

# Alteration of Chromosomal Structure within $\beta$ -Tubulin and Flagellar Calmodulin Genes during Differentiation of *Naegleria gruberi* Amebae into Flagellates

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We have examined DNase I sensitivity of  $\beta$ -tubulin and flagellar calmodulin genes which are transiently and coordinately activated during differentiation of *Naegleria gruberi* amebae into flagellates. The DNase I sensitivity of  $\beta$ -tubulin and flagellar calmodulin genes changed in parallel with the changes in transcriptional activity of the respective genes during differentiation. The two genes were resistant to DNase I in amebae stage when transcription of the two genes was inactive. Forty minutes after initiation of differentiation, when the two genes were most actively being transcribed, the two genes showed the highest sensitivity to DNase I. One hundred and twenty minutes after initiation, the differentiation was completed and transcriptional activity of the two genes decreased to a low level. At this stage, the two genes were resistant to DNase I treatment like the ones at the amebae stage. This change in the DNase I sensitivity of the two genes was not observed when transcription of the two genes was blocked by adding cycloheximide at the beginning of differentiation.

**Key words:** *Naegleria gruberi*,  $\beta$ -tubulin, calmodulin, DNase I sensitivity, cycloheximide

*Naegleria gruberi* amebae differentiate into flagellates rapidly (<2 hr) and synchronously when transferred from a growing medium to a dilute buffer (3, 6). During differentiation, *N. gruberi* changes its shape, and forms new cellular organelles including two basal bodies, a flagellar rootlet, two flagellar axonemes and cytoskeletal microtubules *de novo* (6, 7, 22). Discrete periods of RNA and protein synthesis are required to complete this differentiation (8).

During differentiation,  $\beta$ -tubulin mRNA and flagellar calmodulin mRNA accumulate transiently and synchronously, the differentiation specific (DS) mRNAs (16). These two DS mRNAs are not detectable in amebae, but they begin to accumulate rapidly after initiation of differentiation. The amount of the DS mRNAs reaches a peak level at 70 min after initiation of differentiation, at that time 50% of the cells have visible flagella (16), then it declines to a low level. This regulation in the amount of the  $\beta$ -tubulin mRNA and flagellar calmodulin mRNA is accomplished by both transient transcriptional activation of the genes and changes in the half-life of

the two mRNAs during the differentiation (1, 2, 11, 12). The DS mRNAs are not synthesized in amebae before initiation of differentiation. However, transcription of the DS mRNAs increases rapidly after initiation of differentiation; it reaches a peak level at 40 min after initiation, and then begins to decrease. At 120 min, when differentiation is completed, the two genes are transcribed at a low rate (<20% of the peak value). The mechanism of these transcriptional and post transcriptional regulations of the DS mRNAs is not well understood, but it has been shown that continuous protein synthesis during the differentiation is required to accomplish the regulations (1).

It has been well documented that the chromosomal structure has an important function in the regulation of transcription of a gene (4, 9, 14, 21) and that DNase I is a useful tool to examine the changes in chromosomal structure of a gene (9, 10, 18, 23). It has been reported that in many higher eukaryotic cells, the chromatin structure of a transcriptionally active gene is more sensitive to DNase I digestion than that of an inactive gene, and that the DNase I sensitivity of a gene changes depending on the transcriptional activity of the gene (9, 10, 15, 19,

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20). In yeast *Saccharomyces cerevisiae*, however, the chromatin structure of a transcriptionally active gene and that of an inactive gene showed similar sensitivity to the DNase I (13).

To study the mechanism of the transcriptional regulation of the  $\beta$ -tubulin and flagellar calmodulin genes and to test if there are any differences in DNase I sensitivity of genes with different transcriptional activities of *N. gruberi*, a single cell eukaryote like yeast, we have examined the chromatin structure of the two DS genes and a non-specific (NS) gene, i.e. a gene which is expressed in amoebae and in flagellates, during the differentiation by using DNase I.

## Materials and Methods

### Growth and Differentiation of *Naegleria gruberi*

*N. gruberi* strain NB-1 was grown on NM agar plates at 34°C with *Klebsiella pneumoniae* as described by Fulton (2, 5). Amoebae were harvested and washed three times with ice-cold 2 mM Tris-HCl (pH 7.6 at 20°C) by differential centrifugation. Differentiation was initiated by suspending the washed amoebae in the Tris buffer prewarmed at 25°C. Differentiation was carried out at 25°C in 100 ml flasks in a reciprocating water bath (88 strokes per min). Cycloheximide was added sequentially to the flasks to a final concentration of 100  $\mu$ g/ml at different stages of differentiation (8). Differentiation was monitored every 10 min by fixing a small portion of cells in Lugol's iodine and counting the percent of cells with flagella.

