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Effect of Polyamines on Cellular Differentiation of *N. gruberi*: Inhibition of Translation of Tubulin mRNA

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The effects of a polyamine, spermine, on the differentiation of *Naegleria gruberi* amebas into flagellates were tested. Addition of spermine at early stages of differentiation (until 40 min after the initiation of differentiation) completely inhibited the differentiation. To understand the inhibition mechanism, we examined the effect of spermine treatment on the transcription and translation of differentiation-specific genes during differentiation. Addition of spermine at early stages did not inhibit the accumulation of two differentiation-specific mRNAs, α-tubulin and Class I mRNA, significantly, but rather prevented the rapid degradation of the mRNAs in later stages of differentiation. Addition of spermine at the beginning of differentiation inhibited overall protein synthesis partially and gradually. However, translation of the α-tubulin mRNA was completely inhibited. These data suggest that the inhibition of differentiation of *N. gruberi* by spermine treatment did not result from the inhibition of transcription of differentiation-specific genes but from the specific inhibition of translation of the mRNAs during the differentiation.

Key words: *Naegleria gruberi*, differentiation, spermine, inhibition of translation

Naegleria gruberi is an amebo-flagellate and is found ubiquitously on earth. In laboratory conditions, *Naegleria* amebas differentiate rapidly and synchronously into flagellates (13). In less than two hours after the initiation of differentiation, more than 95% of cells in a population complete this differentiation. During this differentiation, the ameboid *Naegleria* changes its shape into a streamlined flagellate with a regular contour and forms various cellular organelles including basal bodies, flagellar axonemes, cytoskeletal microtubules, *de novo* (13, 33). In the process of this differentiation, *Naegleria* reprograms its gene expression. For example, a group of mRNAs which are not found in ameba, flagellar calmodulin, α- and β-tubulin mRNA, are transiently and coordinately accumulated due to transient activation of the respective genes after the initiation of differentiation, differentiation-specific (DS) mRNAs (22, 23, 27). On the contrary, the amount of actin mRNA which is present at a high concentration in *Naegleria* ameba begins to decrease rapidly after the initiation of differentiation (28). Continuous and programmed synthesis of new proteins and RNAs are required to accomplish this complex regulation of gene expression (5, 14, 33). Inhibition of protein synthesis from the beginning of differentiation prevents the transient accumulation of differentiation-specific mRNAs. Formation of the flagellar apparatus (basal bodies, flagella, and flagellar rootlet) requires protein systhesis for 53 min after the initiation of differentiation. Formation of the cytoskeletal microtubule system requires protein synthesis for 10 min more after the completion of protein synthesis required for the formation of the flagellar apparatus (14, 33).

Starvation and liquid environment are the two well known factors which cause *Naegleria* amebas to differentiate into flagellates (13). However, it is not known how a *N. gruberi* amoeba senses these changes in the growing conditions, by what pathway these signals are transported to the cell nucleus, and how the complex reprogramming of gene expression is accomplished. As a first step to answer these questions, we treated cells with various agents which stimulate signal transduction pathways or provoke changes in gene expression. Polyamines are one group of the agents we have tested.

Polyamines (putrescine, spermidine, and spermine) are found in all studied organisms, from prokaryotes to human cells, and there are nu-

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morous reports which suggest the physiological importance of these compounds. For example, polyamine binds DNA and stabilizes the DNA structure (3, 4, 21, 31). Addition of polyamine affects DNA replication (6, 15), transcription (8), and translation (10, 12, 17, 18, 19). Polyamines increase or decrease protein phosphorylation (1, 11, 25). Binding of polyamine on cell membrane affects the function of cellular membrane and signal transduction pathways (7, 26, 30). In spite of these numerous reports, the action mechanism of polyamine still remains to be understood. In this paper, we report that spermine kept *Naegleria* ameba from differentiating and that this inhibition of differentiation was, at least in part, the result of the inhibition of translation of DS mRNAs.

