

## Regulation of Actin Gene Expression During the Differentiation of *Naegleria gruberi*

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(Received February 27, 2001 / Accepted March 13, 2001)

The regulation of actin gene expression during the differentiation of *Naegleria gruberi* was examined. Actin mRNA concentration was maximal in amoebae and decreased rapidly after the initiation of differentiation. At 20 min after initiation, the concentration of actin mRNA decreased to 55% of the maximal value. The actin mRNA concentration decreased to the minimum at 80 min (15% of the maximum), and then began to increase slightly at the end of differentiation. This decrease of actin mRNA concentration was regulated by the repression of actin gene transcription based on nuclear run-on transcription experiments. The rates of transcription of actin gene in nuclei prepared at 40 and 80 min after the initiation of differentiation were 50 and 28% of that of nuclei prepared at the beginning of differentiation, respectively. The addition of cycloheximide at the initiation of differentiation inhibited both the rapid decrease in the concentration of actin mRNA and the repression of actin gene transcription. These results suggest that the rapid decrease in the concentration of actin mRNA during the differentiation of *N. gruberi* is accomplished by the repression of actin gene transcription and this transcriptional regulation requires continuous protein synthesis during the differentiation.

**Key words:** *Naegleria gruberi*, actin, repression of transcription, transcription regulation

*Naegleria gruberi* amoebae differentiate into flagellates rapidly (<2 h) and synchronously when transferred from a growing medium into a buffer. During differentiation, *N. gruberi* changes its shape, and forms new cellular organelles including two basal bodies, two flagella, and cytoskeletal microtubules. To accomplish this differentiation, *N. gruberi* reprograms its gene expression. Expression of a group of genes that encode flagellar component proteins (e.g.,  $\alpha$ -tubulin,  $\beta$ -tubulin, and flagellar calmodulin) is transiently activated after the initiation of differentiation.  $\alpha$ - and  $\beta$ -tubulin mRNAs are not found in amoebae but begin to accumulate rapidly after the initiation of differentiation. The concentrations of these tubulin mRNAs are maximal at 70 min after the initiation of differentiation, and then decrease. At 120 min after the initiation, the concentrations of tubulin mRNAs decrease to 10~20% of the maximum (16). Different from these genes, the expression of actin gene is repressed after the initiation of differentiation. Sussman and Fulton (23) purified total RNA at different stages of differentiation and translated the RNA *in vitro* and showed that the amount of *in vitro* translated actin decreased rapidly after the initiation of differentiation. Even though these results suggested that the amount of

actin mRNA decreases after the initiation of differentiation, it is not known how the amount of actin mRNA is regulated. To explore the mechanism that regulates the decrease of actin mRNA concentration during differentiation, we cloned a *N. gruberi* actin gene. Using the cloned actin DNA as a probe, we estimated the concentration of actin mRNA and the rate of actin gene transcription during differentiation. In this report, we show that the decrease in the concentration of actin mRNA was regulated, mainly, by the repression of transcription of the gene and that this transcriptional regulation required continuous protein synthesis. We also show that the transcriptional regulation of actin gene is related to actin cytoskeleton organization.

### Materials and Methods

#### *Cell growth and differentiation*

*N. gruberi* strain NB-1 was used throughout this study.  $2.5 \times 10^5$  *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 (6). 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 pelleted

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cells in the Tris buffer pre-warmed to 25°C. Every 10 min, a small portion of 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 (6).

#### RNA preparation

During differentiation, 0.5 ml of differentiating cells ( $\sim 1 \times 10^8$  cells/ml) were taken from each flask. After brief centrifugation, the cells were resuspended in 460  $\mu$ l of 5 M guanidine thiocyanate, 10 mM Na<sub>2</sub>EDTA, 2% Sarkosyl SDS, 25 mM Tris-HCl (pH 7.6). The cell lysate was extracted with 2  $\times$  vol of phenol : chloroform (2:1). After centrifugation, 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 2  $\times$  vol of 100% ethanol (1).

