게 발현하는 것을 확인하였다. mas1+ 결손 돌연변이를 25℃와 36℃에서 배양한 결과, 세포질 분열과정이 늦어진 다중격막 세포의 빈도가 증가하였다. 이를 FACS로 분석하여 DNA 함량이 2C, 4C와 6C등이 형성됨을 확인하였다. *mas1+*결손 돌연변이 세포를 질소 결핍 배양액에서 배양한 결과 다중격막 세포의 형성이 확연히 증가하였는데 이는 질소 결핍에 따른 세포분열의 가속화 단계에서 mas1+의 결손이 특히 부정적 영향을 초래함을 시사한다. *mas1+* 유전자 결손 돌연변이 세포에 *mas1+*을 포함한 plasmid를 형질전환한 후 *mas1+*의 발현을 유도한 결과 정상의 세포 형태로 전환됨을 확인하였다. Mas1 단백질에 EGFP를 융합시켜 발현을 유도한 결과 핵내에서 위치함을 분열형 효모와 인간 배양세포인 HeLa에서 확인하였다. 또한, mas1+ 결손 돌연변이에서 상동성을 가지는 인간 유전자 CIP29/Hcc-1을 발현시킨 결과 multi-septate 세포가 줄어들었다. 한편, 생쥐의 배발달 단계에 따른 CIP29 유전자의 전사체 수준은 세포 분열이 활발한 시기에 증가하였다. 이상의 결과들은 Mas1은 인간의 핵단백질인 유전자 CIP29/Hcc-1과 구조 기능적으로 상동성을 가지며, 세포주기 중 M-G1에 속하는 세포질 분열에 연관되어 있음을 시사한다.