Materials and Methods

Cell growth and differentiation

N. gruberi strain NB-1 was used throughout this study. 2.5×10^5 *N. gruberi* cysts were inoculated on an NM agar plate with 0.1 ml of overnight culture of *Klebsiella pneumoniae* (Kp) and incubated at 34°C (13). For differentiation, cells were harvested with 2 mM ice-cold Tris-HCl (pH 7.6 at 25°C). The cell suspension was centrifuged three times (at 2, 500 rpm for 30 sec) to remove Kp. Differentiation was initiated by resuspending the final pellet in the Tris buffer pre-warmed to 25°C. Every 10 min, a small portion of the cells was fixed with Lugol's iodine and examined under a phase contrast microscope to monitor the differentiation. The differentiation was evaluated by the percentage of cells with visible flagella (13).

RNA preparation

During differentiation, 0.5 ml samples of differentiating cells were taken from each flask. After brief centrifugation, the cells were resuspended in 460 µl of 5 M guanidine thiocyanate, 10 mM Na_2EDTA, 2% Sarkosyl, 25 mM Tris-HCl (pH 7.6). The cell lysate was extracted with 2 volumes of phenol:chloroform (2:1). The aqueous layer was further extracted with a 1:1 solution of phenol and chloroform until the interface was clean. The final aqueous layer was adjusted to 150 mM NaCl with 5 M NaCl, and RNA was precipitated with 2volumes of 100% ethanol (5).

RNA slot blot hybridization

RNA samples (5 µg/slot) were transferred to nylon membranes (Amersham Hybond N^+) according to the manufacturer's manual using a slot blot apparatus (Hoefer Scientific Instrument). Membranes were prehybridized in hybridization buffer (50% formamide, 0.25 M $NaHPO_4$ pH 7.2, 0.25 M NaCl, 1 mM Na_2EDTA, 100 µg/ml wheat germ tRNA, and 7% SDS) for 6 hr at 42°C. For hybridization, the used buffer was drained and fresh hybridization buffer was added to the bag with denatured ^{32}P-labeled cDNA probe. After 16~19 h of hybridization at 42°C, the membrane was rinsed briefly with 2×SSC, 0.1% SDS and then washed two times with 2×SSC, 0.1% SDS for 15 min each. The membrane was further washed twice in 25 mM $NaHPO_4$ pH 7.2, 1 mM Na_2-EDTA, 1% SDS for 15 min each. All washes were carried out at 50°C (5).

Probe preparation

A cDNA fragment of each mRNA (*Eco*RI fragment of α13, 450 bps, for α-tubulin [9], *Pst*I fragment of pcNg 1~8, 520 bps, for Class I [23], and *Pst*I fragment of pcNg 3~28, 700 bps, for a non-specific mRNA [23]) was labeled with ^{32}P-dCTP by using a DIG DNA labeling and detection kit (Boehringer Mannheim, Germany).

Estimation of protein synthesis

The amount of synthesized protein during different stages of differentiation was estimated as follows: *Naegleria* amebas were harvested, equally divided into 8 flasks (flask #1-8), and differentiated at the same time with (flask #1-4) and without (flask #5-8) spermine. At the initiation of differentiation, ^{35}S-methionine was added to a final concentration of 10 µCi/ml to flasks #1 and 5, and after 30 min, 200 µl of the differentiating cells were taken. The cells were harvested by brief centrifugation, resuspended in 200 µl of SDS sample buffer (10% glycerol, 5% mercaptoethanol, 2% SDS, 0.0624 M Tris-HCl, pH 6.8), and boiled for 5 min. To flasks #2 and 6, #3 and 7, #4 and 8, ^{35}S-methionine was added at 30, 60, and 90 min, after the initiation of differentiation, respectively, and 200 µl of differentiating cells were taken 30 min later from each flask and processed as above. To estimate the amount of synthesized protein, 30 µl of each sample were taken and mixed with 0.5 ml of BSA solution (0.1 mg/ml BSA, 0.02% NaN_3). To each sample, 0.5 ml of ice-cold 20% TCA (trichloroacetic acid) were added and mixed thoroughly by vortexing. After 30 min incubation at 4°C, the samples were filtered thwugh GF/C glass fiber filters. The filters were washed with ice-cold 10% TCA and dried. ^{35}S-methionine incorporation was determined by scintillation counting (Beckman, LS-6500).