#### Isolation of nuclei

Nuclei were isolated by the method of Lee and Walsh (12). Differentiating cells ( $\sim 5 \times 10^8$ ) were collected by centrifugation. The cells were gently resuspended in 8 ml of ice-cold buffer 1 (25 mM Hepes, pH 7.5, 20 mM KCl, 20 mM MgCl<sub>2</sub>, 0.6 M sucrose, 10% glycerol, 5 mM dithiothreitol, 0.06% NP-40) and incubated in ice for 3 min. The lysed cells were transferred to a 10-ml centrifuge tube and centrifuged for 2 min at 3,000 rpm in a swinging bucket rotor to remove unlysed cells and cysts. The supernatant was centrifuged again for 5 min at 6,000 rpm in the same rotor to recover nuclei. The nuclear pellet was resuspended in 2 ml of buffer 2 (the same composition as buffer 1 without NP-40) and centrifuged as above. The final pellet was gently resuspended in 0.25 ml of storage buffer (2.5% Ficoll, 0.5 M sorbitol, 0.008% spermidine, 1 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 50% glycerol, 10 mM Tris-HCl, pH 7.5). The nuclear preparation was stored at -70°C.

#### Nuclear run-on transcription

Nuclear run-on transcription assay was carried out as described by Lee and Walsh (10). Nuclei were incubated at a concentration of  $1 \times 10^8$  nuclei/ml in transcription buffer (1.25% Ficoll, 0.25 M sorbitol, 0.004% spermidine, 0.5 mM dithiothreitol, 45 mM Tris-HCl, pH 7.9, 25% glycerol, 150 mM NH<sub>4</sub>Cl, 0.16 mM ATP, GTP, and CTP, 5  $\mu$ M [<sup>32</sup>P]UTP (50 Ci/mmol) with 200 U/ml RNasin at 25°C for 1 hr. Transcription was terminated by adding an equal volume of buffer 3 (0.4 M NaCl, 0.02 M Na-acetate, pH 5.1, 2 mM MgCl<sub>2</sub>, 2% SDS) and proteinase K (150  $\mu$ g/ml) and incubating 30 min at 25°C. RNA was purified by phenol-chloroform extraction and ethanol precipitation.

#### RNA and DNA slot blot hybridization

RNA samples (5  $\mu$ g/slot) and plasmids (2  $\mu$ g/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<sub>4</sub>, pH 7.2, 0.25 M NaCl, 1 mM Na<sub>2</sub>EDTA, 100  $\mu$ g/ml wheat germ tRNA, and 7% SDS) for 6 h at 42°C. For hybridization, the used buffer was drained and fresh hybridization buffer was added with denatured <sup>32</sup>P-labeled cDNA probe (or *in vitro* synthesized RNA). After 16-19 h of hybridization at 42°C, the membrane was rinsed briefly with 2  $\times$  SSC, 0.1% SDS and then washed two times with 2  $\times$  SSC, and 0.1% SDS for 15 min each. The membrane was further washed twice in 25 mM NaHPO<sub>4</sub>, pH 7.2, 1 mM Na<sub>2</sub>EDTA, and 1% SDS for 15 min each. All washes were carried out at 50°C (1).

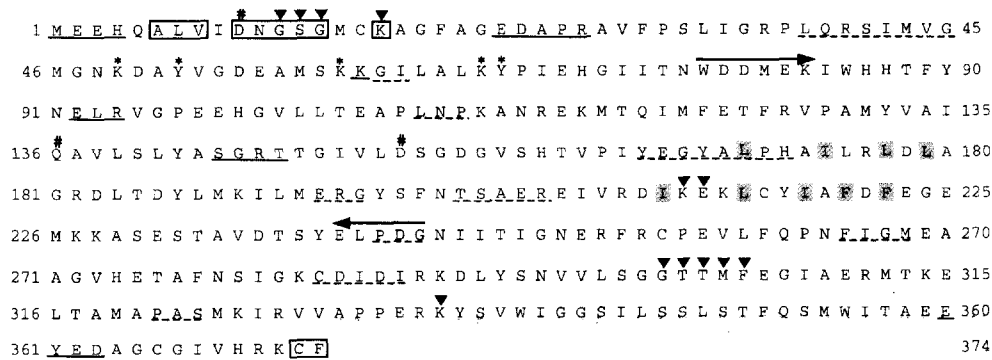
#### Probe preparation for RNA slot blot hybridization

A PCR amplified genomic DNA fragment of actin gene was labeled with <sup>32</sup>P-dCTP by Rediprime kit (Amersham Corp.).

