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Secondary Level Screening of Chlamydomonas Reinhardtii Mutants Defective in Circadian Gene Expression

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SECONDARY LEVEL SCREENING OF *CHLAMYDOMONAS REINHARDTII* MUTANTS DEFECTIVE IN CIRCADIAN GENE EXPRESSION

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TABLE OF CONTENTS

	PAGE
INTRODUCTION	1-7
MATERIALS AND METHODS	8-15
RESULTS	16-45
DISCUSSION	46-55
REFERENCES	56-61

LIST OF TABLES

Tables Page

1. Comparison of reporter enzyme activity for Carni1 and its three single-cell isolates under two different conditions..... 18
2. Assignment of new names to several isolates from the single-point screen... 20
3. Comparison of single-point and multiple-point screen: strains with no significant difference to nonmutagenized Carni1 in the single-point screen...29
4. Comparison of single-point screen and multiple-point screen: strains with significant difference to nonmutagenized Carni1 in the single-point screen...31
5. Peak-to-trough ratios of *CABII-1* and *ARS2* mRNA abundances in Carni1 and the potential mutants.....44

LIST OF FIGURES

Figures Page

1. Arylsulfatase activity pattern in cultures of original Carni1 and its three single-cell isolates..... 19
2. Arylsulfatase activity pattern for strains with similar enzyme activity compared to nonmutagenized Carni1 in the single-point screen..... 22-25
3. Arylsulfatase activity pattern in strains with aberrant enzyme activity compared to nonmutagenized Carni1 in the single-point screen..... 26-28
4. Restriction digest of purified plasmid pHS16 containing a *CABII-1* cDNA fragment..... 33
5. Purified ~300 bp *CABII-1* cDNA fragment..... 34
6. Restriction digest of purified plasmid pJD27 containing a *ARS2* cDNA fragment..... 35
7. Purified ~2000 bp *ARS2* cDNA fragment..... 36
8. Northern blot analysis of potential mutants and nonmutagenized Carni1 under constant dim light..... 37
9. Northern blot analysis of more potential mutants under constant dim light.. 38
10. Northern blot analysis of more potential mutants and nonmutagenized Carni1 under constant dim light..... 39
11. Quantitative analysis of RNA blots from potential mutants and nonmutagenized Carni1 depicted in Figure 8..... 41

12. Quantitative analysis of RNA blots from potential mutants depicted in Figure 9..... 42

13. Quantitative analysis of RNA blots from potential mutants and nonmutagenized Carni1 depicted in Figure 10..... 43

14. Possible mutations in Carni1 affecting *CABII-1* and *ARS2* expression..... 50

SECONDARY LEVEL SCREENING OF *CHLAMYDOMONAS REINHARDTII* MUTANTS DEFECTIVE IN CIRCADIAN GENE EXPRESSION

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To elucidate the signal transduction chain mediating circadian clock control, this work focuses on the isolation of *Chlamydomonas reinhardtii* mutants which are defective in circadian gene expression. In a previous study, the reporter gene *ARS2* encoding the arylsulfatase enzyme was fused to the promoter of the circadian-regulated *CABII-1* gene and transformed into the *Chlamydomonas* nucleus. The *ble* marker was introduced into the genome of this transformant via insertional mutagenesis to generate mutants defective in circadian *CABII-1* expression. Potential mutants were selected based on aberrant single-point accumulative arylsulfatase activity. In this study, the arylsulfatase activity over the entire growth cycle was further investigated in these mutants and the reliability of the single-point screen was assessed. Of the 16 strains whose accumulative arylsulfatase activity did not differ from the nonmutagenized control in the single-point screen, 12 still showed no significant difference in a multiple-point screen. Of the 9

potential mutants with significant difference to the control in the single-point screen, 3 showed no significant difference in the multiple-point screen. Subsequently, 8 of the candidate mutants with aberrant reporter enzyme activity in the multiple-point screen were characterized by the abundance of their mRNA. The peak-to-trough ratio of *CABII-1* and *ARS2* transcript abundance was significantly reduced in 4 of these mutants.

CHAPTER I

INTRODUCTION

A biological rhythm that persists under constant conditions and has a period of about 24 hours is called a "circadian rhythm". Circadian rhythms are found in many organisms, ranging from bacteria to human beings, where they control various biological activities including behavior, metabolism, and gene expression (Pittendrigh 1960). A circadian rhythm shows three basic characteristics (Johnson and Hastings 1986). First, it is a self-sustaining rhythm that continues under constant environmental conditions with a period of approximately 24 hours. The second important feature of a circadian rhythm is that environmental signals reset the phase and entrain the rhythm to the daily environmental changes. The third characteristic is that the period length of a circadian rhythm is temperature-compensated. Because a circadian rhythm can persist without external time cues, it must be driven by an internal clock. The mechanism of the circadian clock is not completely understood yet. A model for the entire timing system states that it consists of at least three components (Aronson et al. 1994, Kreps and Kay 1997): 1) the input pathway which mediates environmental entrainment; 2) a central pacemaker which generates the approximately 24-hour oscillation; and 3) the output pathway through which various biological activities are regulated to show a circadian rhythm.

Within the past decades, extensive studies in several model organisms have

advanced the understanding of the molecular basis of the central oscillator. Thus far, nearly all evidence supports a mechanism based on feedback loops that contain both positive and negative elements. Clock genes yield clock proteins which feed back to repress their own expression (negative elements). Positive elements activate clock gene expression to keep the oscillator from winding down (Dunlap 1999). In the fungus *Neurospora*, the frequency gene (*frq*) encodes the FREQUENCY protein (FRQ) which acts at least indirectly to depress the level of the *frq* transcript (Aronson et al. 1994). Two regulatory genes, *white collar-1* (*wc-1*) and *white collar-2* (*wc-2*), positively regulate *frq* transcription (Crosthwaite et al. 1997). In *Drosophila*, the autorepression of the *period* (*per*) and *timeless* (*tim*) gene by their protein products gives rise to the oscillation (reviewed by Dunlap 1999). The two proteins CLOCK (dCLK) and CYCLE (dCYC) activate *per* and *tim* by binding to an E-box target sequence in their promoters (Hogenesch et al. 1998). In the unicellular cyanobacterium *Synechococcus*, the three genes *kaiA*, *kaiB*, and *kaiC* were identified as essential for circadian phenotypes. While *kaiC* expression is repressed by its own protein product to create a negative feedback, KaiA protein enhances *kaiBC* expression to keep the loop oscillating (Johnson and Golden 1999). In the suprachiasmatic nuclus (SCN), the site of the master clock in the mammalian brain, three *period* genes and two *cryptochrome* (*cry*) genes are involved in the negative feedback loop. The rhythmic transcription of the *mPer* and *mCry* genes is positively regulated by the transcription factors CLOCK and/or BMAL1, a homologue of CYCLE in *Drosophila* (Reppert and Weaver 2000). However, in higher plants, no unequivocal oscillator component has been identified yet. In the plant model organism *Arabidopsis thaliana*, evidence shows that *TIMING OF CAB EXPRESSION-1* (*toc1*) could be a candidate for an oscillator component. In the *toc1-1* mutant, multiple circadian

outputs are altered (Somers et al. 1998) and the *toc1* gene has recently been cloned (Strayer et al. 2000). The *Arabidopsis* pseudo-response regulator 1 (APRR1) was reported to be identical to the *TOC1* gene product, and the APRR1/TOC1 family is proposed to be the molecular basis of the biological clock in higher plants (Matsushika et al. 2000).

Another important aspect of the circadian timing system is the output pathway which encompasses the signal transduction mechanisms the oscillator employs to regulate downstream events causing the rhythmicity of diverse biological processes. Since the 1950s, it has been gradually recognized that a wide variety of behavioral, physiological, and biochemical variables are controlled by the circadian clock, such as leaf movement and photoperiodic flower-induction in plants, eclosion in *Drosophila*, and body temperature in humans. They all show rhythms that persist in constant darkness or constant light with an endogenous period of about 24 hours. Recently, more and more clock-controlled genes (*ccgs*) have been identified. Disruption of central oscillator components could affect the rhythmicity of these genes and the products of these genes are not necessary for clockwork function. The most direct way for the central oscillator to regulate downstream events is to use products from *ccgs* that are regulated (directly or indirectly) by the core feedback loops. It is therefore easily possible that the output pathway from the oscillator to clock-controlled gene expression will be short compared with more complex processes such as plant cell expansion and flowering (Somers 1999).

In *Neurospora*, 11 *ccgs* have been identified using subtractive hybridization and differential screens of time-of-day-specific libraries (Bell-Pedersen 2000). The molecular analysis of *eas* (*ccg-2*) revealed an activating clock element (ACE) within its promoter. The ACE sequences are currently being used to biochemically identify upstream

regulatory factors responsible for *eas* cycling in an attempt to trace the output pathway back to the oscillator (Bell-Pedersen 2000). In *Drosophila*, studies have focused on the pigment-dispersing factor gene (*pdf*). Genetic analysis has shown that *pdf* is clearly involved in circadian ouptut, regulating locomotor activity in flies (Renn et al. 1999). However, directly connecting *pdf* regulation to the central oscillator mechanism has been difficult because *pdf* RNA levels do not oscillate. In mammals, the vasopressin gene, a *ccg*, was the first example identified to be directly regulated by the CLOCK/BMAL1 heterodimer, an essential clock component (Jin et al. 1999). CLOCK also regulates another *ccg*, the albumin D-element binding protein (dbp) gene which is a member of the PAR leucine zipper transcription factor family. CLOCK regulates *dbp* by binding to several E-box motifs within putative enhancer regions located in the first and second intron (Ripperger et al. 2000). Research into the circadian clock in plants revealed circadian regulation of gene expression at many levels: at the level of transcription (Millar and Kay 1991, Liu et al. 1996), transcript abundance (Fujiwara et al. 1996, Zheng et al. 1998), translation (Mittag et al. 1994), and posttranslational processing (Nimmo 1998). Upstream components regulating circadian gene expression are currently being discovered in *Arabidopsis thaliana.* In the *early-flowering 3* (*elf3*) mutant, expression of all circadian-regulated genes examined was disrupted under constant white-light suggesting that the *elf3* phenotype could be due to a defective circadian output pathway (Hicks et al. 1996). The *GIGANTEA* (*GI*) gene mutation leads to an altered period and reduced amplitude in the circadian expression of *ccgs* suggesting that GI is involved in an outer feedback loop essential to maintain circadian amplitude and period length (Park et al. 1999). LHY and CCA1 are both Myb-related proteins with strong homology to each other (Schaffer et al. 1998, Wang and Tobin 1998). Expression of both, *LHY* and *CCA1,*

shows a circadian rhythm. Overexpression of these two genes can repress their own expression and overexpression of *CCA1* can also repress *LHY* expression. A mutation in either of these two genes alters all circadian rhythms examined. Thus, if *LHY* and *CCA1* are not components of the central oscillator, they may be closely associated with it. Expression of the *CAB* gene family which encodes chlorophyll a/b binding proteins of photosystem I and II is circadian regulated (Kay 1993). Loss of CCA1 affects the circadian expression of the *CAB* genes (Green and Tobin 1999). A mutation in *LHY* also leads to arhythmic *CAB* expression (Schaffer et al. 1998). CCA1 protein was demonstrated to interact with the promoter of at least two of the *CAB* genes. (Wang et al. 1997). A protein kinase, CK2, was identified to interact with and phosphorylate the CCA1 protein (Sugano et al. 1998). Overexpression of *CK2* shortens the period of several circadian rhythms of gene expression including *CAB*. These results indicate the shortness of the output pathway between the central oscillator and some *ccgs*.

With the goal of understanding the signal transduction chain from the central oscillator to circadian gene expression, this study further advanced the screening for mutants defective in circadian transcription using the model organism *Chlamydomonas reinhardtii*.

Chlamydomonas reinhardtii is a unicellular, eukaryotic green alga. It has a cell wall, a chloroplast, and two flagella with which it can swim. *Chlamydomonas reinhardtii* shows several advantages as a model organism for molecular studies of the circadian output pathway (Harris 1989). 1) It has well-defined genetics and biochemistry. 2) It can grow in a simple medium which makes the whole study inexpensive and easy to manipulate. 3) The organism is unicellular which provides the most simplified approach to circadian output studies. 4) It can also grow in total darkness if acetate is provided as

an alternative carbon source, so mutants defective in photosynthesis can survive. 5) *Chlamydomonas* shows high amplitude circadian expression from the *CABII-1* promoter even in the dark as long as heterotrophic growth medium is provided (Nikaido et al. 1994). 6) The *Chlamydomonas reinhardtii* transformant Carni1 carrying the reporter gene *ARS2* transcriptionally fused to the *CABII-1* promoter is readily available (Jacobshagen et al. 1996).

ARS2 was chosen as reporter gene to reflect circadian expression of *CABII-1* because it is the only reporter currently available for *Chlamydomonas* that allows detection of quantitative differences in expression via a simple assay (Quinn and Merchant 1995, Jacobshagen et al. 1996, Ohresser et al. 1997). Other reporter genes like the gene encoding β-glucuronidase (*GUS*) are not expressed when integrated into the *Chlamydomonas* genome (Blankenship and Kindle 1992). *ARS2* is a *Chlamydomonas* gene that was developed as a reporter (Davies et al. 1992). It codes for the arylsulfatase enzyme, which is excreted from *Chlamydomonas* cells and its activity can easily be tested in the culture supernatant. The endogenous *ARS2* gene is not expressed under sulfur-sufficient condition, not even at the mRNA level (Davies et al. 1994). In Carni1, *ARS2* exhibits a circadian rhythm of mRNA abundance like that of *CABII-1* consistent with a circadian control of *CABII-1* at the transcriptional level (Jacobshagen et al. 1996). However, the arylsulfatase enzyme is too stable to reflect the circadian rhythm of its mRNA abundance (Jacobshagen et al. 1996). It is a useful reporter at the enzyme level only when accumulative differences in *ARS2* expression are to be determined over a longer time period. Another foreign gene, *ble*, was successfully introduced into the Carni1 genome via insertional mutagenesis (Yuan 1999). The integration may affect functional genes involved in the output pathway regulating circadian expression of

CABII-1. Based on aberrant accumulative arylsulfatase activity determined at a single time point during late logarithmic to early stationary phase, about 1000 transformants were screened for potential mutants in circadian *CABII-1* expression (Yuan 1999).