Western blotting

Two hundred fifty µl of differentiating cells were taken at various stages of differentiation. After brief

centrifugation, the cells were resuspended in 250 µl of SDS sample buffer. The samples were boiled for 5 min and centrifuged for 5 min in an Eppendorf microfuge. Twenty µl of each sample were fractionated by a 10% SDS-PAGE. After electrophoresis, fractionated proteins were transferred to a sheet of nitrocellulose membrane in a transfer buffer (20% methanol, 2.5 mM Tris, 0.195 mM glycine). The amount of α-tubulin in each sample was estimated by using an anti-*Naegleria* α-tubulin monoclonal antibody (33) and ECL Kit from Amersham, USA.

Results

Effect of polyamine treatment on differentiation of *N. gruberi*

Cells were differentiated in the presence of various concentrations (25 µM , 250 µM, and 2.5 mM) of putrescine, spermidine, or spermine. The inhibitory effects of these three polyamines were correlated with their cationic charges. Putrescine, a diamine, had little effect on differentiation. In the presence of 25 µM putrescine, the cells differentiated normally except for a 2 min delay in T_{50} (the time after the initiation of differentiation when 50% of cells have visible flagella). In the presence of 250 µM putrescine, differentiation was further delayed with a T_{50} 75 min, and less than 90% of cells in the population formed flagella. In the presence of 2.5 mM of putrescine, T_{50} was 78 min and about 85% of cells had visible flagella at the end of differentiation (Fig. 1 A). In the presence of 25 µM spermidine, differentiation was delayed a little (T_{50} 72 min) and about 90% of cells formed flagella (Fig. 1B). In the presence of 250 µM of spermidine, T_{50} was 78 min and less than 80% of the cells formed flagella. In the presence of 2.5 mM spermidine, the observed T_{50} was 90 min and only 73% of the cells had flagella. In these flagellated cells; no flagellate shape was observed, the flagellated cells were either spherical or ameboid in shape (Fig. 1 B). Spermine inhibited differentiation the most severely (Fig. 1C). At a low concentration of spermine (25 µM), cells differentiated like control cells except for a little delay in T_{50}. However, in the presence of 250 µM of spermine, less than 30% of cells formed flagella. In the presence of 1 mM spermine, only 2% of the cells formed flagella. In the presence of 2.5 mM spermine, differentiation was completely inhibited. In this condition, all the cells remained as amebas. Because spermine showed the most significant inhibitory effect on differentiation, the effects of spermine were further studied.

Effects of spermine added at various stages of differentiation

The above results showed that the addition of spermine (2.5 mM) inhibited the differentiation of *N. gruberi*. To understand the inhibition mechanism, we examined the effects of spermine added at different stages of differentiation (Fig. 2). Addition of

