Circadian Clock Mutants of Cyanobacteria

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A diverse set of circadian clock mutants was isolated in a cyanobacterial strain that carries a bacterial luciferase reporter gene attached to a clock-controlled promoter. Among 150,000 clones of chemically mutagenized bioluminescent cells, 12 mutants were isolated that exhibit a broad spectrum of periods (between 16 and 60 hours), and 5 mutants were found that show a variety of unusual patterns, including arrhythmia. These mutations appear to be clock-specific. Moreover, it was demonstrated that in this cyanobacterium it is possible to clone mutant genes by complementation, which provides a means to genetically dissect the circadian mechanism.

Circadian rhythms are a fundamental adaptation of living cells to the Earth's daily fluctuation in light and temperature (*1*). The isolation of clock mutants and the molecular genetic analysis of clock-related genes is a powerful approach toward the elucidation of the molecular basis of the circadian mechanism (*2*, *3*). Because the circadian clockwork is undoubtedly constructed of multiple components, the function of many genes will have to be analyzed to understand the operation of the clock. In most species, however, it has not been practical to assay the circadian rhythms of thousands of clones to screen for abnormal phenotypes. Therefore, in spite of extensive efforts, mutants with aberrant periodicity have so far been reported only for *Drosophila*, *Neurospora*, *Chlamydomonas*, mouse, and hamster (*4*), and only two of the genes found (*Drosophila per* and *Neurospora frq*) have been cloned and sequenced (*2*, *3*).

We circumvented the shortcomings of the available systems for the genetic analysis of circadian systems by engineering a genetically tractable prokaryote to carry a reporter gene whose expression can be automatically monitored from many clones simultaneously (*5*). We introduced a bacterial luciferase gene set (*luxAB*) that is controlled by a photosystem II gene promoter (*psbAI*) into the cyanobacterium *Synechococcus* sp. strain PCC 7942. Single colonies of this transgenic strain, AMC149, are bioluminescent with a precise circadian rhythm on agar plates (*6*). In this report, AMC149 will be referred to as wild type. We have used the ability to screen myriad clones on agar plates to identify a large ensemble of circadian mutants.

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To detect mutations that affect the circadian clock of cyanobacteria, we developed an automated monitoring system that can trace the circadian rhythm of each of more than 6000 independent clones concurrently (*7*). Bioluminescence images of an agar plate containing more than 500 colonies of the wild-type strain (AMC149) are shown in Fig. 1. At the peak of the rhythm (Fig. 1A), the intensity of the signals of pixels that represents each cyanobacterial colony was 100 to 300, whereas the background pixel intensity was 3 to 10. At the trough of the rhythm, the intensity of colony pixels decreased to 10 to 20% of the intensity at the peaks (Fig. 1B). Most of the colonies were recognized by the software and defined as areas (represented by ovals) from which bioluminescence data were collected (Fig. 1C).

We mutagenized AMC149 cells with ethyl methanesulfonate (EMS) and subcultured the surviving cells repeatedly in liquid medium for 20 to 60 generations (*8*). Because *Synechococcus* sp. cells are known to contain multiple copies of a single chromosome (*9*), we allowed many cycles of cell division to segregate any mutations that might be recessive. In total, we screened 150,000 clones and initially isolated about 100 clones that displayed abnormal rhythmicity. We rescreened these unusual clones several times to identify consistently abnormal phenotypes. The final mutant phenotypes comprised 12 clones with altered period and five clones with disrupted rhythmicity, which are listed in Table 1. Because screening revealed only these 17 phenotypes, and at least two clones were obtained with each phenotype, we estimate that we have obtained most of the possible EMS-elicited mutants detectable with our present screening conditions.

The rhythms of the period mutants and wild type under the standard assay conditions (*7*) are shown (Fig. 2). The range of periods expressed by the mutants (16 to 60 hours) is larger than that of the clock mutants so far obtained for any eukaryotic species (*4*). The most rapidly cycling mutant (SP16) had a period of 16 hours, whereas the slowest mutant (LP60) had a period of 60 hours. The large deviation of the periods of these two mutants from the wild-type period of 24 hours suggests that the reaction or reactions crucial for the circadian oscillator have been modified by these mutations. The rhythms of SP16 and LP60 tended to damp in continuous light conditions (LL), whereas mutants whose periods were between 20 and 40 hours gen-