To further characterize these potential mutants and to evaluate *ARS2* as a reporter at its enzyme activity level, the pattern of arylsufatase activity over a complete culture growth cycle was determined in this study. Differences in *ARS2* expression may be due to a defect at the transcriptional or the posttranscriptional level. In order to distinguish between these two possibilites, the amount of *ARS2* and *CABII-1* mRNA was determined. As has been demonstrated (Jacobshagen and Johnson 1994), after synchronization by a 12-hour dark pulse, the circadian rhythm in *CABII-1* mRNA abundance of *Chlamydomonas* reaches a trough after about 15 hours in constant dim light at 17°C and a peak after about 27 hours. Consequently, the mRNA amount for *ARS2* and *CABII-1* was measured in each mutant at these two time points, and compared with that of Carni1. Mutants thus identified will be further characterized and their defective gene will be isolated. The approach will allow to elucidate components in the output pathway of the circadian timing system in *Chlamydomonas*.

CHAPTER II

MATERIALS AND METHODS

Strains and Growth Conditions

The transformant Carni1 was derived from *Chlamydomonas reinhardtii* Dangeard stain CC125 (Chlamydomonas Genetics Center at Duke University). Carni1 contains two reporter genes, *ARS2* and *NIT1*, each transcriptionally fused to the promoter of *CABII-1* (Jacobshagen et al. 1996). Three single colony isolates of Carni1, named single1, single2, and single3, had been cultured separately for over 5 years before being used in this study. The 26 potential mutants of Carni1 were obtained by transforming Carni1 with the *ble* marker and screening for aberrant expression of the *ARS2* gene (Yuan 1999).

Liquid stock cultures were obtained by inoculating 50 mL of 0.3 high-salt medium (0.3 HSM) (Sueoka 1960) in 125 mL Erlenmeyer flasks from slants and growing them photoautotrophically on an orbital shaker (Innova 2100 Platform shaker, New Brunswick Scientific, Edison, NJ) at 250 rpm, room temperature and a constant light intensity of 9 $\mu E/m^2s$ from above.

For measuring arylsulfatase enzyme activity excreted into the culture medium, experimental cultures were grown mixotrophically in SGII medium (Sager and Granick 1953) starting at a concentration of 10^4 cells/mL under otherwise the same condition as above.

For RNA analyses, cells were inoculated from the liquid stock into 1 L bottles of

0.3 HSM at a concentration of 10^4 cells/mL. The experimental cultures were grown photoautotrophically in an incubator at a constant temperature of 17 °C and aeration by an aquarium pump. Two 20 W Gro-lux light tubes from each side illuminated the cultures providing a constant light intensity of 55 $\mu E/m^2s$ per side. When cultures reached the late logarithmic phase (1-2.5 x 10^6 cells/mL), they were synchronized by putting them into darkness for 12 hours. Darkness was achieved by wrapping the bottles in aluminum foil. Following synchronization, cultures were put into constant dim light at an intensity of 20 $\mu E/m^2s$ by covering the light tubes with several layers of tissue wipers.

Plasmids

Plasmid pHS16 (Shepherd et al. 1983) contains a 0.3 kb *CABII-1* cDNA fragment originally cloned into pBR322 but later subcloned into the *PstI* site of pSP65 by K. Kindle. The fragment is specific for the *CABII-1* transcript. Plasmid pJD27 was created by inserting an approximately 1.9 kb *ARS2* cDNA fragment into the pUC19 vector (J. Davies, personal communication). The *ARS2* fragment can be released by double digestion with restriction enzymes *SacI* and *HindIII*.

Determination of Cell Density and Arylsulfatase Activity Assay

To determine the cell density of liquid stock cultures for inoculation or of experimental cultures for RNA analysis, 1 mL culture sample was treated with a drop of iodine tincture (0.25 g iodine in 100 mL 95% ethanol) to immobilize the cells (Harris 1989) and the number of cells were counted with a hemacytometer. Cell densities of experimental cultures for arylsulfatase activity assays were determined by measuring

their optical density at 750 nm using a spectrophotometer (UV-1601, SHIMADZU, Columibia, MD).

The activity of arylsulfatase excreted from the cells was assayed in the culture supernatant using p-nitrophenyl sulfate as the substrate as described (Jacobshagen et al. 1996). One unit of arylsulfatase catalytic activity is defined as the amount of product p-nitrophenol formed per 333 μL culture supernatant that leads to an increase in absorbance at 410 nm of 1.0 within 30 min at 30 °C.

Multiple-Point Mutant Screen at the Reporter Enzyme Level

To evaluate the reliability of the previously used single-point mutant screen (Yuan 1999) and to obtain accurate enzyme activity information on the potential mutants, a multiple-point screen was performed. When liquid stock cultures reached their late logarithmic phase (0.5-2 x 10^6 cells/mL), 5 x 10^5 cells were transferred from the stock culture to a 125 mL Erlenmeyer flask containing 50 mL SGII medium so that the starting cell density for each experiment was 10^4 cells/mL. Therefore, the growth curves of different cultures were expected to be similar which made the sampling of several cultures at the same time possible. Sampling started 48 h after inoculation. About 12 hours later, almost all cultures grew to their early or middle logarithmic phase and were sampled the second time. Cultures that grew slowly were not sampled untill they reached the logarithmic phase. During logarithmic phase, samples were taken about every 4-6 hours until the cultures reached their stationary phase. After that, cultures were sampled about every 24 hours. Cell density and arylsulfatase activity of each sample was measured. Independent experiments were performed at least twice for each strain.

Isolation of *CABII-1* and *ARS2* cDNA Fragments

Plasmid pHS16 containing a *CABII-1* cDNA fragment and plasmid pJD27 containing an *ARS2* cDNA fragment were amplified in *E. coli* strain XL1 blue and isolated using the QIAfilter Plasmid Midi/Maxi kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. Isolated plasmid pHS16 was digested with the restriction enzyme *PstI* (New England Biolabs, Beverly, MA) while plasmid pJD27 was subjected to a double digest using *HindIII* and *SacI* (New England Biolabs, Beverly, MA). For each 20 μL restriction digest volume, 5 μg plasmid and 10 units of each restriction enzyme was added. The digestion products were separated by electrophoresis on a 1.5% and 0.8% agarose gel, respectively. All DNA agarose gels were prepared in 1 x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.5) with 500 ng/mL ethidium bromide. Electrophoresis was performed in 1 x TAE buffer. DNA bands were visualized on a transilluminator (Transilluminator FBTI- 614, Fisher Biotech, Pittsburgh, PA) and images taken by a digital camera (DC40, Kodak Scientific Imaging Systems, New Haven, CT). The amount of DNA in each band was estimated by comparison to DNA mass standards using a computer program (Kodak 1D 3.0 Imaging System, Kodak Scientific Imaging Systems, New Haven, CT). The ~300 bp *CABII-1* and ~2000 bp *ARS2* bands were cut out of the gels and purified using QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. To judge the purity and determine the concentration of the isolated DNA fragments, 4 μL of the 30 μL *CABII-1* fragment solution and 2 μL of the 30 μL *ARS2* fragment solution were subjected to electrophoresis on a 1.5% and 1% agarose gel, respectively.

Northern Blot Analysis

A. Sampling and RNA Extraction

Chlamydomonas strains were grown as described (see growth conditions). Samples were taken at 15 hours and 27 hours in constant dim light which corresponds to the trough and peak, respectively, of the circadian *CABII-1* and *ARS2* mRNA abundance rhythm in Carni1 (Jacobshagen et al. 1996). Sixty gram of ice was added immediately to each 150 mL sample to keep it in its original physiological state. Samples were centrifuged at 4,900 g for 5 min at 4 °C. Cells were resuspended in a small volume of supernatant and transferred to a 50 mL centrifuge tube (Corning Inc., Corning, NY). Cells were collected by centrifugation at 1,900 g for 10 min at 4 °C, frozen in liquid nitrogen and stored at –70 °C.

Total RNA was isolated from cell pellets using TRIzol Reagent (Life Technology , Rockville, MD). Isolation was performed as suggested by the manufacturer except that 4 mL TRIzol reagent was used for 1 to 4 x 10^8 *Chlamydomonas* cells. At the end of the procedure, dried RNA pellets were dissolved in diethylpyrocarbonate-treated water in different volumes (30 to 80 µL) based on RNA yields. The concentration of RNA in the samples was determined spectrophotometrically by measuring the absorbance at 260 nm (Ultrospec 3000 by Amersham Pharmacia Biotech, Uppsala, Sweden). RNA samples were stored at -70°C.

B. Electrophoresis and Membrane Blotting

RNA samples were denatured by adding formamide/formaldehyde mix. To obtain 157 µL formamide/formaldehyde mix, 20 µL 10 x MOPS (0.4 M MOPS, 100 mM

sodium acetate, and 10 mM EDTA, pH 7.0), was combined with 37 μL formaldehyde (37%) and 100 μL formamide. Each 4 μL RNA sample containing 10 μg of RNA was mixed with 15 μL formamide/formaldehyde mix and heated to 65 °C for 15 min. Following this treatment, 2 μL of 10 x loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, and 0.4% xylene cyanol) was added to each sample.

Each RNA sample at an amount of 2.5 μg (5 μL) was loaded three times onto a 1% agarose gel containing 1 x MOPS and 6.3% formaldehyde, and subjected to electrophoresis in 1 x MOPS at 3 V/cm gel for ~3.5 hours. After electrophoresis, one part of the gel was stained with ethidium bromide (0.5 μg/mL) to evaluate whether all samples were loaded equally. The remaining gel was first incubated in water for 1 hour to remove the formaldehyde and then equilibrated in 10 x SSC solution (1 x SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) for 20 minutes. After equilibration, the RNA was transferred to neutral nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Upsala, Sweden) through capillary blotting over night in 10 x SSC (Ausubel et al. 2000). Following transfer, RNA was cross-linked to the membrane using a UV-crosslinker (UV Stratalinker 1800, Stratagene, La Jolla, CA) at its automatic setting.

C. Preparation of Radioactively Labeled Probes

Using the isolated *CABII-1* and *ARS2* cDNA fragments as templates, radioactively labeled *CABII-1* and *ARS2* probes were synthesized in vitro using the Prime-a-Gene® labeling system (Promega, Madison, WI) and α-^{32}P-dCTP (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instruction. Unincoporated nucleotides were removed by NICK Sephadex G-50 column chromatography (Amersham Pharmacia

Biotech, Uppsala, Sweden) as described by the manufacturer (Promega, Madison, WI). The specific activity of the probes was about 1.3 x 10^9 cpm/μg DNA as determined in a scintillation counter (1500 TRI-CARB liquid scintillation anlyzer, Packard, Downers Grove, IL).

D. Hybridization and Autoradiography

Membranes were cut into two parts, one part for hybridization with the *CABII-1* probe and the other for hybridization with the *ARS2* probe. Membranes (12 cm x 4 cm or 8 cm x 4 cm) which would be hybridized with the same probe were put into the same glass bottle (35 mm diameter, 15 cm length) and prehybridized in 10 mL of hybridization solution at 44°C for 2 hours in a hybridization oven (Autoblot Mini Hybridization Oven, Bellco Glass Inc., Vineland NJ). The hybridization solution consisted of 50% formamide, 6 x SSPE (0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.4), 5 x Denhardt's reagent (0.01 g each of Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin in 10 mL H_2O), 0.5% sodium dodecyl sulfate (SDS) and 0.1 mg/mL salmon sperm DNA. The hybidization solution was discarded and 10 mL new hybridization solution with 1.5-2.5 x 10^7 cpm of either the *CABII-1* or *ARS2* probe was added to the bottle for hybridization at 44 °C overnight. Membranes were washed twice in 2 x SSC for 5 min at room temperature, twice in 2 x SSC, 1% SDS for 30 min at 68 °C and twice in 0.1 SSC for 30 min at room temperature. After washing, blots were wrapped with Saran wrap and exposed to autoradiography film (Hyperfilm MP, Amersham Pharmacia Biotech, Piscataway, NJ). Films were developed as follows: 4 min in developer solution (Kodak GBX Developer and Replenisher) without shaking, 30 sec in water with constant

shaking and 4 min in fixer solution (Kodak GBX Fixer and Replenisher) with 5 seconds of shaking for every 30 seconds interval.

E. Quantitative Analysis

Computer images of the ethidium bromide stained gels under UV light (Transilluminator FBTI- 614, Fisher Biotech, Pittsburgh, PA) were captured by a digital camera (DC40, Kodak Scientific Imaging Systems, New Haven, CT), and the 16S rRNAs were quantified using the KODAK 1D 3.0 program (Kodak Scientific Imaging Systems, New Haven, CT).

Autoradiograms of the Northern blots were scanned with a UC840 scanner (MacVersion, UMAX) using the Adobe photoshop program, and the intensity of the bands was quantified using the NIH Image program version 1.6.

CHAPTER III

RESULTS

Variability of Reporter Gene Expression in Nonmutagenized Carni1

The criterion that had been used in a previous mutant screen based on the *ARS2* reporter was for potential mutants to show aberrant accumulative reporter enzyme activity compared with nonmutagenized Carni1. Therefore, the stability of *ARS2* expression at the arylsulfatase activity level is very important in Carni1. In order to judge this stability, the arylsulfatase enzyme activity in Carni1 was compared with the activity in three single-cell isolates of Carni1 that had been maintained separately for over five years.

Carni1 and its single-cell isolates were first grown on an orbital shaker with speeds of 125 rpm, at room temperature and a constant light intensity of 20 $\mu E/m^2s$ (condition 1). But since the supernatant of the cultures was yellow, it was suspected that the condition led to some cell lysis. To avoid this problem, cultures were instead grown on an orbital shaker with speeds of 250 rpm, at room temperature and a constant light intensity of 9 $\mu E/m^2s$ (condition 2). No yellow supernatant was observed under this condition in any culture. In order to compare *ARS2* expression between different strains, the highest arylsulfatase activity during culture growth was chosen as criterion, because it should provide the greatest sensitivity. All arylsulfatase activities obtained were standardized to 1 OD cell density at 750 nm to exclude the effect of variations in cell

amount during culture growth. Highest arylsulfatase activities for Carni1 and its isolates under the two different culture conditions are shown in Table 1. Under condition 1, single isolate2 showed significantly lower aylsulfatase activity compared to all other cultures, while under condition 2, no significant difference was observed among the original Carni1 and its three single isolates ($p = 0.87$). The results indicate that condition 2 is more optimized and that under this condition *ARS2* expression at the enzyme activity level is stable in Carni1. Further experiments were all exclusively performed under condition 2.