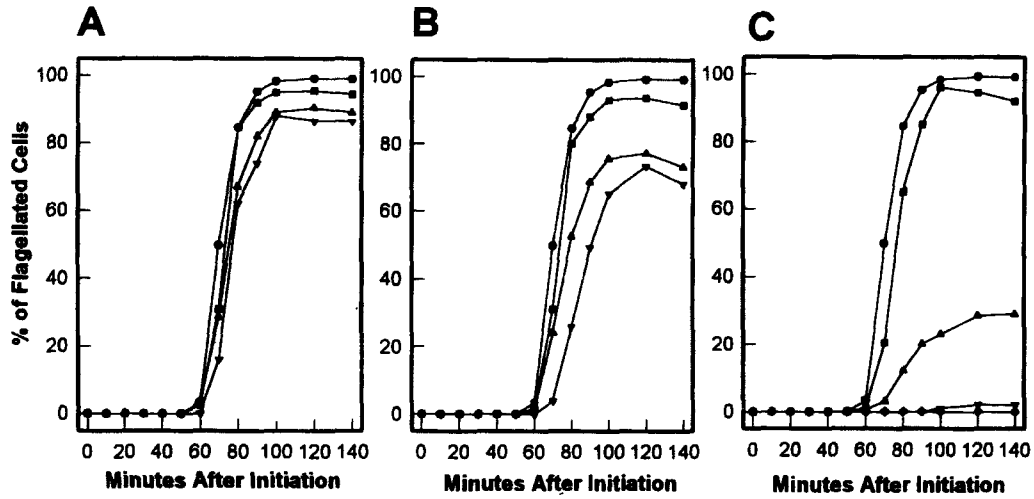

Fig. 1. Effects of three polyamines on the differentiation of *N. gruberi*. *N. gruberi* amebas were differentiated in the presence of varying concentrations of putrescine, spermidine or spermine. Differentiation was monitored as described in Materials and Methods. Each graph represents an average of the results of five independent experiments. A. Effect of putrescine on differentiation of *N. gruberi*. Symbols: -●-, control differentiation; -■-, 25 µM; -▲-, 250 µ -◆-, 2.5 M. B. Effect of spermidine on differentiation of *N. gruberi* Symbols: -●-, control differentiation; -■-, 25 µM; -▲-, 250 µM; -◆-, 2.5 mM. C. Effect of spermine on differentiation of *N. gruberi*. Symbols: -●-, control differentiation; -■-, 25 µM; -▲-, 250 µM; -◆-, 1 mM; -▼-, 2.5 mM.

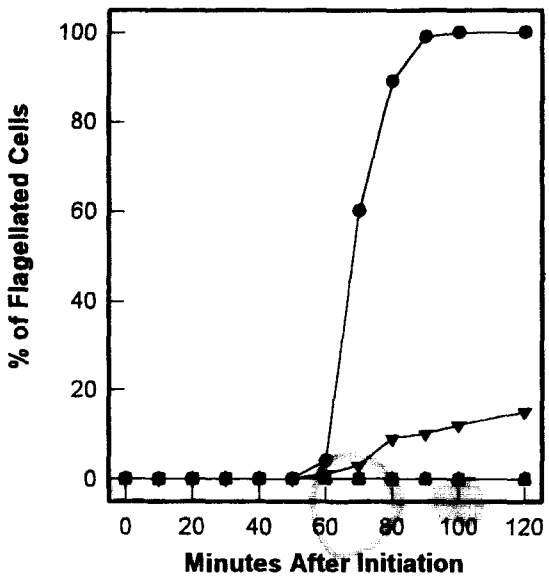

Fig. 2. Effect of spermine added at various stages of *N. gruberi* differentiation. After initiation of differentiation, spermine was added to the differentiating cells to a final concentration of 2.5 mM at 0 min (-■-), 40 min (-▲-), and 60 min (-▼-). -●-, control cells. Differentiation was monitored as in Fig. 1.

spermine to a concentration of 2.5 mM 40 min after the initiation of differentiation inhibited flagellar formation completely. When spermine was added 60 min after initiation, 10% of the cells formed flagella. Addition of spermine at the end of differentiation (120 min) decreased the number of flagellated cells rapidly (data not shown).

Effect of spermine on the accumulation of DS mRNA

Because the addition of spermine at early stages of differentiation (0 and 40 min) inhibited differentiation completely, we examined the effects of spermine on the accumulation of two differentiation-specific mRNAs: α-tubulin mRNA, which encodes flagellate-specific α-tubulin, and Class I mRNA, a ~7 kb mRNA which encodes an unidentified protein. In a control differentiation, α-tubulin mRNA was not detected at the beginning of differentiation (23). The amount of the tubulin mRNA increased rapidly after the initiation of differentiation, reached at its peak value at 70 min, and then began to decline rapidly. At 120 min when the differentiation was completed, a very low level of the tubulin mRNA was detected (Fig. 3A, 23). Addition of spermine (2.5 mM) at the beginning of differentiation partially inhibited the accumulation of α-tubulin mRNA. Until 40 min after initiation, the pattern of α-tubulin mRNA accumulation was similar to that of the control cells. The α-tubulin mRNA began to accumulate rapidly after the initiation of the differentiation. However, after 40 min, the amount of tubulin mRNA increased very slowly until 80 min, reaching about 50% of the maximum value of the control cells. After 80 min, the amount of α-tubulin mRNA began to decrease slowly. At 120 min, the amount of the tubulin mRNA was about 40% of the maximum of the control. The addition of spermine at 40 min had a similar effect on the accumulation of the mRNA. After addition