### Isolation of Nuclei

Nuclei were isolated by the method of Lee and Walsh (12). Differentiating cells were pelleted 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% Nonidet P-40) and incubated on 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 (RPRS-14) 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.

### DNase I digestion and DNA purification

Nuclei were resuspended in a DNase I digestion buffer (1.25% Ficoll, 0.25 M sorbitol, 0.004% spermin, 0.5 mM DTT, 12.5 mM MgCl<sub>2</sub>, 45 mM Tris-HCl pH 7.9, 25% glycerol [w/v]) to a concentration of  $0.25 \times 10^8/0.25$  ml and treated with different amounts of DNase I for 3 min at 37°C. DNase I digestion was stopped by adding SDS and EDTA to a final concentration of 0.5% and 0.1 M, respectively. DNA was purified by using the method of Sambrook *et al.* (18).

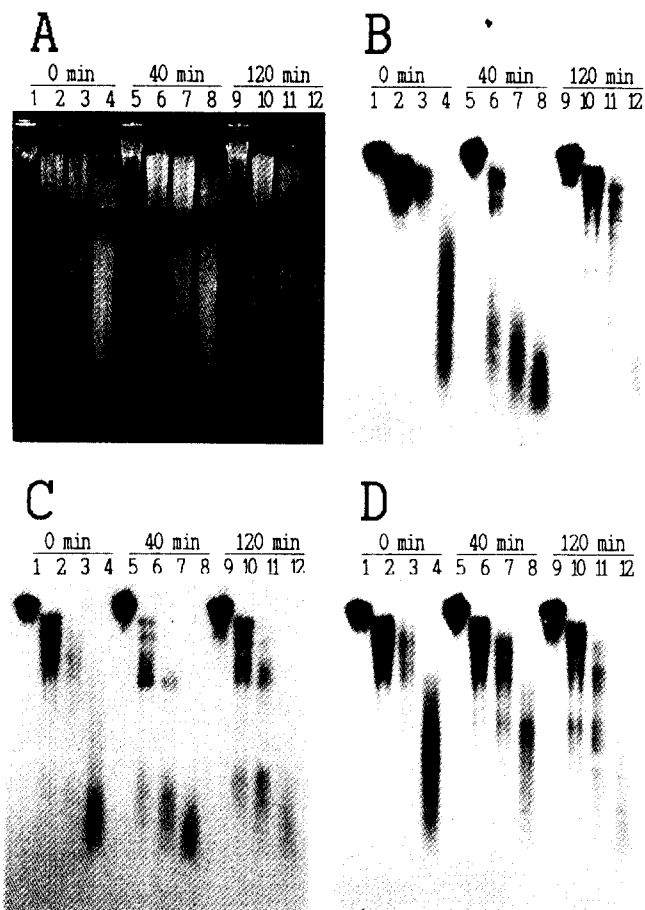
### DNA electrophoresis and Hybridization

DNA was fractionated by agarose gel electrophoresis and transferred to nylon membranes (Amersham, Hybond<sup>+</sup>) using the methods of the manufacturer's. 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 hr at 42°C. For hybridization, the prehybridization buffer was discarded and fresh hybridization buffer was added to the bag with denatured DNA probe. *Pst*I digested cDNA fragment of pcNg 8-5 (580 bp), pcNg 44 (375 bp), and pcNg 3-28 (700 bp) were used as probes for  $\beta$ -tubulin, flagellar calmodulin, and a non-specific gene, respectively (16). DNA probes were prepared by using a random primer extension kit from Boehringer Mannheim and <sup>32</sup>P-dCTP from Amersham. After 16-19 hr of hybridization at 42°C, the membrane was rinsed briefly with 2 $\times$ SSC, 0.1% SDS, then washed two times with 2 $\times$ SSC, 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, 0.1% SDS for 15 min each, and twice in 25 mM NaHPO<sub>4</sub>, pH 7.2, 1 mM Na<sub>2</sub>EDTA, 1% SDS for another 15 min each. All washings were carried out at 50°C (2).