## Results and Discussion

#### Cloning of an actin gene from *N. gruberi*

Even though Sussman and Fulton, by *in vitro* translation assay, suggested the decrease in the concentration of actin mRNA during differentiation (23), the actual changes in the concentration of actin mRNA during differentiation has not been reported. To examine changes in the concentration of actin mRNA in detail, we decided to clone the actin gene. Because actin is one of the most conserved proteins in eukaryotic organisms, we prepared degenerated primers based on the a.a sequences of conserved regions of actins of various organisms (Fig. 1). Using these primers and genomic DNA of *N. gruberi*, we carried out polymerase chain reaction and cloned a 338 bp long DNA fragment (Ng-a1). The deduced a.a sequence of this DNA fragment showed 90% sequence homology to actins from various organisms. Using this PCR fragment, we screened a genomic library of *N. gruberi* (2), and identified two clones (ph4 and 6). The *N. gruberi* DNA in ph4 was subcloned and characterized. One of the subclones (Ng-A4) harbored a 1.8 kb DNA fragment containing a continuous open reading frame of 1,125 bp (GenBank accession # AF101729) encoding a protein of 374 a.a. The deduced amino acid sequence of this open reading frame is 95% homologous to that of the actin of *Naegleria fowleri* (8) and of other organisms including *Diphylobothrium dendriticum* (92%, 25), *Schistosoma mansoni* (91%, 17), and *Dictyostellium discoideum* (91%, 20). This protein contains N-terminal 8 a.a (Ala-Leu-Val-X-Asp-Asn-Ser-Gly-X-X-Lys) that is conserved in most actins and also has conserved a.a involved in interaction with ATP, Ca<sup>2+</sup>, DNase I, myosin, and actin (Fig. 1). Based on these facts and the previously reported results showing



**Fig. 1.** The characterization of *N. gruberi* actin. The residues in *N. gruberi* actin that interact with ATP (▼), calcium (#), DNase I (\*), and myosin (solid lines) are marked. The residues that are implicated in actin-actin interaction are marked with dashed lines. Gray boxes represent NES (nuclear export signal) and white boxes indicate highly identical or conserved residues on various actins (5, 10, 22, 24). Arrows indicate the orientations and the positions of the two peptides used for the synthesis of oligonucleotide primers for polymerase chain reaction.

lack of introns in most of the cloned *N. gruberi* genes (11), we concluded that we cloned a genomic actin gene from *N. gruberi*.

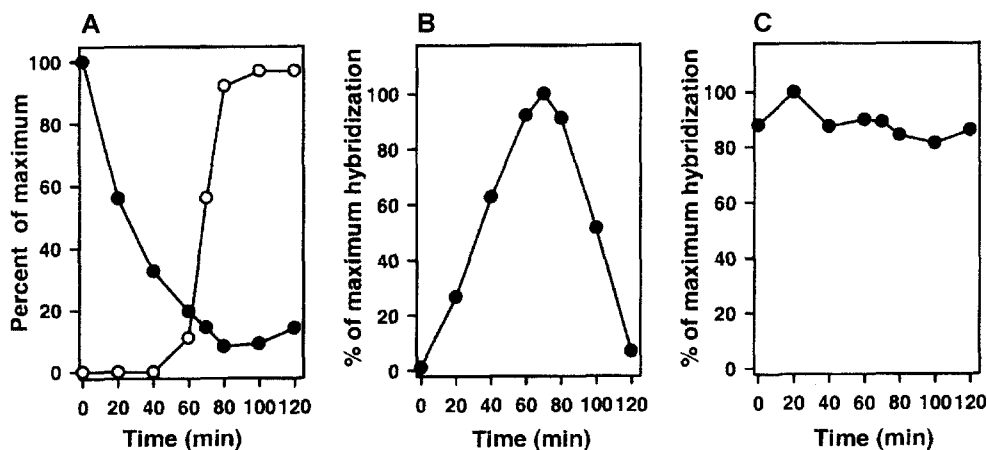
#### *The rapid decrease in the concentration of actin mRNA after the initiation of differentiation*