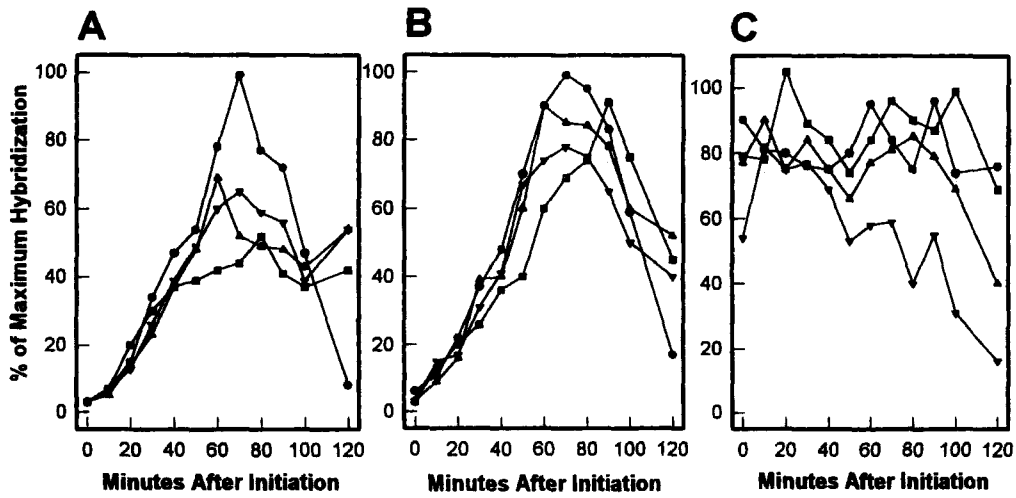

Fig. 3. Effect of spermine on the accumulation of differentiation-specific mRNAs. Spermine was added at 0, 40, and 60 min after initiation of differentiation. During the differentiation, 0.5 ml of cells were collected every 10 min from each flask to prepare RNA samples were transferred to nylon membranes (5 μg/slot) by using a slot blot apparatus and hybridized with cDNA probes labeled with ^{32}P by random primer extension method. After hybridization and fluorography, each radioactive slot was excised and the amount of radioactivity was estimated by scintillation counting. Each figure represents an average of three independent experiments. A: Accumulation of α-tubulin mRNA. B: Accumulation of Class I mRNA. C: Accumulation of a non-specific mRNA. Symbols: -●-, control; -■-, spermine was added at 0 min; -▲-, at 40 min; -▼-, at 60 min.

of spermine, the amount of tubulin mRNA increased continu-ously for 20 min at a rate similar to that of the control cells. After 60 min, the amount of the mRNA decreased rapidly for 10 min, and then very slowly until 100 min. At 120 min, the amount of tubulin mRNA was about 55% of the maximum of the control cells. When spermine was added at 60 min after initiation, we observed a similar result. The amount of tubulin mRNA increased for 10 min after the addition of spermine, and then began to decrease until 100 min. At 120 min, the amount of the tubulin mRNA was about 55% of the maximum of the control cells (Fig. 3A).

Addition of spermine at the beginning of differentiation did not block accumulation of Class I mRNA but changed the pattern of the mRNA accumulation. The Class I mRNA accumulated continuously after the initiation of differentiation but at a slower rate than that of the control. The amount of Class I mRNA reached its peak, 90% of that of the control, at 90 min after initiation, and then declined. At 120 min, the amount of Class I mRNA was about 45% of the maximum of the control cells. Addition of spermine at 40 min showed a slightly different effect. The mRNA accumulated in the differentiating cells at a rate very similar to that of the control cells until 60 min , and then began to decline. At 120 min, the amount of Class I mRNA was near 50% of the maximum. When spermine was added 60 min after the initiation, the amount of Class I mRNA increased for 10 min, and then began to decrease slowly. At 120 min, the amount of Class I mRNA in the spermine treated cells was about 40% of the maximum value of the control cells (Fig. 3B).