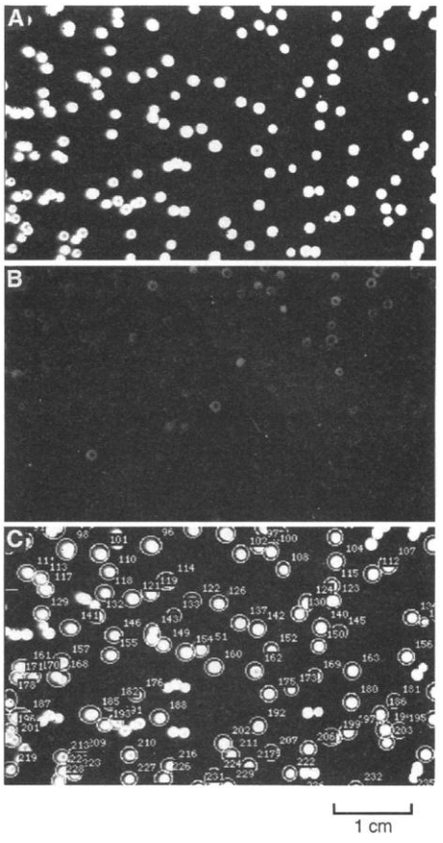

Fig. 1. Bioluminescence images of an agar plate of AMC149 colonies. AMC149 was cultured in BG-11 medium in the presence of spectinomycin sulfate (40 μg/ml). Five hundred to 1000 cells were inoculated onto agar plates [1.5% agar, 30 ml of BG-11 medium supplemented with 1 mM sodium thiosulfate (*16*) and spectinomycin (40 μg/ml)]. Plates were incubated in LL from white fluorescent lamps (46 $\mu E\ m^{-2}\ s^{-1}$) at 30°C until colonies grew to a diameter of 0.5 to 1 mm. At that time, each plate was subjected to a 12-hour dark pulse, and a small dish (8 mm in diameter) that contained 0.3 ml of decanal solution (3% V/V in oil) was placed within each plate. The plates were monitored by the CCD camera apparatus under the standard assay conditions (*7*). Bioluminescence images of a part of a plate obtained by 3-min exposures at the peak of the rhythm (hour 12 of LL) (**A**) and at the trough (hour 24 of LL) (**B**) are shown. (**C**) Regions of colonies that are recognized by the software (ovals). Images are represented in 256 gray scales (pixel of 0 count = black, that of 500 counts = white).

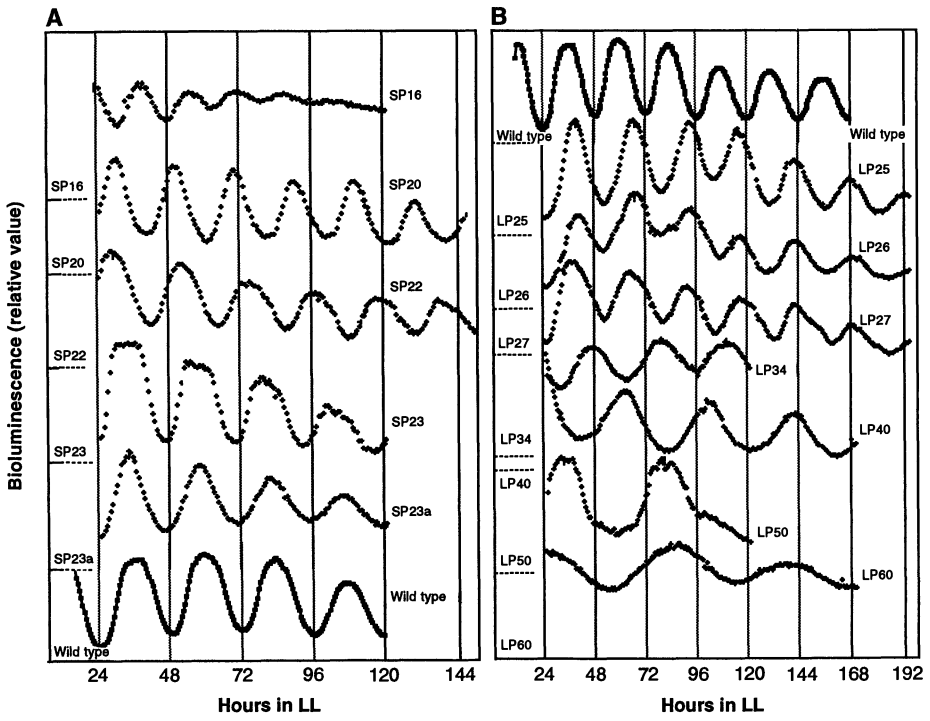

Fig. 2. Bioluminescence rhythms of clock mutants that display altered periods. Free-running bioluminescence rhythms of period mutants together with that of wild type under the standard assay conditions (36 min of white light at 46 $\mu E\ m^{-2}\ s^{-1}$ followed by 9 min of darkness, 30°C) are shown. (**A**) Short-period mutants and wild type. (**B**) Long-period mutants and wild type. For all experiments, colonies were grown in LL, given a 12-hour dark pulse, then returned to LL for the measurement of rhythmicity depicted here. Vertical scales for bioluminescence intensity are approximately equivalent. The baseline (dark) levels for the traces are shown by dashed lines on the left vertical axes that are labeled with the name of the mutant.