Figure 1 shows the arylsulfatase activity pattern throughout an entire culture growth cycle for nonmutagenized Carni1 and its isolates under condition 2. The enzyme activity increases during the logarithmic phase, reaches its highest level during early stationary phase and decreased slowly afterwards. The slow decrease during stationary phase is probably due to proteases released from the cells.

Characterization of Potential Mutants at the Reporter Enzyme Activity Level

The previous selection of potential mutants based on reporter enzyme activity was carried out as a "single-point screen" (Yuan 1999). Each culture was tested at only a single time point, usually when it reached about stationary phase, and only a single assay was performed. It made the screening of about one thousand mutagenized strains possible. The arylsulfatase activity at the single time point was compared to the respective activity in a nonmutagenized Carni1 culture of the same cell density as determined from a standard curve. Strains that had at least 50% lower or 50% higher arylsulfatase activity were considered significantly different and therefore potential mutants. As shown in Table 2, some of the isolated strains were renamed in order to

Table 1: Comparison of reporter enzyme activity for Carnil and its three single-cell isolates under two different conditions. *ARS2* reporter expression was determined as arylsulfatase enzymatic activity excreted into the culture medium. The data show the highest enzyme activities during a culture growth cycle relative to the culture density and represent the average of two independent experiments. SD indicates the standard deviation, * indicates that arylsufatase activity was tested only once. Condition 1: shaker speeds of 125 rpm at room temperature and a constant light intensity of 20 $\mu E/m^2s$. Condition 2: shaker speeds of 250 rpm at room temperature and a constant light intensity of 9 $\mu E/m^2s$.

Strains	Condition 1		Condition 2	
	Arylsulfatase activity/cell density (U/OD_{750nm})	SD	Arylsulfatase activity/cell density (U/OD_{750nm})	SD
Carnil	2.69	0.086	2.46	0.22
single1	2.75	0.24	2.59 *	____
single2	1.72	0.15	2.7*	____
single3	2.55	0.05	2.62*	____

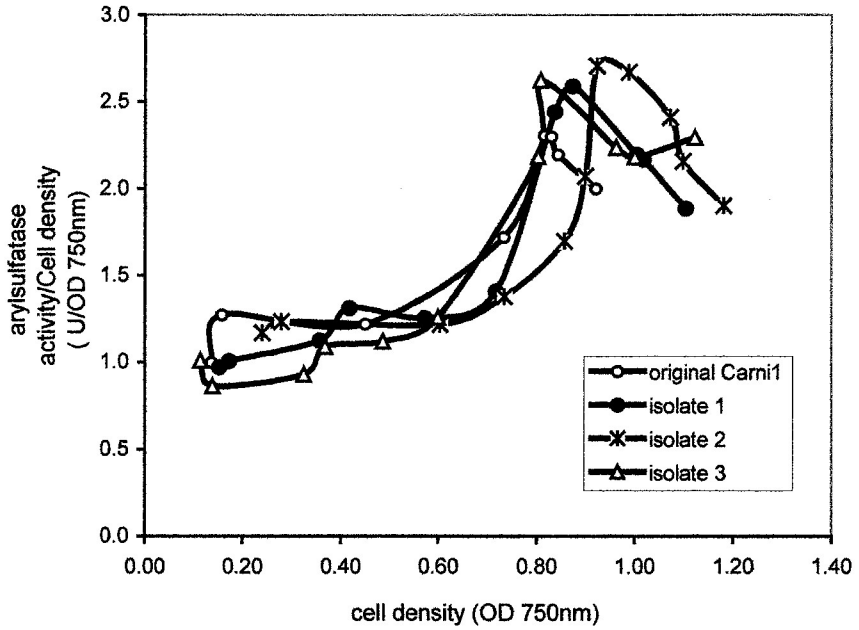

Figure 1: Arylsulfatase activity pattern in cultures of original Carni1 and its three single-cell isolates. Enzymatic activities of the arylsulfatase reporter released into the culture medium were examined over a complete culture growth cycle. Cell density is indicated by the optical density at 750 nm. Arylsulfatase activity is standardized to the optical density of the culture. Cultures were grown in mixotrophic medium under condition 2 (at room temperature, a constant light intensity of 9 $\mu E/m^2s$, and a shaker speed of 250 rpm).

Table 2: Assignment of new names to several isolates from the single-point screen. To avoid confusion, the new names are used throughout this thesis.

Original name	New name
34 5/13/99	#24
102 6/18/99	#26
79 7/14/99	#25
23 7/14/99	#23
17 7/14/99	#21
20 7/14/99	#22
118???	#28
2 7/4/99	#19
118s 6/13/99	#29
118 6/13/99	#27
7 7/4/99	#20

avoid confusion.

To examine the efficiency of the single-point screen and to further characterize the mutants at their reporter enzyme level, the arylsulfatase activity of 16 strains without significant difference to Carni1 (Figure 2) and 9 strains with significant difference (Figure 3) were examined using a "multiple-point screen." In this screen, the arylsulfatase activity of each strain was monitored at multiple time points throughout the logarithmic and stationary phase of the liquid culture. As depicted in Figure 2 and 3, enzyme activity patterns show only low variation between the two independent experiments in most strains examined. When comparing the highest arylsulfatase activity of the two experiments, the values vary at an acceptable range (between 0% and 30%). The overall pattern of mutants #4, #6, #9, #7, #24, #23, and #29 in Figure 2 and #1, #10, #11, #14, #16, and #21 in Figure 3 differ prominently from nonmutagenized Carni1. Among them, #1 and #21 in Figure 3 clearly show a much higher arylsulfatase activity while #6, #9, and #24 in Figure 2 and #10, #14, and #16 in Figure 3 clearly show a significantly lower activity.

In order to allow for a simple comparison with the single-point screen, the highest value of enzyme activity for each strain was calculated as a percentage to that of a Carni1 control culture. As shown in Table 3, 12 out of the 16 strains with no significant difference in the single-point screen still showed no significant difference in the multiple-point screen. However, significantly lower enzyme activity was detectable in 3 strains (#9, #6, and #24) and significantly higher activity in 1 strain (#7).

Of the 9 potential mutants with significant difference to Carni1 in the single-point screen, 3 showed no significant difference to Carni1 in the multiple-point screen (#12, #13, #18 in Table 4). Four of the mutants (#10, #11, #14, and #16) still showed

Figure 2. Arylsulfatase activity pattern for strains with similar enzyme activity compared to nonmutagenized Carni1 in the single-point screen. Enzymatic activities of the arylsulfatase reporter released into the culture medium were examined over a complete culture growth cycle. Cell density is indicated by the optical density at 750 nm and arylsulfatase activity is standardized to the optical density of the culture. Cultures were grown in mixotrophic medium under condition 2 (at room temperature, a constant light intensity of 9 $\mu E/m^2s$, and a shaker speed of 250 rpm). Thin lines with open circles represent the Carni1 control while bolt lines with solid diamonds represent the potential mutants. Two independent experiments are depicted for each strain.

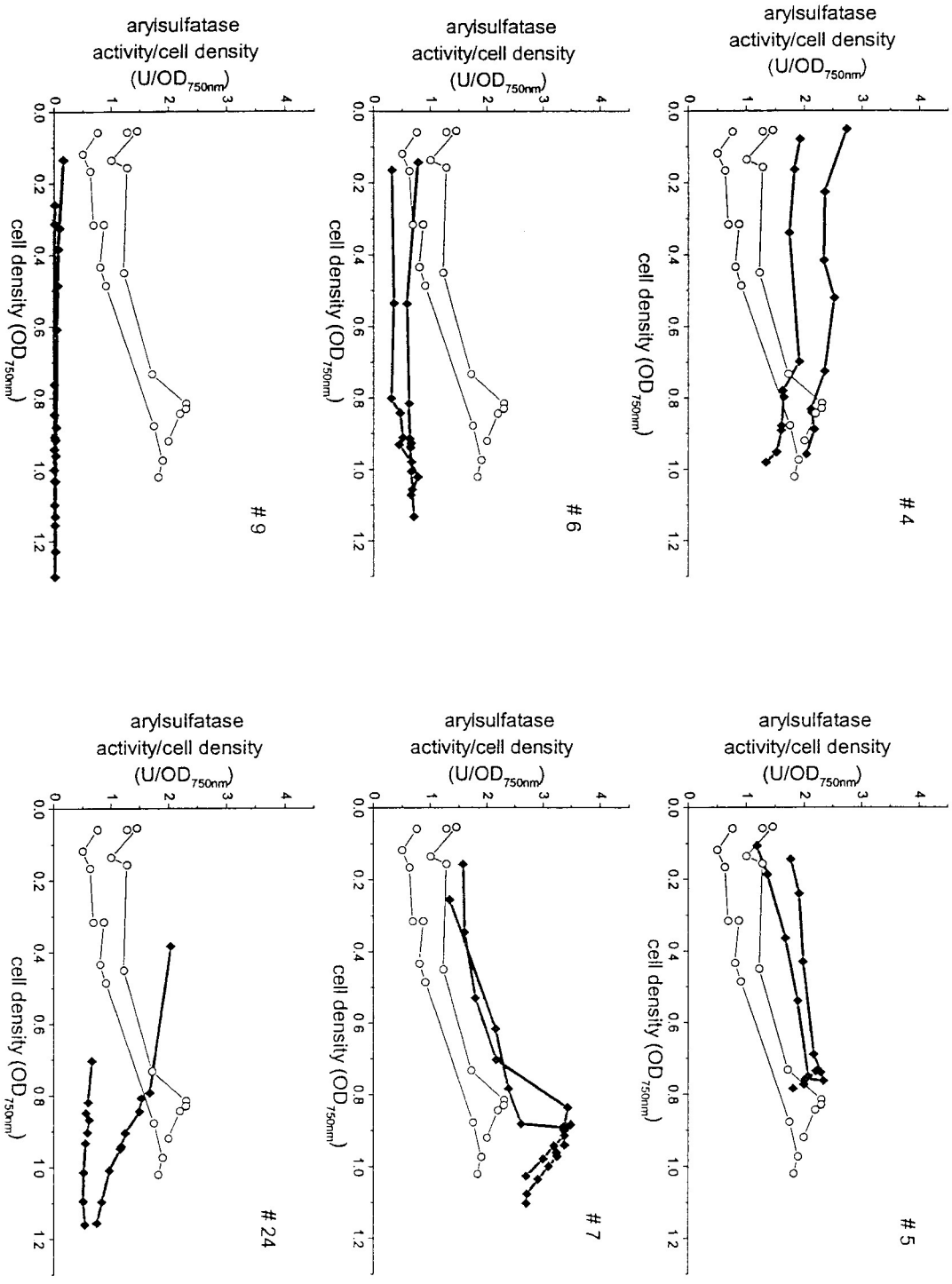
arylsulfatase activity/cell density (U/OD750nm)
cell density (OD750nm)
4
5
6
7
9
24

Figure 2-1

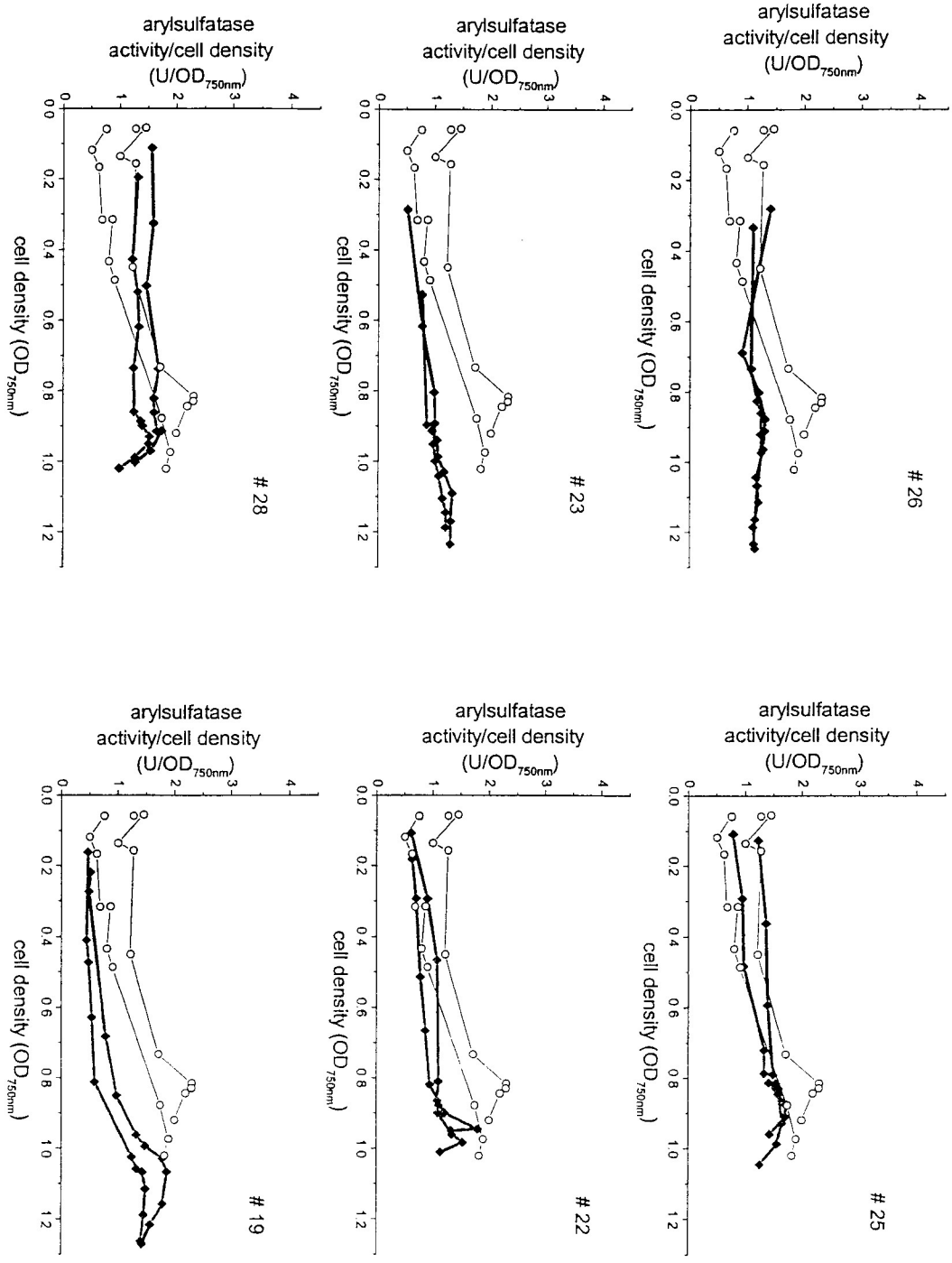
arylsulfatase activity/cell density (U/OD750nm)
cell density (OD750nm)
26
23
28
25
22
19