These spermine-induced effects were specific to the DS mRNAs. We examined the effect of spermine on the amount of a non-specific mRNA, i.e., an mRNA which is present both in amebas and in differentiating cells (23). Addition of spermine at various stages of differentiation showed no specific effect on the levels of a differentiation non-specific mRNA (Fig. 3C).

Inhibition of translation by spermine

The above results show that the inhibition of differentiation of *Naegleria* by spermine treatment was not the result of the inhibition of the accumulation of differentiation-specific mRNAs. Because it has been reported that translation of an mRNA could be inhibited or increased by spermine treatment (2, 10), we examined the possible effects of spermine treatment on protein synthesis during differentiation. Spermine-treated cells and control cells were pulse-labeled with ^{35}S-Methionine for 30 min at four stages during differentiation (0~30 min, 30~60 min, 60~90 min, and 90~120 min), and the amount of ^{35}S-methionine incorporation was measured. In control cells, the rate of protein synthesis increased slowly, reaching its peak in the last 30 min (90~120 min) of differentiation. However, the rate of protein syn-

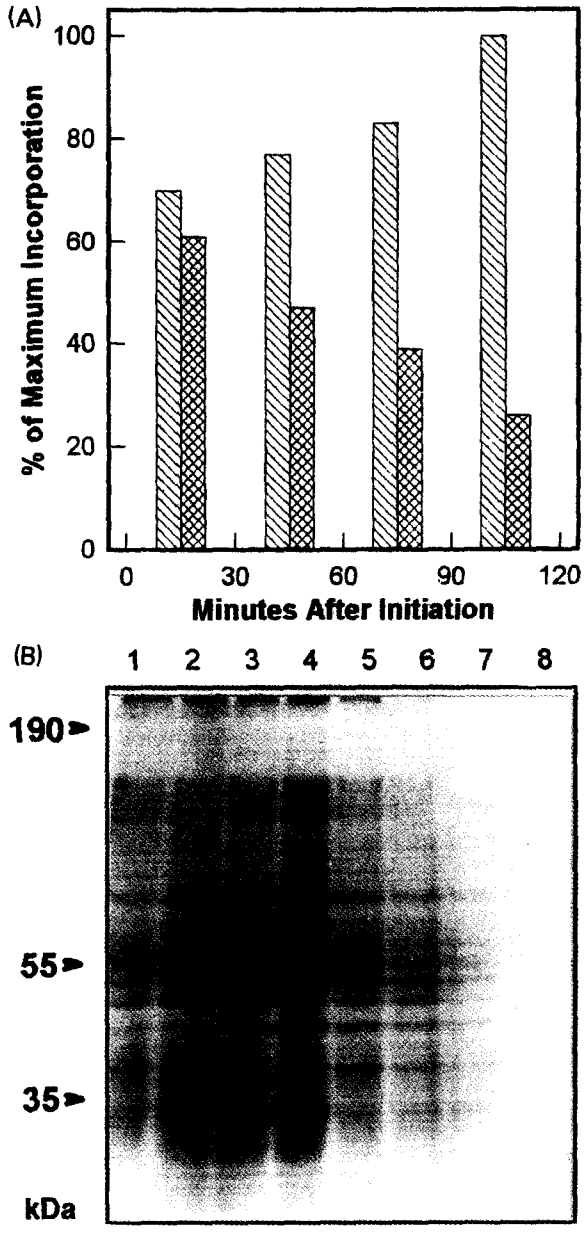

Fig. 4. Effect of spermine on total protein synthesis during differentiation of *N. gruberi*. Differentiating cells were fed with ^{35}S-methionine (40 μCi/flask) for 30 min at four different stages (0~30, 30~60, 60~90, and 90~120 min) of differentiation. Two hundred μl of differentiating cells were taken and amount of incorporated ^{35}S-methionine was estimated by scintillation counting (A) and SDS-PAGE (B) as described in Materials and Methods. Symbols: ▨, control; ■, spermine treated. Lane: 1 and 5, ^{35}S-methionine was added at 0 min; 2 and 6, 30 min; 3 and 7, 60 min; 4 and 8, 90 min.