## Results

### Change in the DNase I sensitivity of $\beta$ -tubulin and flagellar calmodulin genes during differentiation of *N. gruberi*

To test whether the changes in transcriptional activity of the two differentiation specific (DS) genes during differentiation of *N. gruberi* are accompanied with changes in chromosomal structure of the respective genes, we have prepared nuclei from 3 different stages of the differentiation; amoebae stage (0 min), 40 min, and 120 min after initiation of differentiation. Each nuclear preparation was digested with various concentrations of DNase I and DNA was purified. The purified DNA was fractionated on 1% agarose gel. From Fig. 1, it was evident that,



**Fig. 1.** Changes in DNase I sensitivity of the differentiation specific and the non-specific genes during differentiation of *Naegleria gruberi*. Nuclei isolated at 0, 40, 120 min after initiation of differentiation were incubated at 37°C for 3 min with 0 (lane 1, 5, and 9), 0.5 (2, 6, and 10), 1 (3, 7, and 11), or 3  $\mu$ g (4, 8, and 12) of DNase I. DNA was then extracted and fractionated on a 1% agarose gel (10  $\mu$ g of DNA/lane). Gel electrophoresis pattern of DNase I digested total chromosomal DNA is shown in the A panel. The DNA was transferred to a nylon membrane and hybridized with [ $^{32}$ P]  $\beta$ -tubulin cDNA (B), flagellar calmodulin cDNA (C), and differentiation non-specific gene (D). 0 min: nuclei isolated at beginning of differentiation. 40 min: nuclei isolated at 40 min after initiation of differentiation. 120 min: nuclei isolated at 120 min after initiation of differentiation.

as a whole, DNase I sensitivity of the *N. gruberi* chromatin did not change during differentiation. The DNase I treated and fractionated DNAs were transferred to a nylon membrane, and hybridized with  $^{32}$ P labeled cDNA probe to examine DNase I sensitivity of specific genes.

The two DS genes showed a very similar change in DNase I sensitivity during differentiation.  $\beta$ -tubulin gene was relatively resistant to DNase I at 0 min in the nuclei the gene was inactive (Fig. 1). After digestion with 0.5  $\mu$ g and 1.0  $\mu$ g of DNase I for 3 min,  $\beta$ -tubulin gene was still found in large DNAs. In the nuclei at 40 min, DNase I sensitivity of the  $\beta$ -tubulin gene increased dras-

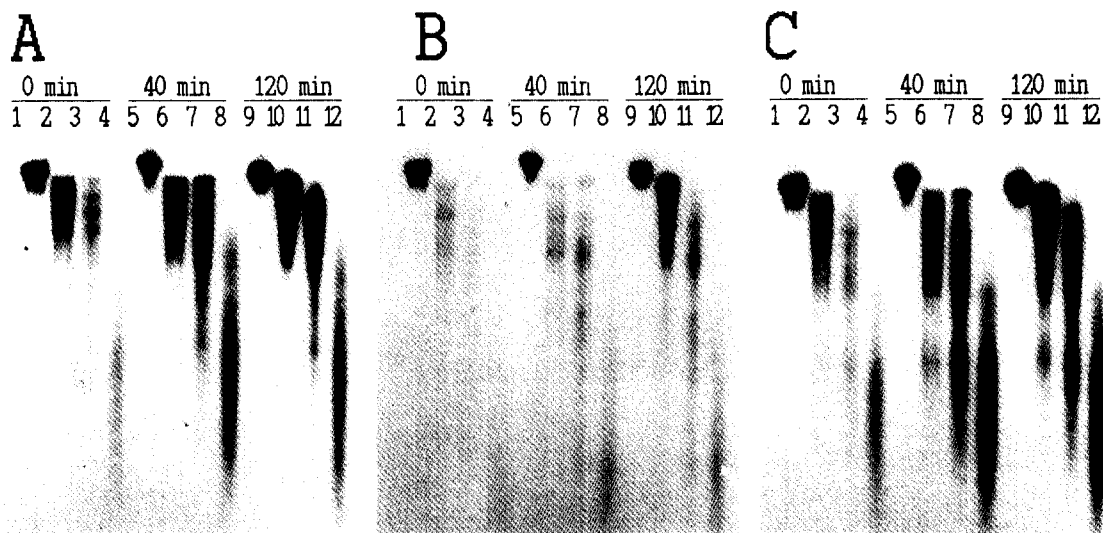
tically. Digestion with 0.5  $\mu$ g of DNase I produced significantly digested  $\beta$ -tubulin DNA. When the nuclei were digested with higher concentrations (1  $\mu$ g or 3  $\mu$ g) of DNase I, the  $\beta$ -tubulin DNA was found to be in small fragments. In the nuclei at 120 min, where transcription of the tubulin gene is decreased to a low level (12), the tubulin gene was far less sensitive to DNase I compared to the 40 min nuclei. After digestion with 0.5  $\mu$ g of DNase I, most of the  $\beta$ -tubulin gene was found in very large DNA fragments. When the nuclei were treated with 1  $\mu$ g of DNase I,  $\beta$ -tubulin gene was still found in large DNA fragments. The flagellar calmodulin gene was also resistant to DNase I at the 0 min. However, like the  $\beta$ -tubulin gene, the flagellar calmodulin gene became very sensitive to the enzyme digestion at the 40 min and DNase I sensitivity of the flagellar calmodulin gene decreased significantly in the 120 min nuclei (Fig. 1). This change in the DNase I sensitivity was specific to the two DS genes. DNase I sensitivity of a non-specific gene did not change noticeably throughout the differentiation (Fig. 1).