Figure 2-2

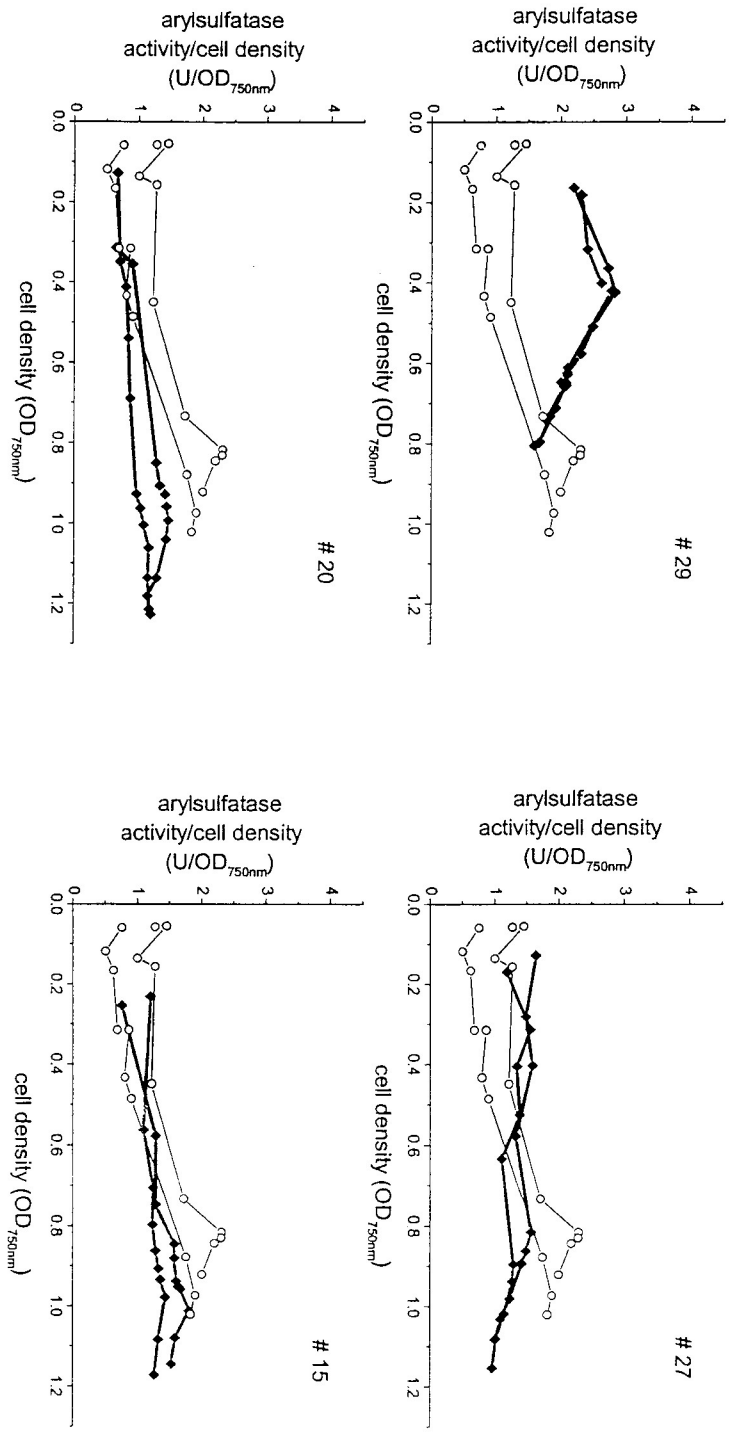
arylsulfatase
activity/cell density
(U/OD750nm)
cell density (OD750nm)
29
20
27
15

Figure 2-3

Figure 3. Arylsulfatase activity pattern in strains with aberrant enzyme activity compared to nonmutagenized Carni1 in the single-point screen. For explanation see legend to Figure 2.

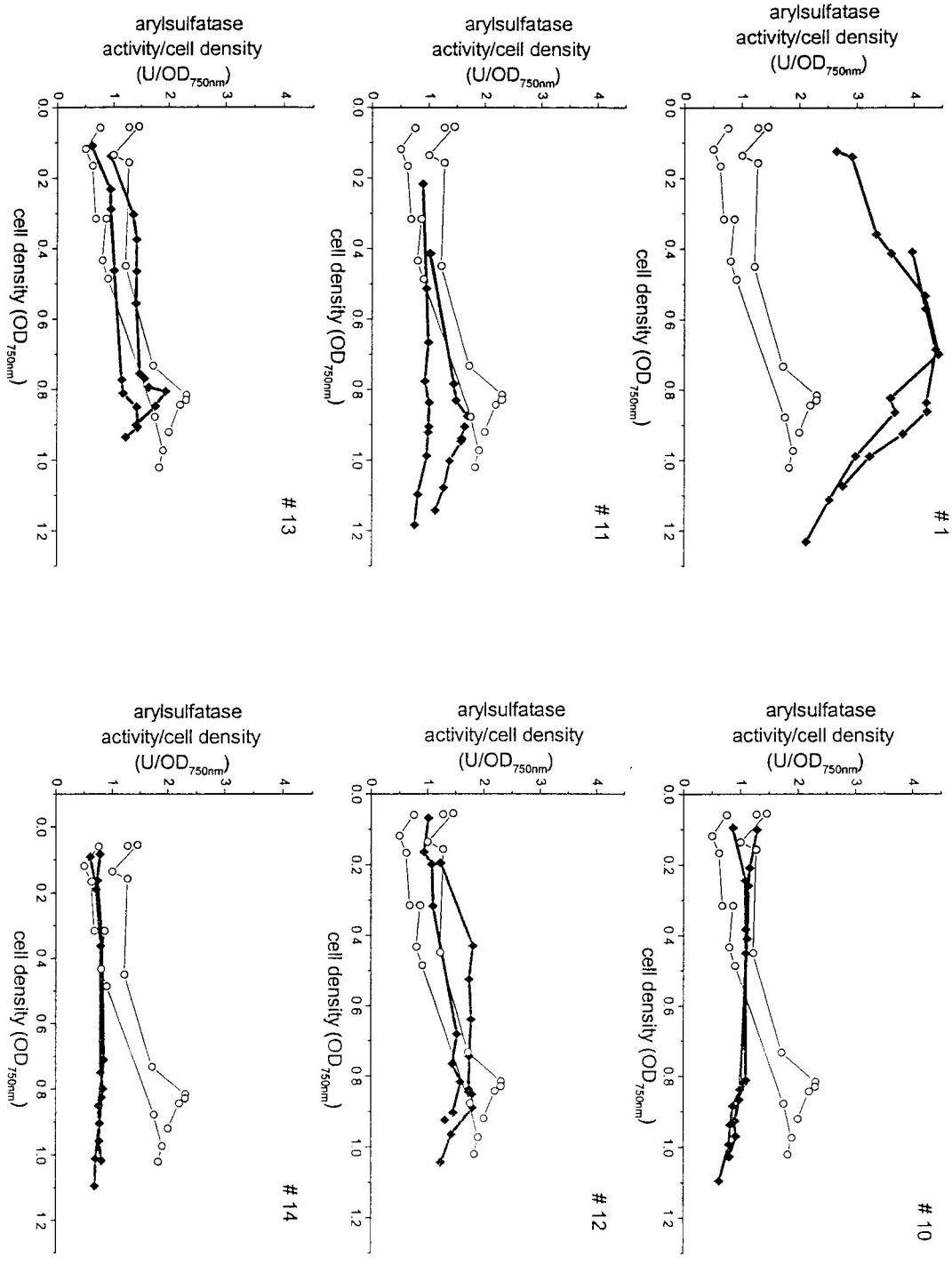
arylsulfatase activity/cell density (U/OD750nm)
cell density (OD750nm)
1
11
13
10
12
14

Figure 3-1

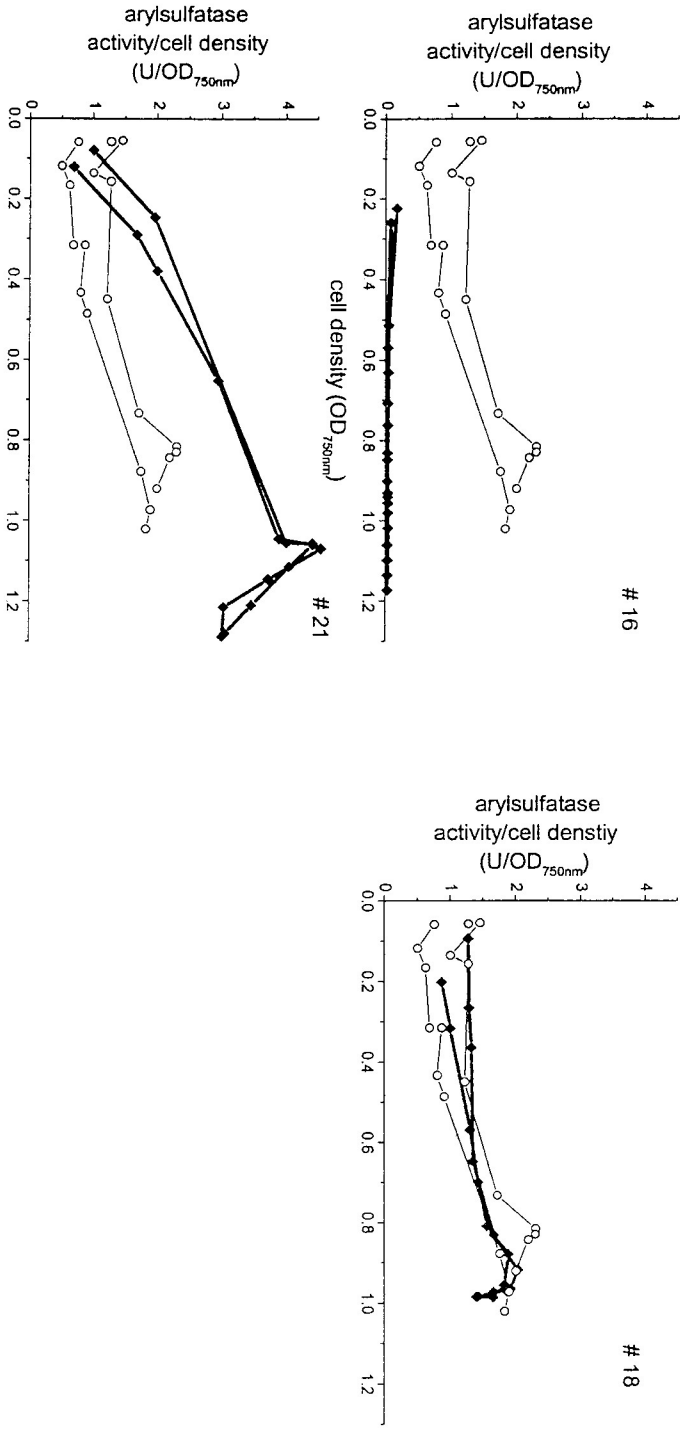
arylsulfatase
activity/cell density
(U/OD750nm)
cell density (OD750nm)
16
21
18

Figure 3-2

Table 3: Comparison of single-point and multiple-point screen: strains with no significant difference to nonmutagenized Carnil in the single-point screen. The previous single-point screen involved a single measurement of reporter activity at a single time point. The reporter arylsulfatase activity in the culture medium is expressed as a percentage to that of nonmutagenized Carnil at the same culture density. In the multi-point screen, several time points were taken and the highest arylsulfatase activity value which was standardized to the cell density at 750 nm is expressed as a percentage of the respective value for a Carnil control culture. The data for the multi-point screen represent the average of two independent experiments. For some single-point screen data (≥) the true value is probably higher, since measurements were taken at absorbances greater than 2.0 and therefore beyond the linear range of the spectrophotometer.

Mutant	Single-point screen	Multi-point screen	Remarks
#4	61.5%	83%	The ratio of enzyme activity to culture density remains constant.
#5	60.7%	100%	Culture grows to only low density
#6	64.3%	33%	The ratio of enzyme activity to culture density remains constant.
#7	63.3%	151%	
#9	70.9%	0 %	No arylsulfatase enzyme activity.
#24	≥119%	40.7%	Cannot grow in autotrophic medium.
#15	57.0%	71%	
#26	≥124%	56.6%	
#25	≥120%	74.6%	
#23	≥120%	55.7%	
#22	≥124%	73.7%	
#28	≥125%	72.4%	The ratio of enzyme activity to culture density remains constant.
#19	≥120%	74%	
#29	≥118%	92.4%	Culture grows to only low density
#27	≥118%	63.3%	The ratio of enzyme activity to culture density remains constant.
#20	≥138%	56%	

significantly lower enzyme activity in the multiple-point screen, and one (#21) still showed significantly higher enzyme activity. Surprisingly, mutant #1 which exhibited significantly lower enzyme activity in the single-point screen (46.6%) showed significantly higher enzyme activity in the multiple-point screen (195%). Compared with the multiple-point screen, about 44% of the isolated mutants in the single-point screen could not be verified.