Using the cloned actin DNA as a probe, we estimated the concentration of actin mRNA at different stages during differentiation by RNA slot blot hybridization. The concentration of actin mRNA decreased rapidly after the initiation of differentiation (Fig. 2). The concentration of actin mRNA in cells at 20 min after the initiation of differentiation was 55% of that of the mRNA in amoebas. The actin mRNA concentration was at its minimum (15%) in the 80 min cells, and then began to increase slightly. To examine whether this decrease in actin mRNA concentration is specific to the mRNA, we examined the concentrations of  $\alpha$ -tubulin

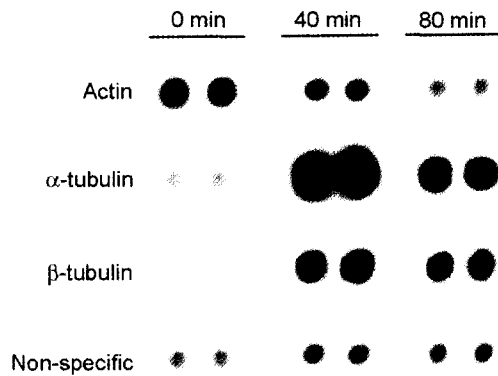
mRNA and a non-specific mRNA (an mRNA which is present both in amoebas and in differentiating cells) with the same RNA samples. The concentration of  $\alpha$ -tubulin mRNA transiently increased during the differentiation as reported and the concentration of non-specific mRNA did not change significantly (16). These data suggest that the decrease in concentration of the actin mRNA is a specifically regulated process during differentiation.

#### *Repression of actin gene transcription during differentiation*

Changes in the concentration of an mRNA in a cell could be achieved by regulating its synthesis and/or degradation. Because the above data showed that the concentration of actin mRNA specifically decreased after the initiation of differentiation of *N. gruberi*, we examined the rate of transcription during differentiation



**Fig. 2.** The decrease in the concentration of actin mRNA during the differentiation of *N. gruberi*. Cells were collected at 20 min intervals to prepare RNA. 10  $\mu$ g of each RNA sample were separated on a formaldehyde agarose gel and transferred to a piece of nylon membrane. After hybridization with  $^{32}$ P-labeled probes (Ng-a1 for actin, c $\beta$ -1 for  $\beta$ -tubulin [3], and pcNg3-28 for non-specific gene [16]) and fluorography, hybridized RNA bands were sliced out and the amount of hybridization was estimated by scintillation counting. (A) The percentage of flagellated cells (○) and the change in the concentration of actin mRNA (●). (B) and (C): Changes in the amount of  $\beta$ -tubulin mRNA and non-specific mRNA.