#### Effect of inhibition of protein synthesis on the DNase I sensitivity of the $\beta$ -tubulin and flagellar calmodulin genes.

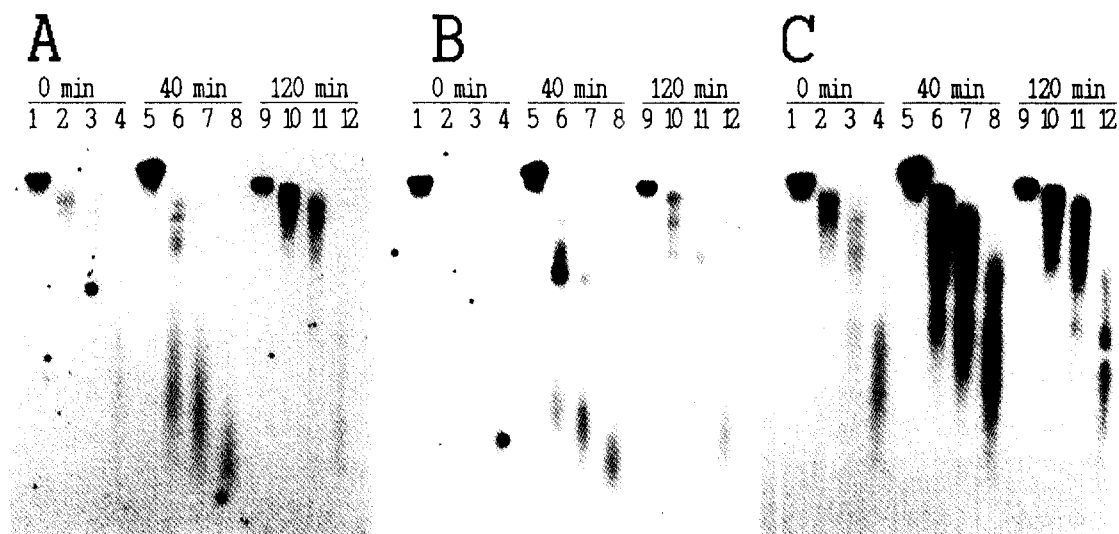
Addition of cycloheximide at the beginning of differentiation inhibited activation of transcription of the DS genes and, hence, the accumulation of the DS mRNAs (1). To examine whether this inhibition is also reflected in the chromosomal structure of the DS genes, we examined DNase I sensitivity of the DS genes after addition of cycloheximide.

Addition of cycloheximide had a significant effect on DNase I sensitivity of the  $\beta$ -tubulin and flagellar calmodulin genes. DNase I sensitivity of the two DS genes did not change throughout the differentiation in the presence of the drug (Fig. 2). This effect of cycloheximide treatment was specific to the DS genes. Inhibition of protein synthesis by cycloheximide treatment did not cause any noticeable change in the DNase I sensitivity of the non-specific gene (Fig. 2).