Based on results of the multiple-point screen, 8 potential mutants were selected for further screening at their mRNA abundance level. Among them were mutants #9 and #16 which showed no arylsulfatase activity in the multiple-point screen, #10, #14 and #6 which showed significantly lower enzyme activity, and #1 which showed significantly higher enzyme activity. Strains #4 and #23 were also selected because their arylsulfatase activity showed an aberrant pattern compared to Carnil although their highest arylsulfatase activity value did not significantly differ from Carnil. Instead of increasing gradually during the logarithmic phase, the arylsulfatase activity in mutant #4 was maintained at a constant level. The enzyme activity of mutant #23 was still increasing even after the culture reached its stationary phase.

Mutant #21 which showed significantly higher enzyme activity could not be analyzed because it grew too slow under the conditions used for RNA sampling. Further experiments need to be performed under appropriate conditions to obtain mRNA data and to elucidate the reason for the growth difficulty. Mutant #24 was not analyzed at its mRNA level because it cannot grow under autotrophic conditions which were the conditions used for RNA sampling. Mutant #7 and #11 which showed 151% and 50% arylsulfatase activity, respectively, were not selected in this study. However, they are still interesting and further characterizations of these mutants are needed.

Table 4. Comparison of single-point screen and multiple-point screen: strains with significant difference to nonmutagenized Carnil in the single-point screen. For explanation, see legend to Table 3. The data for the multiple-point screen represent the average of two independent experiments.

Mutant	Single-point screen	Multiple-point screen	Remarks
#1	46.6%	195%	Highest enzyme activity appears during mid-logarithmic phase.
#10	13.1%	46%	The ratio of enzyme activity to culture density remains constant.
#11	13.0%	50%	The ratio of enzyme activity to culture density remains constant.
#12	24.8%	74.5%	
#13	35.6%	75%	
#14	36.3%	37%	The ratio of enzyme activity to culture density remains constant.
#16	9.5%	0 %	No arylsulfatase enzyme activity
#18	24.3%	86.7%	
#21	≥148%	198%	

Preparation of Templates for Probe Synthesis

To prepare templates for probe synthesis, the *CABII-1* and *ARS2* fragments were isolated from plasmid pHS16 and pJD27, respectively. Both plasmids were isolated from the *E. coli* strain XL1 blue. The ~300 bp *CABII-1* cDNA fragment was released from pHS16 by digestion with *PstI* restriction enzyme and separated from the vector by 1.5% agarose gel electrophoresis (Figure 4). The ~300 bp fragment was isolated from the agarose gel and dissolved in 30 µl buffer. Four µl of this extract were subjected to electrophoresis on a 1.5% agarose gel in order to judge its purity and determine the concentration. As shown in Figure 5, the fragment was pure, since no other nucleic acid fragment was visible in the gel. The concentration of the isolated *CABII-1* fragment was estimated at about 10 ng/µl and the yield at about 38%. Plasmid pJD27 was double digested with *HindIII* and *SacI* to release the *ARS2* fragment from the vector. After separation by electrophoresis on a 0.8% agarose gel (Figure 6), the ~2000 bp *ARS2* fragment was purified from the gel using the same method as for the *CABII-1* fragment. Two µl of the 30 µl extracted solution were subjected to electrophoresis on a 1% agarose gel. As shown in Figure 7, the *ARS2* fragment was pure and the concentration was estimated at about 15 ng/µl. The yield was about 45%.

Northern Blot Analysis of *CABII-1* and *ARS2* mRNA Abundance

Figures 8 to 10 show that Carni1 and all potential mutants examined synthesize mRNAs that hybridize with probes for *CABII-1* and *ARS2*. Therefore, although no arylsulfatase activity was detectable in the culture supernatant of mutants #9 and #16, both retain their ability to synthesize *ARS2* transcript. The results suggest that the defect

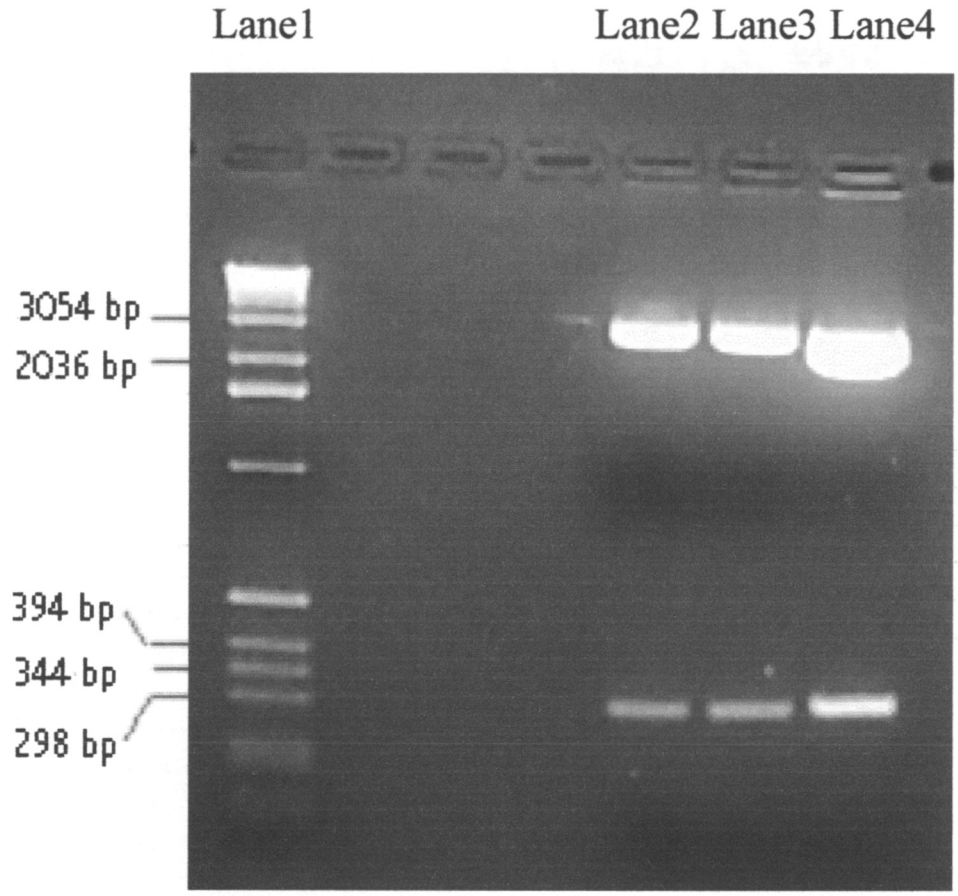

Figure 4. Restriction digest of purified plasmid pHS16 containing a *CABII-1* cDNA fragment. The plasmid pHS16 was digested with *PstI* to release the ~300 bp *CABII-1* fragment from the ~3000 bp vector. The fragments were separated by electrophoresis on a 1.5% agarose gel. Lane 1: 1 μg of 1 kb molecular weight ladder. Lanes 2, 3 and 4: restriction digest products of 2.5 μg, 2.5 μg, and 5 μg plasmid, respectively.

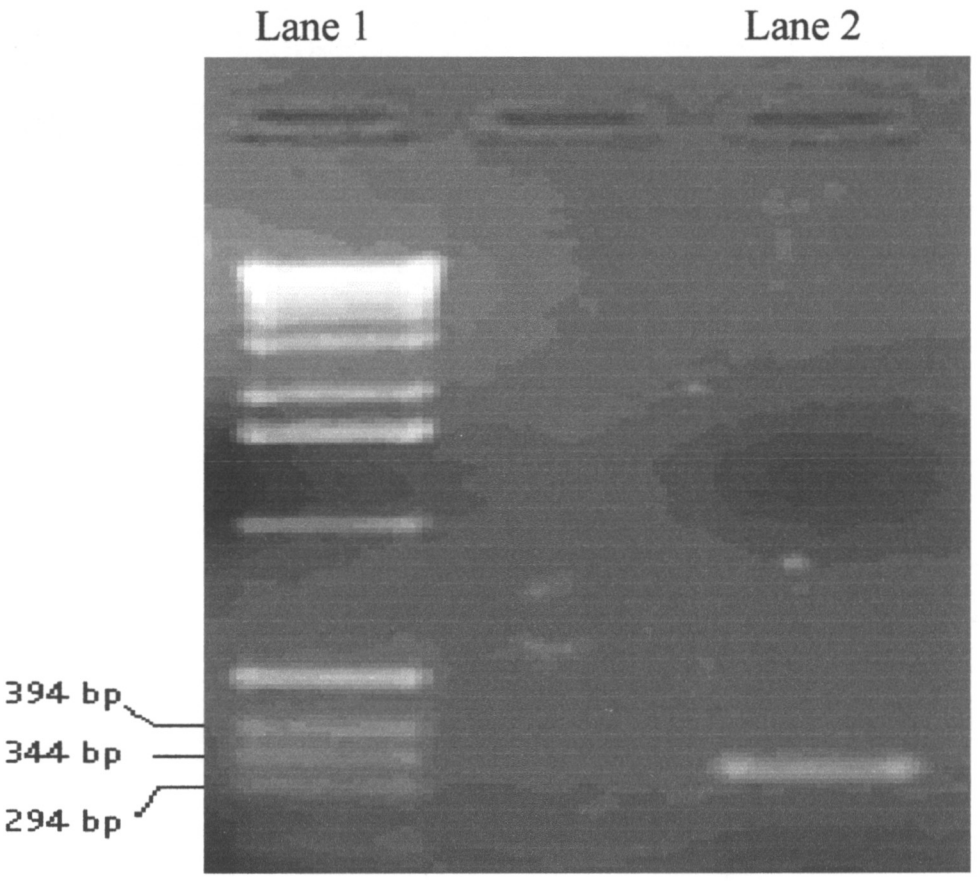

Figure 5: Purified ~300 bp *CABII-1* cDNA fragment. Four μL of the isolated *CABII-1* cDNA solution was subjected to electrophoresis on a 1.5% agarose gel. Lane 1: 1 μg of 1 kb molecular weight ladder. Lane 2: 4 μL (~40 ng) extracted *CABII-1* fragment.

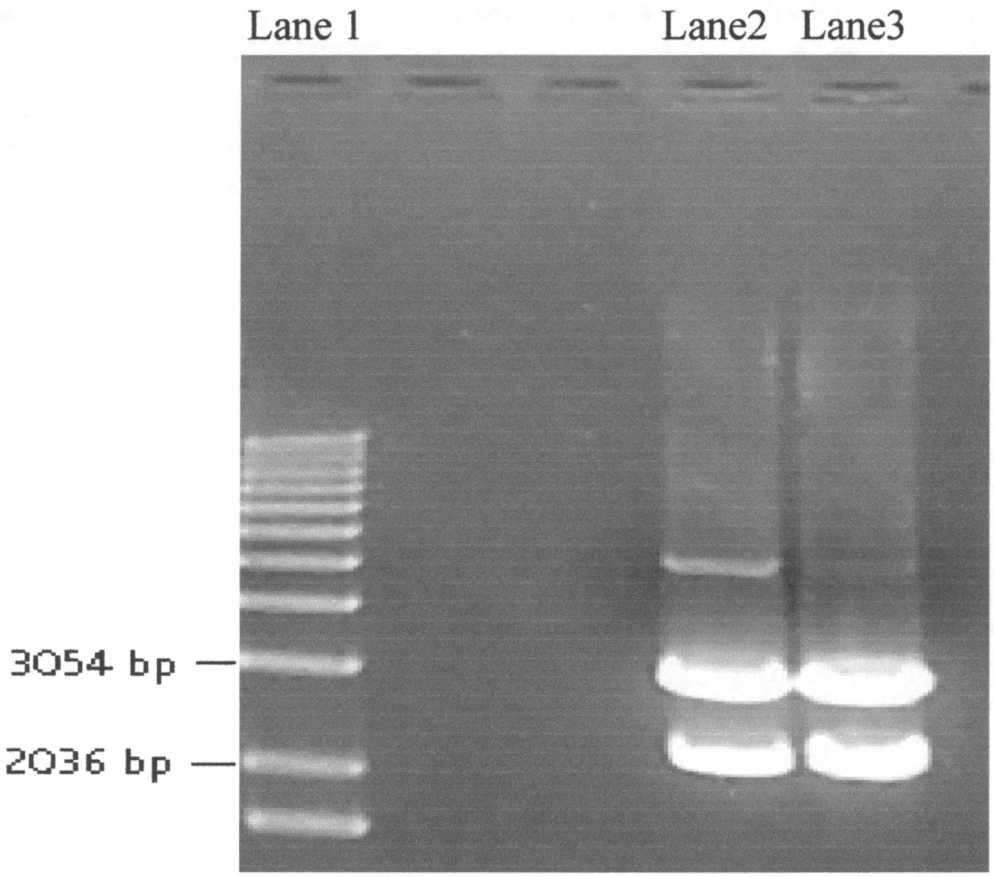

Figure 6. Restriction digest of purified plasmid pJD27 containing a *ARS2* cDNA fragment. The plasmid pJD27 was double digested with *HindIII* and *SacI* to release the ~2000 bp *ARS2* fragment from the ~3000 bp vector. The fragments were separated by electrophoresis on a 0.8% agarose gel. Lane 1: 1 μg of 1 kb molecular weight ladder. Lanes 2 and 3 : restriction digest products of 5 μg plasmid each.