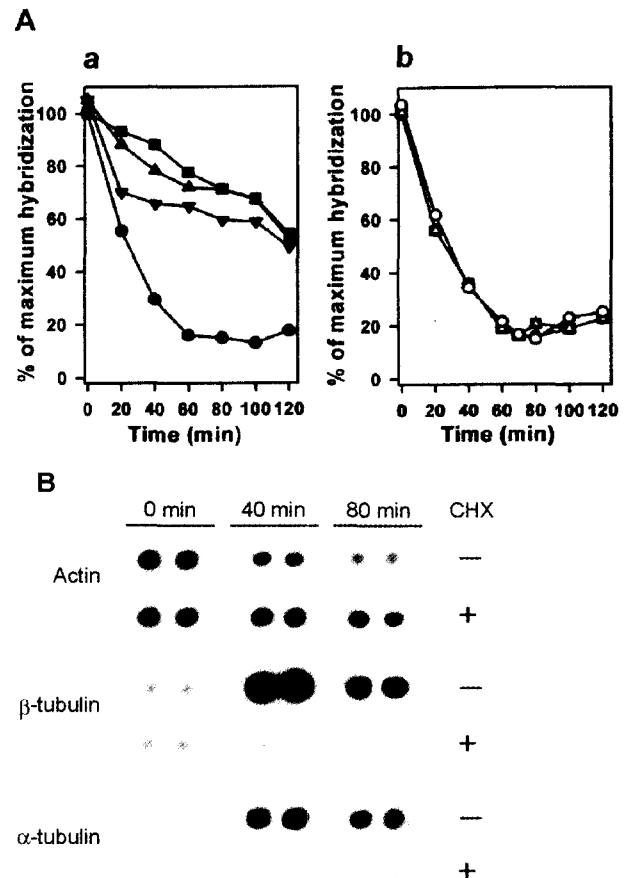
**Fig. 3.** The rate of transcription of actin,  $\alpha$ -,  $\beta$ -tubulin, and non-specific genes at different stages of differentiation. Nuclei were isolated at 0, 40, and 80 min after initiation. These nuclei were incubated in run-on transcription reactions for 60 min. *In vitro* synthesized RNA was hybridized on nylon membrane containing cDNA clones (2  $\mu$ g/dot) of actin,  $\alpha$ -,  $\beta$ -tubulin, and a non-specific gene (pcNg3-28). Duplicates of each plasmid were present on each filter. After hybridization, the amount of hybridization was visualized by fluorography.

**Table 1.** The rate of transcription of actin,  $\alpha$ -,  $\beta$ -tubulin, and non-specific genes during differentiation.<sup>a</sup>

Genes	Time (min)	Synthesis (cpm)	% of maximum hybridization	Time of maximum synthesis (min)
Actin	0	688.26	100.00	0
	40	351.01	51.00 $\pm$ 1.0	
	80	192.71	28.00 $\pm$ 1.6	
$\alpha$ -tubulin	0	89.05	2.90 $\pm$ 0.7	
	40	1382.12	45.00 $\pm$ 1.0	
$\beta$ -tubulin	0	44.27	3.50 $\pm$ 0.9	40
	40	126.87	100.00	
	80	860.15	68.00 $\pm$ 1.8	
Non-specific	0	126.16	58.03 $\pm$ 1.3	40
	40	217.41	100.00	
	80	206.54	95.00 $\pm$ 1.4	

<sup>a</sup>After *in vitro* transcription and fluorography as in Fig. 3, each spot was sliced out and the amount of radioactivity bound to each plasmid was determined by scintillation counting. The numbers in synthesis (cpm) represent averages of the amount of bound radioactivity to each spot in Fig. 3. The values in the percentage of maximum hybridization represent the average of three independent experiments.

by nuclear run-on transcription. The rate of transcription decreased after the initiation of differentiation. The transcription rates of actin gene in nuclei from 40 and 80 min cells were 50 and 28% of that of nuclei from 0 min cells, respectively (Fig. 3 and Table 1). These decreases in the rates of transcription in 40 and 80 min nuclei were similar to the decreases in the concentration of the mRNA in 40 and 80 min cells (Fig. 2). To examine whether this



**Fig. 4.** The effect of CHX added at various stages of differentiation on the decrease of actin mRNA concentration. (A) Changes in the amount of actin mRNA in CHX-treated cells. a, symbols: (●), Control; (■), CHX was added at 0 min; (▲), 5 min; and (▼), 10 min. b, symbols: (○), Control; (□), CHX was added at 65 min; and (△), 85 min. (B) The effect of CHX on the change in the rate of transcription of different genes. At the beginning of differentiation. After nuclear run-on transcription, *in vitro* synthesized RNA was purified and hybridized to actin,  $\alpha$ -, and  $\beta$ -tubulin DNA probes as in Fig. 3. Symbol: -, transcription in control; +, transcription in nuclei from CHX treated cells.

observed change in the transcription rate was a result of authentic transcriptional regulation, we examined the change in the rate of transcription of  $\alpha$ - and  $\beta$ -tubulin genes in the same nuclear preparations. The rate of transcription of  $\alpha$ - and  $\beta$ -tubulin genes increased drastically in 40 min nuclei and then decreased in 80 min nuclei as reported previously (12). These results suggested that the rate of transcription of actin gene specifically decreased after initiation and that the decrease in the rate of transcription might be the main reason for the observed decrease in the concentration of the mRNA.