thesis decreased continuously in spermine treated cells (Fig. 4). In the first 30 min, the amount of synthesized protein in the spermine treated cells was 85% of that of the control cells. The amount of synthesized protein in the second, third and fourth 30 min periods were 60, 50, and 30% of that of the control cells at the respective stages. These data show that the spermine treatment inhibited protein synthesis by 50%.

It has been reported that the effect of spermine on translation was variable depending on the mRNA studied. Based on these observations, we examined the amount of α-tubulin during differentiation by western blot analysis using an anti α-tubulin monoclonal antibody (33). In a control experiment, α-tubulin was not detected in amebae as was expected from the fact that the tubulin mRNA was never detected in amebae, but only during later stages of differentiation (60, 90, 120 min) when tubulin mRNA was accumulated in the cells. However, α-tubulin did not accumulate at all in the spermine treated cells (Fig. 5).

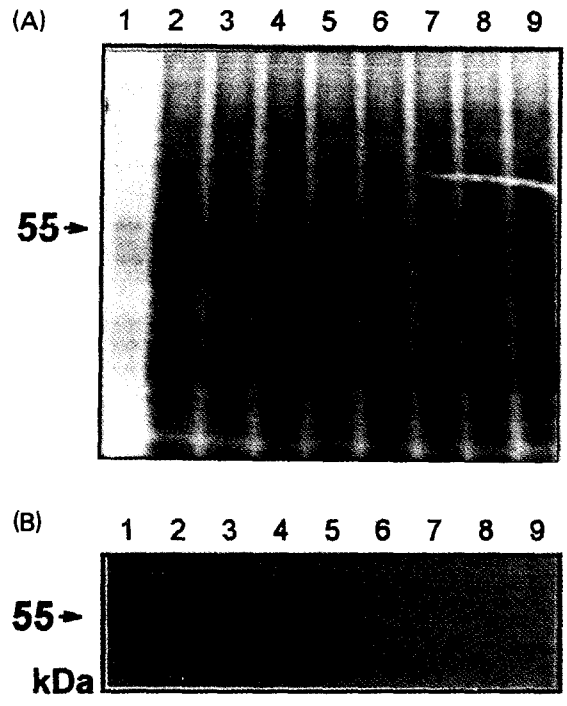

Fig. 5. Synthesis of α-tubulin during differentiation was completely inhibited by spermine treatment. Cells were differentiated in two flasks with or without spermine. Two hundred μl of cells were taken at 0 min (lane 2 and 6), 60 min (lane 3 and 7), 90 min (lane 4 and 8), and 120 min (lane 5 and 9) from each flask. The cells were lysed in SDS sample buffer and fractionated by 10% SDS-PAGE. Lane 1, purified *N. gruberi* flagellar tubulin. Lane 2~5; control, lane 6~9; spermine treated. A, Coomassie brilliant blue staining. B. Western blot with anti α-tubulin antibody.

Discussion

Polyamines (putrescine, spermidine, and spermine) are found in all life-forms. Because of this ubiquitous presence, the physiological roles of these compounds have been studied extensively in organisms ranging from bacteria to human cells. Results from these studies have shown that polyamines are indeed involved in many vital phenomena of life, including maintenace of chromosmal structure (3, 4), DNA replication (6, 15), transcription (8), translation (10, 11, 16, 18, 19, 29) and signal transduction (25, 26, 30, 32). Despite these numerous reports, how polyamines function in these various physiological roles is not clear. We examined the effect of polyamines on the differentiation of *N. gruberi*. The three examined polyamines inhibited differentiation of *N. gruberi* to degrees, which correlated with the number of charges on the respective polyamines; the greater the charge on the polyamine, the more effective it was at inhibiting differentiation. This increasing effectiveness of polyamines with increasing number of charges has been observed in many other systems (7, 26).