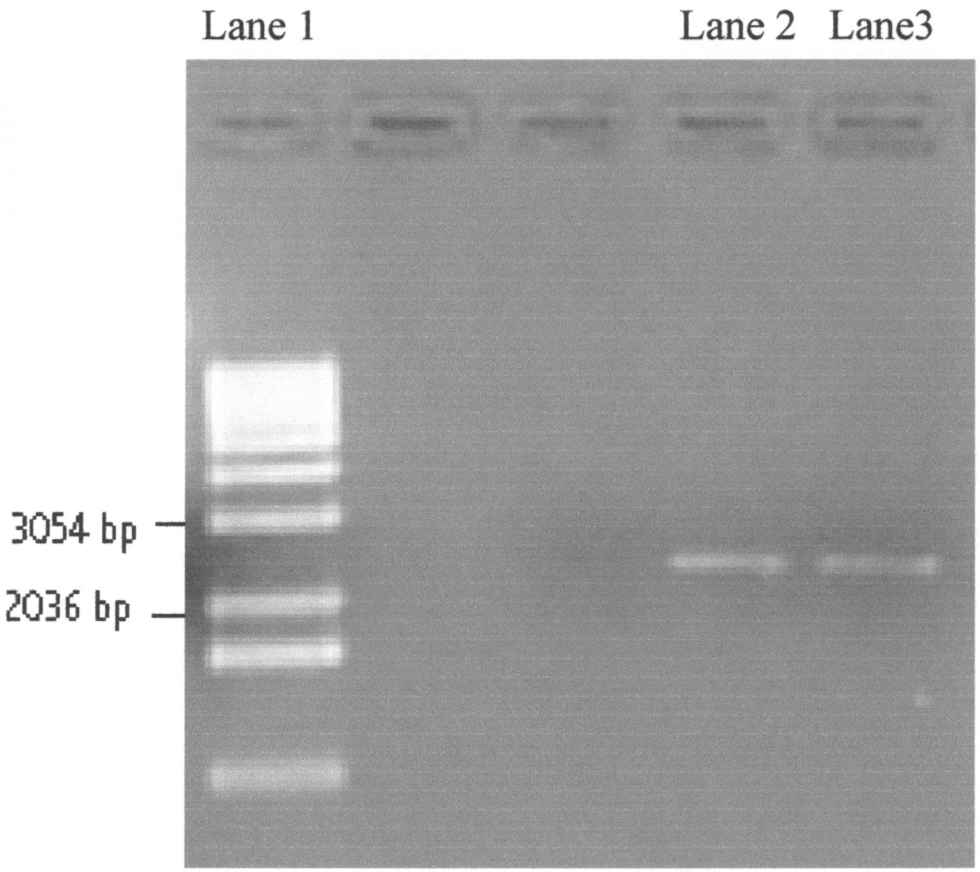

Figure 7: Purified ~2000 bp *ARS2* cDNA fragment. Two μL of the isolated *ARS2* cDNA solution was subjected to electrophoresis on a 1% agrose gel. Lane 1: 1 μg of 1 kb molecular weight ladder. Lanes 2 and 3: 2 μL (~30 ng) extracted *ARS2* fragment each.

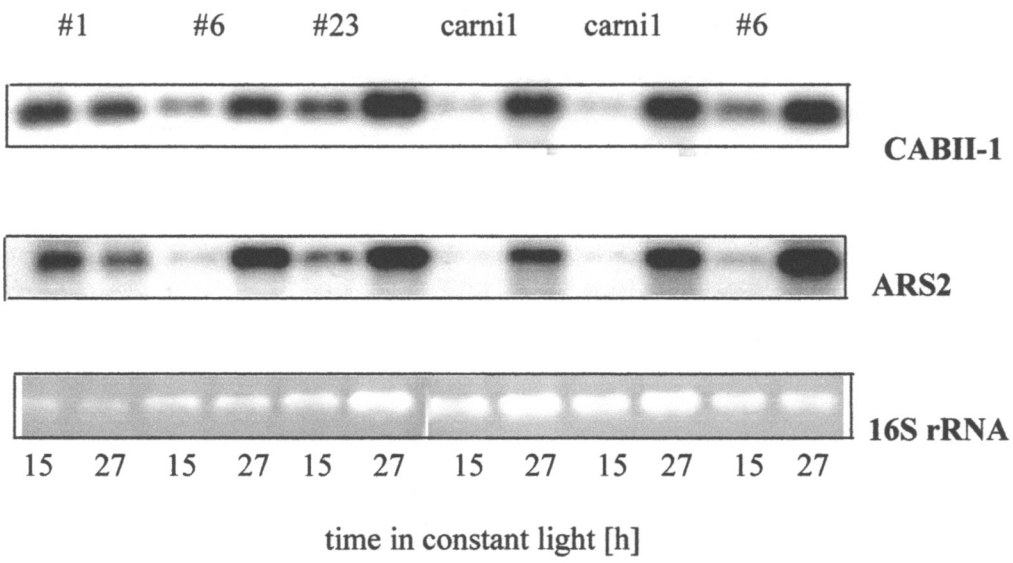

Figure 8: Northern blot analysis of potential mutants and nonmutagenized Carni1 under constant dim light. The expected trough (15h) and peak (27h) time point for the circadian rhythm in *CABII-1* and *ARS2* mRNA abundance was tested for the strains indicated above. The upper panel shows the single autoradiograph obtained when hybridizing with a probe for the *CABII-1* mRNA and the middle panel the single autoradiograph obtained with the reporter probe. The lower panel shows the 16S rRNA portion of the ethidium bromide-stained gel as an indicator for the amount of total RNA loaded in each lane.

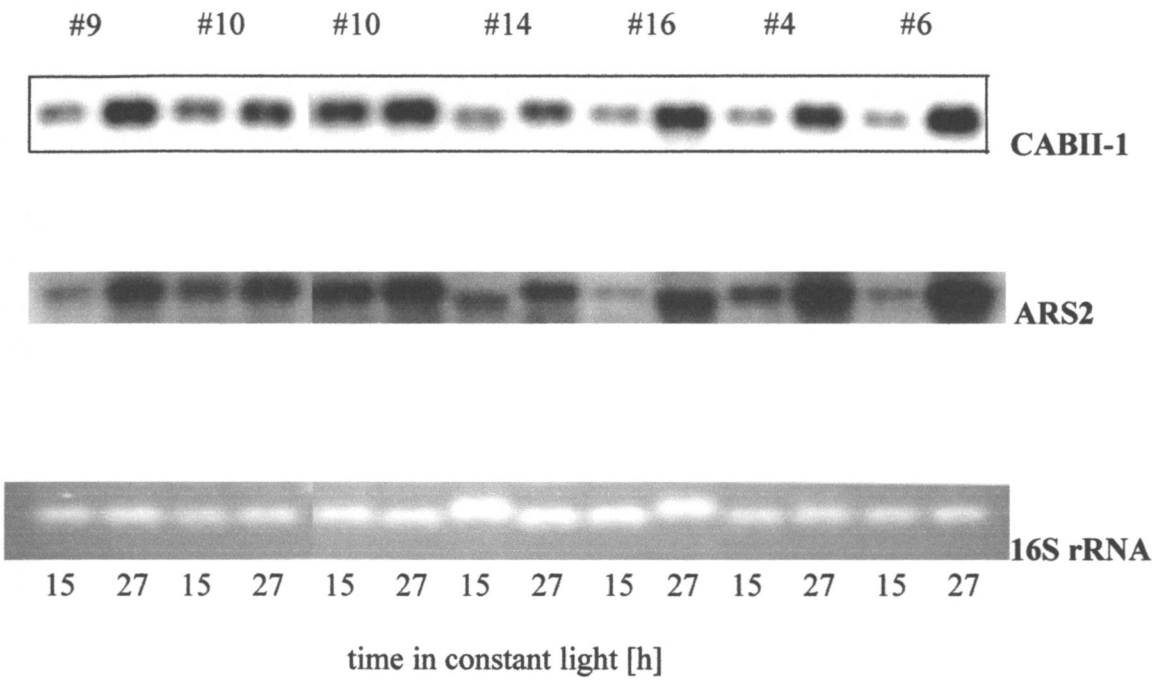

Figure 9: Northern blot analysis of more potential mutants under constant dim light. For explanation see legend to Figure 8. The upper and middle panel each represents a single autoradiograph.

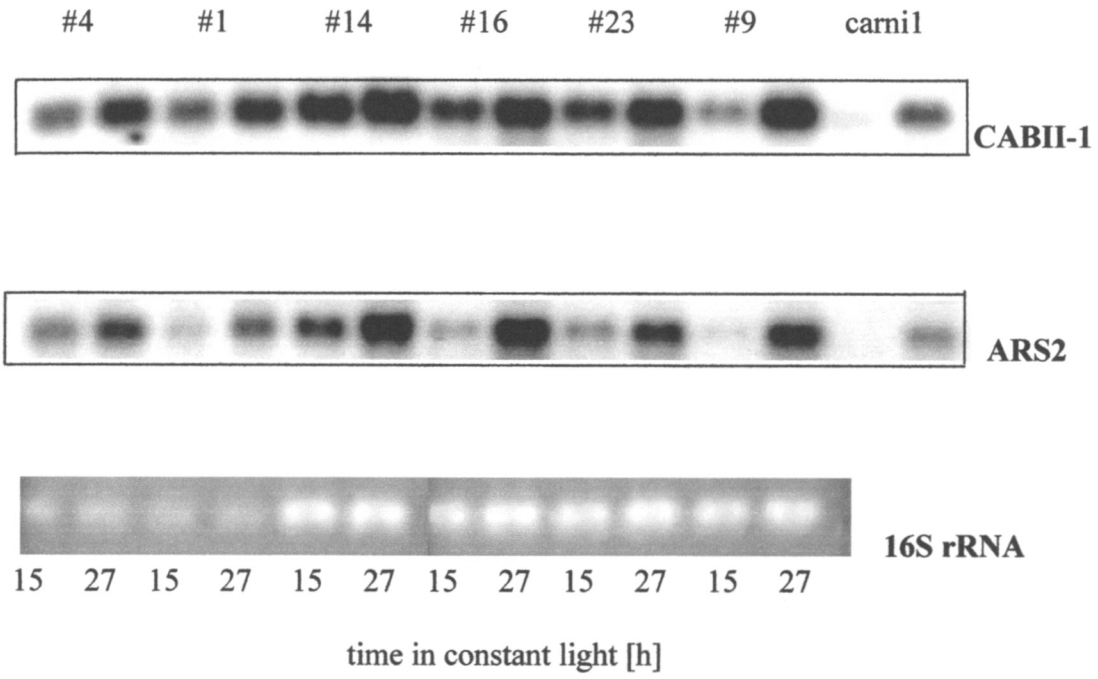

Figure 10: Northern blot analysis of more potential mutants and nonmutagenized Carni1 under constant dim light. For explanation see legend to Figure 8. The upper and middle panel each represents a single autoradiograph.

in *ARS2* expression in these mutants occurs at the posttranscriptional level rather than the transcriptional level and therefore should not affect *CABII-1* expression. In Carni1, the *CABII-1* and *ARS2* transcripts both show low amounts at 15 h in constant dim light and high amounts at 27 h as expected. The difference is due to the circadian rhythm in *CABII-1* promoter activity which drives both genes in Carni1 (Jacobshagen et al. 1996). The ethidium bromide stained 16S rRNA in Figures 8 to 10 makes it possible to assess whether all samples were loaded at equal amounts.

CABII-1 and *ARS2* mRNA abundances in Carni1 and the potential mutants were quantified as shown in Figures 11, 12 and 13. Expression of *ARS2* was in accordance with *CABII-1* expression in all strains examined. A strain whose peak-to-trough ratio of *CABII-1* and *ARS2* mRNA abundances was less than one third of the respective ratio for Carni1 was considered significantly different. As shown in Table 5, the peak-to-trough ratio for *CABII-1* of Carni1 was on average 5.11. A significant decrease would therefore represent a peak-to-trough ratio of less than 1.70. Results in Table 5 indicate that the peak-to-trough ratio of *CABII-1* for mutant #10, #14 and #23 shows a significant decrease from that of Carni1. The mRNA abundance in mutant # 1 is also aberrant. In one experiment, the peak-to-trough ratio was significantly reduced while in the other independent experiment the trough value was even larger than the peak value (Table 5). The expression of *CABII-1* in mutants #4, #6, #9, and #16 is not significantly different from Carni1 in accordance with their most likely defect at the posttranscriptional level of *ARS2* expression. However, the results for mutant #4 and #16 need to be confirmed, especially since the data for one experiment could not be normalized to the amount of 16S rRNA.

Surprisingly, no overt relationship between mRNA abundance and enzyme

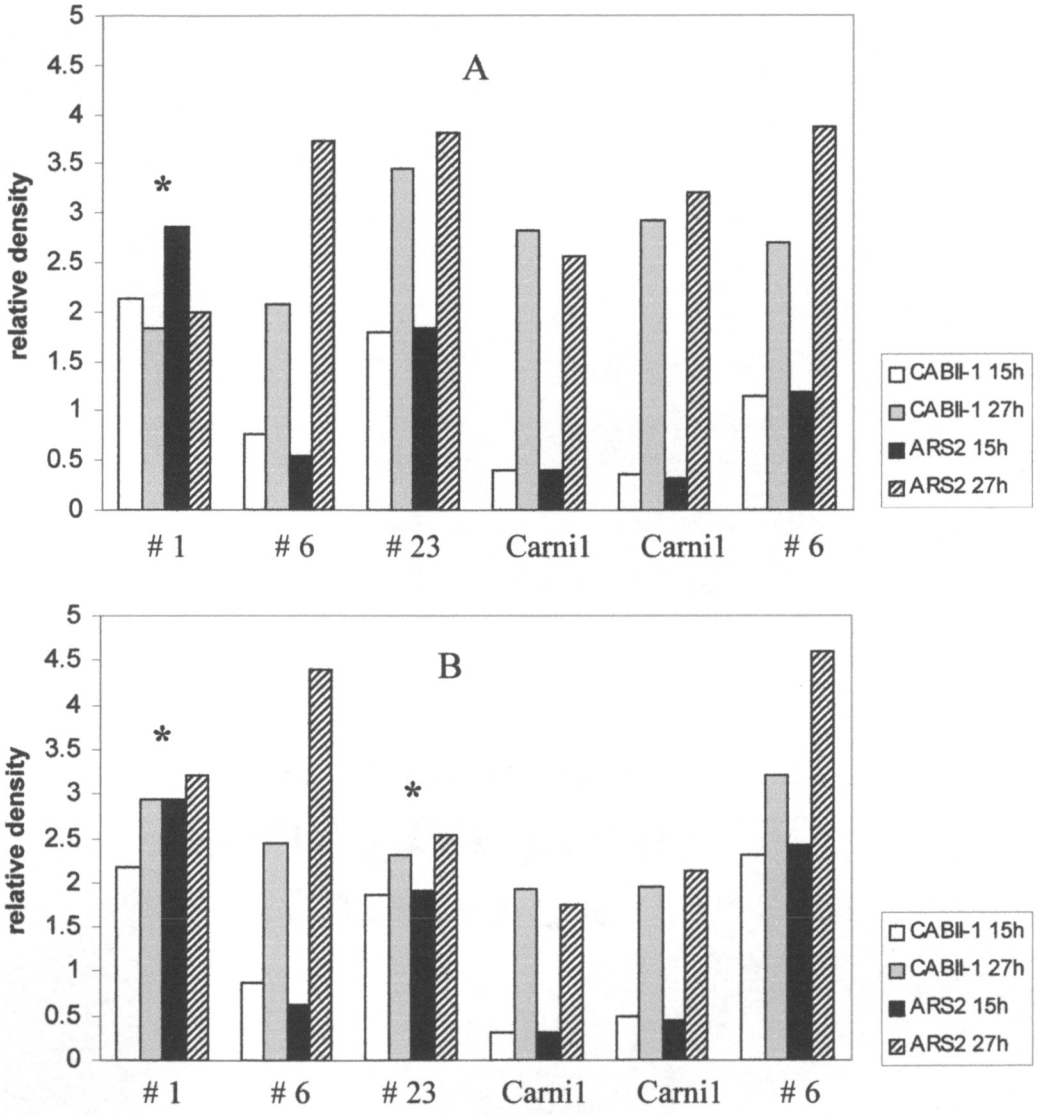

Figure 11: Quantitative analysis of RNA blots from potential mutants and nonmutagenized Carni1 depicted in Figure 8. A. Relative amount of *CABII-1* and *ARS2* mRNA at the expected trough (15 hour in constant dim light) and peak (27 hour in constant dim light) time point during their circadian rhythms. B. Same amount of *CABII-1* and *ARS2* mRNA as in "A" but normalized to the16S rRNA abundance. "*" indicates that the strain has aberrant *CABII-1* and *ARS2* mRNA amounts.