#### *The requirement of continuous protein synthesis for the transcriptional repression of actin gene*

The above results revealed that actin gene transcription

was repressed after the initiation of differentiation. To explore the repression mechanism, we examined whether continuous protein synthesis is required for the repression of actin gene expression. Cycloheximide (CHX; final concentration, 100 µg/ml), which inhibits protein synthesis of *N. gruberi* almost completely and immediately (7), was added at different stages of differentiation and the concentration of actin mRNA was estimated. In the cells treated with cycloheximide at the beginning of differentiation, the concentration of actin mRNA decreased very slowly (Fig. 4A). At 80 min, when the concentration of actin mRNA decreased to 28% of the maximal concentration in control cells, the mRNA concentration was 70% of the maximum and then, at 120 min, the mRNA concentration was 50% of the maximum. The delay in the decrease of actin mRNA concentration was also evident when the drug was added at 5 or 10 min after the initiation of differentiation. However, addition of cycloheximide at later stages (65 or 85 min) when actin mRNA concentration had already decreased to a low level had no significant effect on the concentration of actin mRNA (Fig. 4A). Addition of cycloheximide had no specific effect on the concentration of a non-specific mRNA, which is present in amoebas and in differentiating cells.

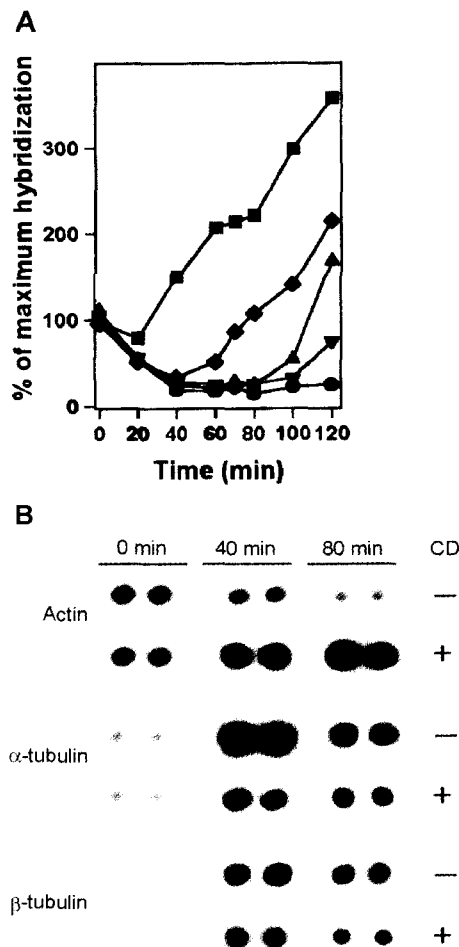
The above results revealed that continuous protein synthesis after the initiation of the differentiation is required for the rapid decrease in the concentration of actin mRNA during differentiation and suggested that the synthesis of the protein after the initiation of differentiation might be required for the observed repression of transcription. To examine this possibility, we tested the effect of cycloheximide treatment on the observed repression of actin gene expression by nuclear run-on transcription. Cycloheximide was added at the beginning of differentiation and nuclei were prepared at 40 and 80 min. After *in vitro* transcription, the rate of transcription was estimated as above. Cycloheximide treatment prevented the repression of actin gene transcription (Fig. 4B). In the two nuclear preparations, the actin gene was transcribed as actively as in nuclei from amoebas (0 min). In contrast, the transcription of tubulin genes was completely inhibited in the nuclei from cycloheximide treated cells as reported previously (1). These results suggest that continuous protein synthesis is required for the repression of actin gene expression and for the activation of transcription of tubulin genes (1). The nuclear run-on experiments with nuclei from cycloheximide treated cells showed that actin mRNA was transcribed actively at 40 and 80 min after initiation. However, RNA slot blot hybridization experiments showed a slow but steady decrease in actin mRNA concentration in cycloheximide treated cells. These data suggest that actin mRNA is rapidly degraded after the initiation of differentiation with or without the synthesis of the protein after the initiation of differentiation. The more rapid decrease of actin mRNA concentration in control cells might be the

synergistic effects of the transcriptional repression of the gene and the rapid degradation of the mRNA.