From the results in Fig. 1 and 2, it was clear that chromatin structure of  $\beta$ -tubulin and flagellar calmodulin genes changed from a DNase I insensitive structure at amebae stage to a very sensitive one at 40 min after initiation, and that this change required protein synthesis. It was not clear, however, whether protein synthesis was also necessary to restore the chromatin structure of the DS genes to a DNase I resistant form at the end of differentiation. To answer this question, we added cycloheximide at 40 min after initiation of the differentiation and examined DNase I sensitivity of the genes



**Fig. 2.** Effect of protein synthesis inhibition at the beginning of differentiation on the change of DNase I sensitivity of the DS and NS genes. Cycloheximide (100  $\mu\text{g}/\text{ml}$ ) was added at the beginning of the differentiation. Nuclei were isolated at 0, 40, 120 min during differentiation. DNase I digestion, gel electrophoresis, and Southern hybridization were carried out as in legend of Fig. 1. A: hybridization with  $\beta$ -tubulin cDNA. B: hybridization with flagellar calmodulin cDNA. C: hybridization with non-specific cDNA.



**Fig. 3.** Effect of protein synthesis inhibition at 40 min after initiation of differentiation on the change of DNase I sensitivity of the DS and NS genes. Cycloheximide (100  $\mu\text{g}/\text{ml}$ ) was added at 40 min after initiation of differentiation. Nuclei were isolated at 0, 40, 120 min during differentiation. DNase I digestion, gel electrophoresis, and Southern hybridization were carried out as in legend of Fig. 1. A: hybridization with  $\beta$ -tubulin cDNA. B: hybridization with flagellar calmodulin cDNA. C: hybridization with non-specific cDNA.

at 120 min. As shown in Fig. 3 chromatin structure of the DS genes was restored to the DNase I resistant form at the end of differentiation without protein synthesis.

### Discussion

Using DNase I, we have examined the chromatin structure of two DS genes,  $\beta$ -tubulin and flagellar calmodulin genes, and one non-specific gene at various stage

of *N. gruberi* differentiation. The results showed that, in *N. gruberi*, genes with different transcriptional activity had different sensitivity to DNase I. In this regard, chromatin structure of *N. gruberi* is more closely related to that of higher eukaryotes than that of yeast (13). DNase I sensitivity of the  $\beta$ -tubulin and flagellar calmodulin gene changed during the differentiation and this change was parallel to the observed changes in the transcription of the respective genes (12). The two genes were relatively insensitive to DNase I in amebae stage, before tran-

scription of the two genes was activated. However, the two DS genes were very sensitive to DNase I in the 40 min nuclei when transcription of the  $\beta$ -tubulin and flagellar calmodulin were most active. In the 120 min nuclei when the differentiation was completed and the two genes were being transcribed at a low rate, the two genes became resistant to DNase I. This change in DNase I sensitivity of the DS genes was inhibited by addition of cycloheximide at the beginning of differentiation, which also blocked activation of transcription of the DS genes (1). Transcription of the DS genes begins to decline after 40 min of differentiation and protein synthesis is required to facilitate this process in the later stages of differentiation (1). However, chromatin structure of the DS genes was restored to the DNase I resistant form at the end of differentiation when cycloheximide was added at 40 min after the initiation of differentiation (Fig. 3). This implies that protein synthesis may not be required to restore the chromatin structure of the DS genes in later stages.

These results suggest that the observed changes in the chromosomal structure of the two DS genes are closely related to the change in the transcriptional activity of the genes, and that protein synthesis is required to cause the changes in the chromosomal structure and to activate transcription of the DS genes at an early stage of differentiation. However, it is not clear whether protein(s) synthesized in the beginning of differentiation is necessary for the change in chromosomal structure to facilitate the binding of transcriptional factor(s), or it is a transcriptional factor which activates transcription of the DS genes by an unknown mechanism and thus changing the DNase I sensitivity of the genes by this activation. There have been many reports that DNase I sensitivity of a gene changes in accordance with the change of its transcriptional activity, e.g. fibroin gene of silk worm, *Bombyx mori* (10). It has been also reported that the binding of a protein causes a change in chromatin structure and this change allows the binding of a transcription factor(s) which in turn activates gene transcription of a gene. In mouse mammary tumor virus (MMTV), binding of the hormone-receptor complex to the hormone responsive element causes changes in the chromosomal structure of the promoter region. This change allows the NF-1 to bind to the regulatory region, which facilitates transcription from the promoter (17).

DNase I sensitivity of the NS gene did not change during the differentiation. Addition of cycloheximide at two different stages of differentiation had no noticeable effect on the chromatin structure of the NS gene. These results suggest that transcription of the NS gene is regulated by a distinct mechanism from that of the DS

genes.

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