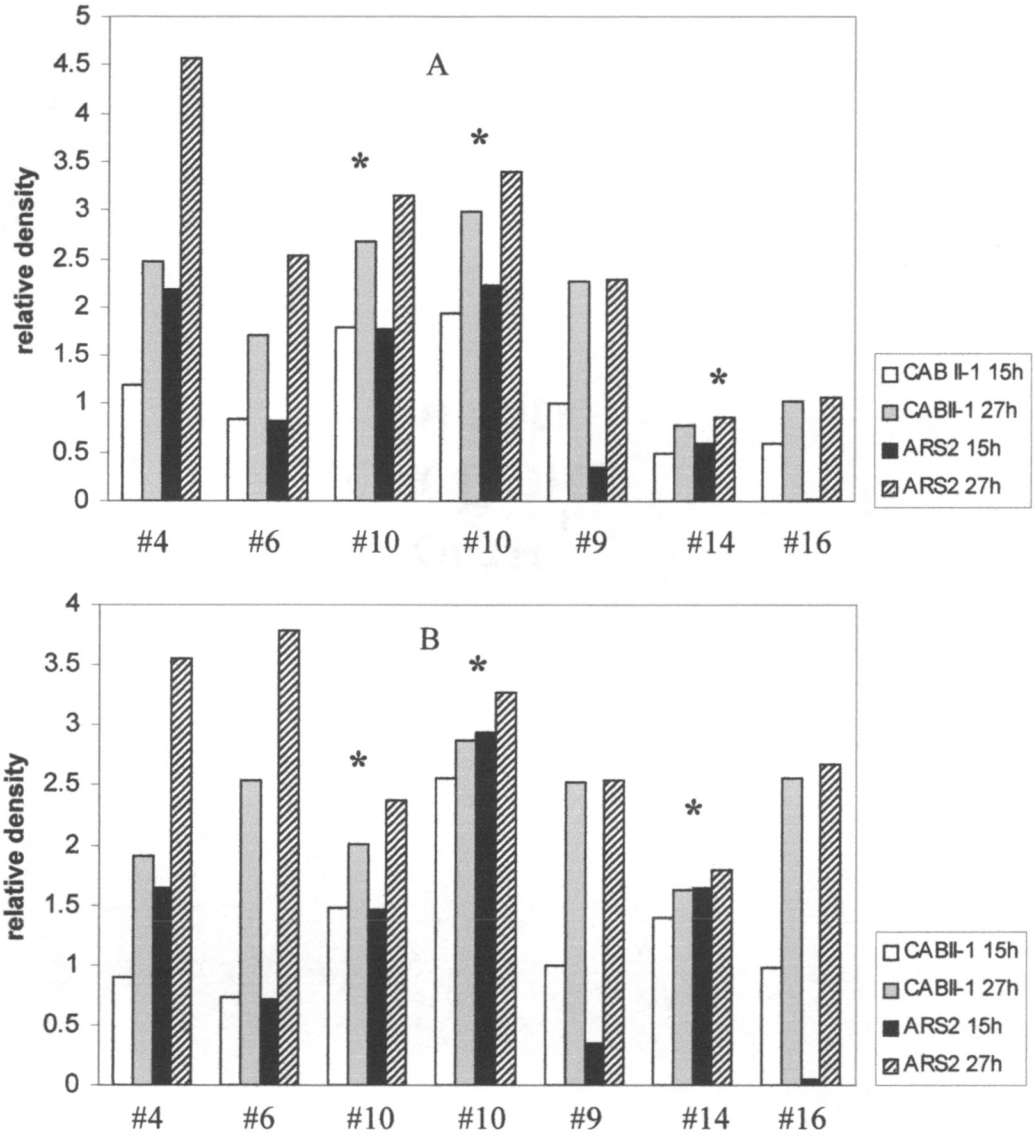

Figure 12: Quantitative analysis of RNA blots from potential mutants depicted in Figure 9. For explanation see legend to figure 11.

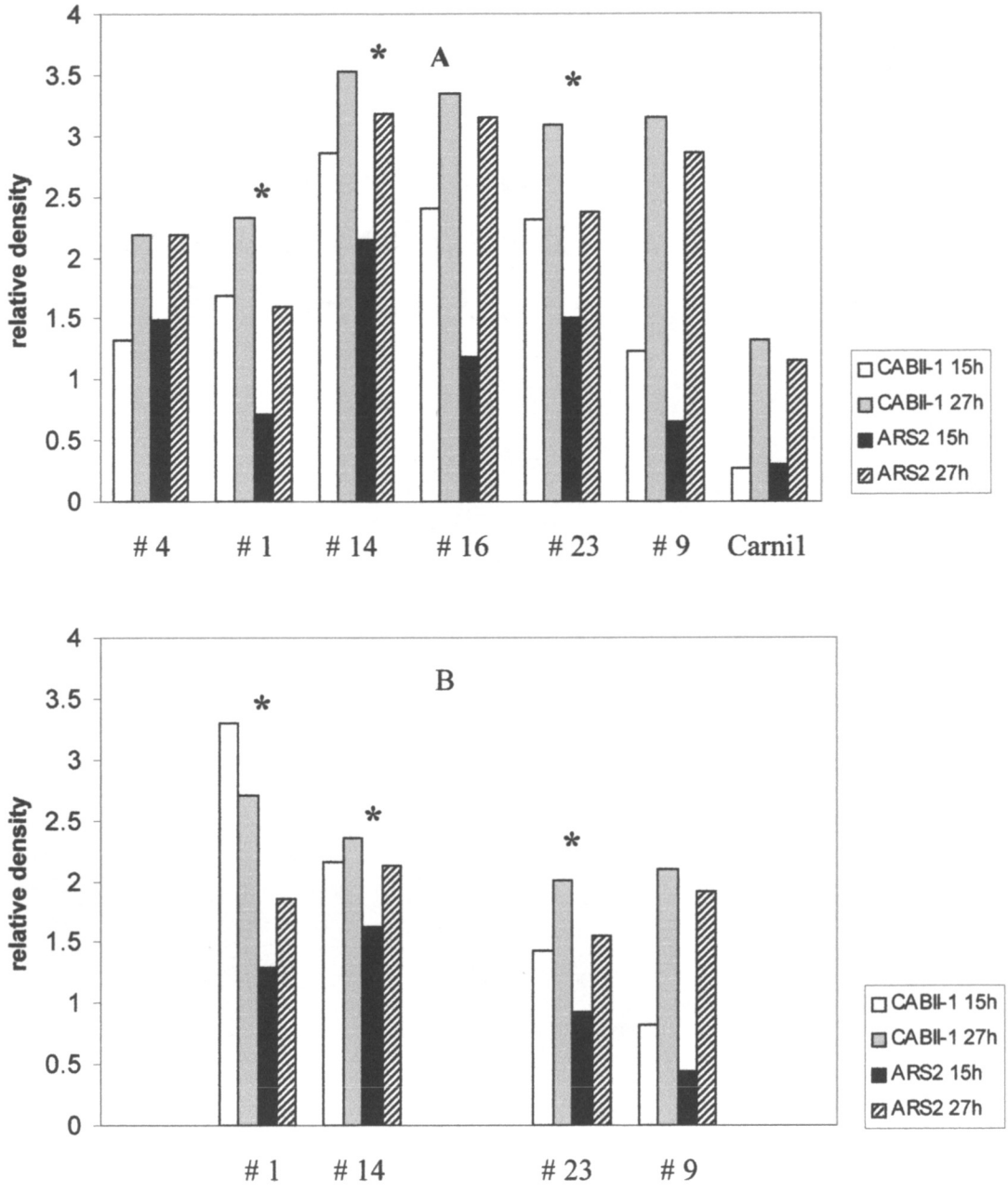

Figure 13: Quantitative analysis of RNA blots from potential mutants and nonmutagenized Carni1 depicted in Figure 10. For explanation see legend to figure 11. In mutants # 4, # 16 and Carni1, the 16S rRNA band in ethidium bromide-stained gel was damaged and could not be used to normalize mRNA abundances in these strains.

Table 5. Peak-to-trough ratios of *CABII-1* and *ARS2* mRNA abundances in Carni1 and the potential mutants. The peak value was defined as the amount of mRNA after 27 h in constant dim light and the trough value after 15 h in constant dim light. The ratios shown are derived from the amount of mRNA normalized to the 16s rRNA. * indicates not normalized data. "1" shows the results of the first experiment and "2" of the second.

CABII-1 Peak-to-trough ratio			ARS2 Peak-to-trough ratio		
Strain	1	2	Strain	1	2
Carni1	6.33	3.88	Carni1	5.72	4.80
#1	1.35	0.82	#1	1.10	1.43
#4	2.06	* 1.66	#4	2.10	* 1.47
#6	2.04	2.78	#6	3.10	7.01
#9	2.27	2.55	#9	6.61	4.33
#10	1.50	1.55	#10	1.79	1.53
#14	1.58	1.09	#14	1.47	1.31
#16	1.75	* 1.39	#16	37.07	* 2.64
#23	1.25	1.41	#23	1.34	1.66

activity was observed. Although mutant #10 and #14 showed significantly lower reporter enzyme activity, their *CABII-1* and *ARS2* mRNA abundances were consistently higher than the control (Figures 12 and 13). The mRNA abundance of mutant #1 which showed very high enzyme activity was maintained at a level similar to that of #10 and #14 (Figures 11 and 13).

Overall, the results indicate that mutants #1, #10, #14 and #23 are the most likely candidates for a defect in circadian *CABII-1* expression. The mRNA abundance pattern of mutant #21, #24, #7 and #11 still needs to be analyzed under appropriate conditions.

CHAPTER IV

DISCUSSION

Chlamydomonas reinhardtii Strain Carni1 As A Useful Model Organism to Study the Circadian Output Pathway

Most circadian oscillator components have been identified by genetic and biochemical analyses of mutants defective in rhythmic outputs. In *Drosophila*, *per* and *tim* were isolated and further characterized from mutants that showed long-period, short-period or arythmic locomotor activity (Young 1998). In *Neurospora* mutants defective in the conidiation rhythm, several loci were identified using forward genetic approaches. These loci include *frq*, *wc-2*, *chr*, and *prd-1* (Bell-Pedersen 2000). Similar studies were performed in rodents. For instance, *clock* was isolated from a mouse mutant with a long-period wheel-running activity (Antoch et al. 1997). And *CKIε* was identified through genetic analysis of the short-period hamster mutant called *tau* (Lowrey et al. 2000).

As circadian gene expression is probably closer to the central oscillator compared with complicated circadian behaviors, it should be easier to identify mechanisms of signal transduction in the output pathway through the study of mutants defective in rhythmic gene expression (Somers 1999). In an effort to identify intermediate factors in the circadian output pathway, this study focused on screening of *Chlamydomonas reinhardtii* mutants defective in circadian *CABII-1* expression. *CABII-1* is a member of the *CABII* gene family encoding the chlorophyll a,b-binding proteins of photosystem II.

In *Chlamydomonas* strain Carni1, a promoterless *ARS2* gene is fused downstream of the *CABII-1* promoter. In this transformant, *ARS2* mRNA abundance shows a similar period and amplitude as that of the endogenous *CABII-1* gene (Jacobshagen et al. 1996). The result indicates that the circadian clock in *Chlamydomonas* regulates the expression of *CABII-1* at the transcriptional level.

Reporter genes provide a convenient method for monitoring circadian rhythms of transcription. When a particular promoter is controlled by the circadian clock, a reporter gene inserted downstream of such a promoter should be expressed under clock control. Currently, the most ideal reporter is luciferase. Rhythmic control over the target promoter by the circadian clock in this case is simply reflected by the oscillations in intensity of bioluminescence with high resolution. Luciferase has been extensively used in the study of *ccgs* in cyanobacteria (Liu et al. 1995). It has also been successfully applied in *Arabidopsis* (Somers et al. 1998) and *Drosophila* (Stanewsky et al. 1998). Mutants in other aspects of the circadian clock were also isolated by using luciferase as a reporter. However, this gene and other heterologous genes are generally difficult to express in *Chlamydomonas* possibly due to codon mismatches. *ARS2* is a *Chlamydomonas* gene. It encodes the enzyme arylsulfatase which is synthesized in response to sulfur limitation (Lien and Schreiner 1975). Arylsulfatase is secreted into the medium where it is readily assayed using a chromogenic substrate. Davies and coworkers (1992) developed *ARS2* as a reporter under sulfur-sufficient condition. Under this condition, endogenous *ARS2* is not expressed even at the mRNA level. In the last few years, it has been successfully used as a reporter in *Chlamydomonas* and the evolutionary related alga *Volvox* (Kucho et al. 1999, Hallmann and Sumper 1994). However, arylsulfatase is not an ideal reporter for circadian studies. Although *ARS2* mRNA abundance in Carni1 exhibits the same

circadian rhythm as that of *CABII-1*, arylsulfatase activity released into the medium shows a monotonic increase due to its long half-life (Jacobshagen et al. 1996). Therefore, arylsulfatase was used in this study as a reporter of accumulative changes over several circadian cycles as the primary level of mutant screening.