Among the three tested polyamines, spermine has the strongest inhibitory effect on the differentiation of *N. gruberi*. Addition of spermine (2.5 mM) at the beginning of differentiation or 40 min after the initiation blocked the differentiation completely. When spermine was added at 60 min, less than 20% of the cells formed visible flagella. This inhibition of differentiation was not a result of the inhibition of synthesis of differentiation-specific mRNAs. Addition of spermine at the beginning of differentiation or at 40 min after initiation had a similar effect on the accumulation of α-tubulin mRNA. The α-tubulin mRNA continued to accumulate for 20~40 min in the presence of spermine; aftherwards the amount of the mRNA did not change significantly. The α-tubulin gene is transcriptionally inactive before the initiation of differentiation (21). Hence these data showed that spermine treatment did not block the transcriptional activation of the gene after the initiation of differentiation. Addition of spermine had a slightly different effect on the accumulation of Class I mRNA depending on the time of spermine treatment. When the drug was added at the beginning of differentiation, the amount of the Class I mRNA continued to increase but at a slower rate. The amount of the mRNA increased until 90 min, and then decreased. When spermine was added at 40 min, the Class I mRNA concentration increased until 60 min and then slowly decreased. Because the

expression of the Class I gene is not active like that of the α-tubulin gene before the initiation of differentiation (22), these data also show that spermine treatment did not inhibit the transcriptional activation of the gene. These observed differences between the accumulation of α-tubulin mRNA and that of the Class I mRNA in spermine-treated cells might be related to the fact that the transcriptional regulation mechanisms of these two genes during differentiation are different (5). The partial inhibition of the differentiation-specific mRNA accumulation can not explain the complete inhibition of differentiation. When RNA synthesis was inhibited by the addition of actinomycin D 40 min after initiation of differentiation, at which time the amount of differentiation-specific mRNA was at about 40% of its peak value, approximately 50% of cells in the population formed visible flagella (14).

Addition of spermine at the beginning of differentiation of *N. gruberi* inhibited total protein synthesis gradually. In the last 30 min (90 to 120 min), the amount of protein synthesised in spermine treated cells was about 30% of that of the control cells in the same period. However, in the presence of spermine, synthesis of α-tubulin was completely inhibited. These data suggested that the inhibition of *N. gruberi* differentiation by spermine was a result of the inhibition of α-tubulin synthesis. The effects of spermine on translation has been studied in several systems. The results from these studies show that spermine treatment increases or decreases the rate of translation depending on the mRNAs (2, 20). Our result shows that translation of α-tubulin mRNA was specifically inhibited by spermine. This specific inhibition of α-tubulin synthesis by spermine also explains the stabilization of the α-tubulin mRNA in later stages. As previously reported, inhibition of protein synthesis by cycloheximide during differentiation stabilizes the mRNA completely (5). Addition of spermine at the beginning of differentiation had a similar effect on the accumulation of β-tubulin and flagellar calmodulin mRNAs (data not shown). These data suggest that the translation of these mRNAs might also be specificallly inhibited. Unlike the DS mRNAs, spermine treatment had no specific effect on the levels of non-specific mRNA during differentiation. Inhibition of protein synthesis by cycloheximide had no specific effect on the levels of the non-specific mRNA either (5). These data imply that spermine treatment at the initiation of differentiation inhibits translation of the DS mRNAs specifically, thereby inhibiting the cellular differentiation of *N. gruberi*. However, these data do not explain how the addition of spermine at 60 min, when the protein synthesis required for the formation of flagella has already been completed (14, 33), could inhibit flagellar formation. This result suggests that spermine inhibited the differentiation of *N. gruberi* not only by preventing translation of the differentiation-specific mRNAs but also by other unknown mechanism(s).

Acknowledgments

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