#### ***Disruption of the actin cytoskeleton increased the concentration of actin mRNA***

It has been reported in several systems that concentration of actin mRNA is closely related with actin cytoskeleton organization and its disruption increased (15) or decreased (13, 14, 18, 19, 21) the concentration of actin mRNA. Recently we observed that the actin cytoskeleton of *N. gruberi* reorganized during differentiation. After the initiation of differentiation, a new F-actin spot was transiently formed at the cell's periphery and basal bodies and flagella were formed from the F-actin spot. The addition of cytochalasin D (CD) inhibited the formation of F-actin spot and the formation of basal bodies and flagella (Lee *et al.*, submitted for publication). To explore the possible relationship between actin reorganization and the change in the concentration of actin mRNA during the differentiation of *N. gruberi*, we examined the effect of cytochalasin D. Cytochalasin D binds to plus ends of actin filaments and causes depolymerization of the actin cytoskeleton, hence increasing the amount of G-actin (4).

The addition of cytochalasin D (final concentration, 50 µg/ml) caused rapid changes in cell shape. When cytochalasin D was added at the beginning, the cells became round within 10 min and remained round until the end of differentiation. When these cytochalasin D treated cells were transferred onto glass slides, these cells were unable to form a pseudopodium and remained round in contrast to control cells which initiated amoeboid movement almost immediately when transferred onto glass slides. When the cells in cytochalasin D buffer were harvested, resuspended in Tris buffer without cytochalasin D, and then transferred onto glass slides, the cells resumed amoeboid movement. These results suggested that cytochalasin D treatment disturbed the actin cytoskeleton of *N. gruberi*. Cytochalasin D treatment caused a dramatic increase in the concentration of actin mRNA. When cytochalasin D was added at the beginning of differentiation, the concentration of actin mRNA began to increase within 10 min after the drug treatment. At the end of differentiation (120 min), the concentration of actin mRNA in the cytochalasin D treated cells increased about 4 times to the concentration of actin mRNA in amoebas. The addition of cytochalasin D at 30, 65, or 85 min after the initiation of differentiation had the same effect; the actin mRNA concentration began to increase within 10 min after the drug addition. To examine whether this increase in the concentration of actin mRNA was the result of the increase in the rate of transcription, we carried out nuclear run-on transcription. Cytochalasin D was added at the beginning of differentiation, and nuclei were prepared at 40 min and 80 min. After *in vitro* transcription, the rate was estimated as above. The rates at 40 and 80 min nuclei from cytoch-



**Fig. 5.** The effect of CD added at various stages of differentiation on the accumulation of actin mRNA. (A) Changes in the amount of actin mRNA in CD-treated cells. Symbol: (●), control; (■), CD was added at 0 min; (▲), 30 min; (▼), 65 min; and (◆), 85 min. (B) The effect of CD on the transcription of different genes. At the beginning of differentiation, CD was added to the differentiating cells. Nuclei were isolated at 0, 40, and 80 min after the start of differentiation. After nuclear run-on transcription, *in vitro* synthesized RNA was purified and hybridized to actin,  $\alpha$ -,  $\beta$ -tubulin as in Fig. 4. Symbol: —, transcription in control; +, transcription in nuclei from CD treated cells.

alasin D treated cells were 2.3 and 3.8 fold higher, respectively, than that of the gene in amoebas (Fig. 5A). These results suggested that actin cytoskeleton disruption increased the rate of actin gene transcription. These effects of cytochalasin D on the transcriptional activation of actin gene were specific. The rates of tubulin gene expression in the two nuclear preparations (40 and 80 min) were decreased to 60 and 30%, respectively, to that of the control (Fig. 5B). These results, the decrease in the rate of transcription of tubulin genes in the cytochalasin D treated cells, were consistent with our previously reported results that the addition of the drug decreased the accumulation of  $\beta$ -tubulin mRNA (9).

## Acknowledgment

This work was supported by a grant from KOSEF (961-0501-010-2) to JooHun Lee.

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