Assessment of ARS2 As A Reporter At the Enzyme Activity Level in A Screen for Circadian Output Mutants

There are certainly advantages for using arylsulfatase as a reporter when screening for circadian output mutants. Although the input pathway was shown to affect the free-running period (Kay 1993), mutants in the input pathway that exhibit a shorter or longer free-running period will not show a significantly higher or lower accumulative arylsulfatase activity under constant conditions. Similarly, mutations in the oscillator were often reported to lead to a short-period, long-period or arythmic phenotype of circadian outputs. But a mutant with a longer or shorter period will not show a difference in accumulative transcriptional activity over several circadian cycles compared to wild-type. Therefore, mutations in the input pathway or the oscillator will not be picked up by the applied screen in most cases.

The kind of screening method used makes the selection of potential mutants from thousands of transformants possible. But screening at the arylsulfatase activity level also has several limits. As shown in Figure 14, there are a number of sites where mutations can occur within and outside the circadian timing system. Due to this fact in combination with the low time resolution of the arylsulfatase reporter, some of the isolated mutants will not necessarily be true circadian output mutants. 1) The *CABII-1* promoter may contain response elements regulated by signaling pathways other than the circadian clock

(site 6 in Figure 14). In *Arabidopsis*, both the circadian oscillator and a direct phototransduction pathway control the *CAB* promoter (Millar and Kay 1996). Light activation at the transcriptional level has also been reported for the *Chlamydomonas CABII-1* gene (Jasper et al. 1991). Therefore, a mutation in components of the phototransduction pathway may also lead to an aberrant accumulative arylsulfatase activity. 2) If a mutation occurs in the regulatory mechanism of the endogenous *ARS2* that leads to an activation of the gene under sulfur-sufficient conditions, accumulative arylsulfatase activity would be increased (site 7 in Figure 14). But since the *CABII-1* gene would not be affected by such a mutation, this kind of mutant will be excluded by analysis of *CABII-1* transcript abundance. 3) Mutations at the posttranscriptional level of *ARS2* expression may occur such as a defective excretion of arylsufatase to the medium. Or the promoter or coding region of the *ARS2* reporter might be affected by a mutation (site 5 in Figure 14) leading to abolishment of enzyme activity. However, these mutants can also be ruled out by subsequent mRNA level screening. 4) A mutation could occur in the oscillator such that an arhythmic phenotype with significantly higher or lower accumulative arylsulfatase activity arises (site 2 in Figure 14). This kind of mutation could be identified by further studies like the examination of other circadian outputs. 5) Some mutants truly defective in the output pathway could be missed (site 3 in Figure 14) like phase-shifting mutants which will show wild-type accumulative arylsulfatase activity.

Insertional Mutagenesis in Carni1

Insertional mutagenesis has been successfully used to generate mutants in a wide variety of organisms (Lee et al. 1995, Cummings et al. 1999, Ermilova et al. 2000, Prieto

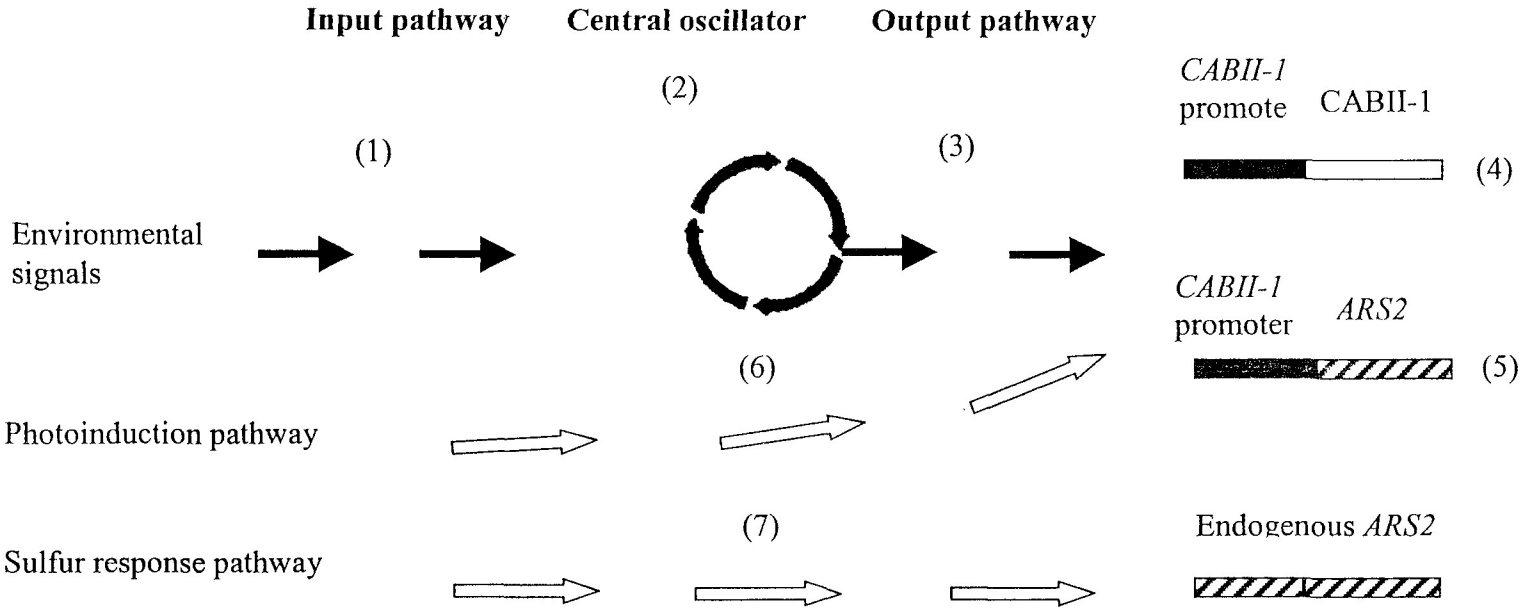

Figure 14: Possible mutations in Carni1 affecting *CABII-1* and *ARS2* expression. Site 1 indicates a possible mutation in the input pathway of the circadian timing system, site 2 in the circadian oscillator, and site 3 in the circadian output pathway. Sites 4 and 5 indicate possible mutations in the promoter or coding region of *CABII-1* and the *ARS2* reporter construct, respectively. Site 6 indicates a possible mutation in pathways that regulate *CABII-1* transcription other than the circadian clock such as a light induction pathway, and site 7 a possible mutation in the regulation of the endogenous *ARS2* gene.

et al. 1996, Amsterdam et al. 1999). Depending on the recipient organism and the specific biological phenomenon under investigation, the frequency of mutants that were recovered varies considerably. In *Coprinus cinereus*, a screen of REMI-generated transformants yielded sporulation-defective mutants at a frequency of 1.2% (Cummings et al. 1999). In *Chlamydomonas reinhardtii*, 5 out of 8630 arginine-independent transformants were defective in their chemotaxis towards various sugars (Ermilova et al. 2000) while 2 regulatory mutants for nitrate assimilation were obtained out of 8975 transformants in another experiment (Prieto et al. 1996).

In this study, Carni1 had been mutagenized via insertional mutagenesis with the *ble* marker (Yuan 1999). This kind of mutagenesis generally leads to a complete loss of function of the affected gene (Rochaix et al. 1998). It can be assumed that the circadian clock acts on *CABII-1* and on *ARS2* reporter expression through either circadian activation or repression. Disruption of components of the circadian transduction chain should therefore lead to either constant high or constant low transcription of both genes. As a consequence, the accumulative arylsulfatase activity in mutants would be significantly higher or lower compared with nonmutagenized Carni1. Since selection of prospective mutants is based on aberrant reporter enzyme activity, the stability of *ARS2* expression in nonmutagenized Carni1 is very important. In our experiments under optimized conditions, arylsulfatase activity of nonmutagenized Carni1 and its three single-cell isolates showed a stability of *ARS2* expression that is acceptable ($p = 0.87$, SAS version 8). Therefore, variations of arylsulfatase activity in mutagenized Carni1 can reasonably be assumed to be due to a genetic defect.

Comparison of Single-Point Screen with Multiple-Point Screen

Of the 16 strains whose arylsulfatase activity did not differ significantly from Carni1 in the single-point screen (Table 3), 12 also showed no significant difference using the multiple-point screen. The reliability of the single-point screen in identifying wild-type expression level is therefore about 75% in this particular study. However, since the selection of strains was not random but in favor of those with activities just below the level that would make them significantly different, the reliability of the single-point screen is most likely higher. The four strains that showed aberrant enzyme activity are: #6, #7, #9, and #24. Mutant #9 showed no detectable enzyme activity in the multiple-point screen but 70.9% in the single-point screen. Mutant #6 showed a significantly lower arylsulfatase activity of 33% in the multi-point screen compared with 64.3% in the single-point screen. Mutant #7 showed significantly higher arylsulfatase activity (151% in the multiple-point screen as compared to 63.3% in the single-point screen) and mutant #24 showed significantly lower arylsulfatase activity (40.7% in the multiple-point screen as compared to ≥119% in the single-point screen). Interestingly, mutant #24 was unable to grow under photoautotrophic conditions. The different results between the two screening methods are either due to the single-point screen being more unreliable or a genetic change occurred in the strain after it had been characterized by the single-point screen.

Of the 14 potential mutants that were significantly different from Carni1 in the single-point screen, 9 were only available for further studies. Of these 9 mutants, 3 showed no significant difference to nonmutagenized Carni1 in the multiple-point screen (#12, #13, #18 in Table 4) and one the opposite mutant phenotype (#1 in Table 4. Notice the significantly lower activity in the single-point screen compared to the significantly

higher activity in the multiple-point screen.). The selection error in favor of mutants that could not be verified is therefore about 44% in the single-point screen.

Overall, the single-point screen compared to the multiple-point screen is a reasonably reliable and certainly rapid way to screen for potential mutants as a first step. The multiple-point screen is still necessary to verify mutant phenotypes and thereby reduce the number of potential mutants that need to be screened at the mRNA level.

Analysis of *CABII-1* and *ARS2* mRNA Abundance

Although no enzyme activity was detectable in mutants #9 and #16 in the multiple-point screen, *ARS2* mRNA abundance in these two strains was similar to nonmutagenized Carni1 in northern blot analyses. The most likely explanation for this result is that the mutation in #9 and #16 occurred at the posttranscriptional level of *ARS2* reporter expression. The mutants are therefore not useful for circadian studies but could be useful for studies of the sulfur-response system in *Chlamydomonas* (Grossman 2000).

The *ARS2* and *CABII-1* mRNA abundance of strain #4 was also measured. Although highest arylsufatase activity of this strain is not significantly aberrant compared with Carni1, its enzyme activity pattern differs considerably. However, the mRNA abundance rhythms are similar to Carni1. Mutant #6 which exhibits significantly decreased arylsulfatase activity also shows a similar transcript abundance pattern as Carni1. Aberrant activity patterns in these strains should therefore be caused by a mutation that acts at the posttranscriptional level of *ARS2* reporter expression.

For mutants #10 and #14 which showed significantly reduced reporter enzyme activity, northern blot analysis revealed that their mRNA abundance pattern taken at two time points is also aberrant. The peak-to-trough ratio for the amount of *CABII-1* transcript

was 1.53 for mutant #10 and 1.34 for mutant #14 compared to 5.11 for nonmutagenized Carni1. Mutant #23 which has 55.7% reporter enzyme activity and a somewhat different overall pattern also showed a decreased peak-to-trough ratio (peak/trough = 1.33). It indicates that #10, #14 and #23 show either a greatly reduced amplitude or an arhythmic, erratic expression pattern. An unexpected phenotype is shown by mutant #1 since it exhibited significantly lower enzyme activity in the single-point screen (46.6%) but very high enzyme activity in the multiple-point screen (195%). Results from the mRNA level screen revealed that the mRNA abundance pattern in this strain is aberrant. The *CABII-1* mRNA abundance peak value is less than the trough value (peak/trough = 0.82) in one experiment and slightly larger than the trough value (peak/trough = 1.35) in the other experiment. But although the two experiments differ somewhat in their results, both are similar in revealing a considerably reduced peak-to-trough ratio. One possibility is that the expression of *CABII-1* is constant in this mutant and the difference between the two experiments is due to experimental variability. Further research is needed to better assess this mutant.

We also noticed that *ARS2* and *CABII-1* transcript abundances show a similar pattern in all strains investigated, i.e., when the amount of *ARS2* mRNA is aberrant, the amount of *CABII-1* mRNA is aberrant in the same way. It indicates that none of the mutations affect *CABII-1* or the *ARS2* reporter by interfering directly with the coding region or their *cis*-acting elements. It further indicates that the endogenous *ARS2* gene was not affected either. The results also show that the expression of *CABII-1* and the *ARS2* reporter gene is highly repeatable between the two independent experiments in most strains investigated. It suggests that also at the transcriptional level *ARS2* shows a high enough stability to make it a valuable reporter.

In summary, this study has shown that mutants #1, #10, and #14 and #23 are the most promising for harboring a defect in the circadian output pathway, because they exhibit aberrant reporter expression at both the enzyme and mRNA level with concurrent expression of the *CABII-1* gene. In the future, the precise circadian pattern of *CABII-1* mRNA abundance in these mutants needs to be analyzed by high-resolution sampling. Although the most promising mutant phenotype might be considered to show a completely abolished rhythm of *CABII-1* and *ARS2* expression, residual rhythmicity will necessarily occur if *CABII-1* expression is under hierarchical circadian regulation. Mutants #1, #10, #14 and #23 are therefore very interesting, even though they might still show some low amplitude rhythm in *CABII-1* expression.

Conclusion

Of the 8 strains with aberrant arylsulfatase activity in the more rigorous multiple-point screen that were analyzed further in this study, 4 exhibited aberrant mRNA patterns as well. The selection efficiency at the laborious *CABII-1* mRNA level was therefore greatly increased with the use of the prescreen. The major conclusion of this work is that *ARS2* at the level of arylsulfatase activity is a valuable reporter especially since reporter systems with high time-resolution are so difficult to use in *Chlamydomonas reinhardtii.*

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