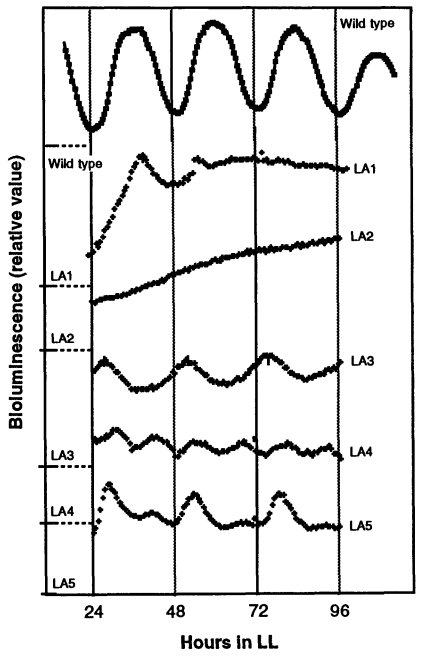

Fig. 3. Bioluminescence rhythms of clock mutants that display disrupted patterns. Free-running bioluminescence rhythms of low amplitude mutants are plotted as in Fig. 2. Experiment performed as in Fig. 2.

erally sustained a high amplitude rhythm. In particular, LP40 showed a well-sustained rhythm, even at the exceptionally long period of 40 hours, which is a 60% extension of the 24-hour wild-type period. Because the clock mutants for other systems deviate no more than 30% from 24 hours (*3*, *4*), it was unexpected that a circadian clock mechanism could be slowed to such an extent without impairing its robust expression. Of the seven mutants with periods between 20 and 28 hours, the amplitudes, persistence, and periods of their rhythms were differentially affected by changing the light intensity in LL (Table 1). This result suggests that different processes of the circadian mechanism have been altered in these various mutants.

Phase resetting by light or dark signals (or both) and temperature compensation of the period are key characteristics of circadian rhythms. A single 12-hour dark pulse will reset the phase of each mutant that expresses sustained rhythmicity (*10*), as we previously found for wild-type cells (*5*). Moreover, the Q_{10} values of the circadian frequency (1 per period) of the mutants ranged between 0.95 and 1.3 (Table 1). Therefore, phase resetting by dark pulses and temperature compensation of the period were not impaired in these mutants.

Traces of bioluminescence from five clones that were named LA for rhythms of low amplitude are shown in Fig. 3. Both LA1 and LA2 were arrhythmic mutants; LA1 was possibly rhythmic during the first 1 or 2 days, whereas LA2 appeared to be arrhythmic from the beginning of measurement. LA3, LA4, and LA5 were low amplitude mutants that expressed a variety of abnormal phenotypes. The rhythm of LA3 had a symmetrical waveform, with a peak that was earlier than that of wild type by 8 hours. LA4 sometimes expressed what appeared to be an approximately 12-hour period. The waveform of LA5 was distorted to a bimodal form and exhibited a minor peak and a major peak. The phase relation of the LA5 major peak was earlier than that of wild type by 6 hours. LA mutants may be defective in components of the central clockwork or may represent examples in which components of the output pathway are disrupted, as in (*11*).

Growth was not affected by these mutations (Table 1). Because these mutants were isolated after repeated subculturing in liquid medium, they were selected to grow at the same rate as wild-type cells, as can be seen from the doubling times in liquid culture (Table 1). Mutant colonies on agar also grew at approximately the same rate as did wild-type colonies. In particular, the growth rates of SP16 and LP60 were not affected even though the circadian clock oscillates at radically different rates in these strains. These results suggest that the clock mutations described here are not in genes essential for growth or cellular housekeeping and that these mutations are specific to the clock mechanism.

The average level of bioluminescence of all mutants was comparable with that of wild type. This suggested that the mutant phenotypes did not result from EMS-induced damage to the luciferase reporter construct in AMC149. To confirm this, we removed the P*psbAI-luxAB* reporter gene from some of the mutants, then reintroduced a cloned luciferase reporter construct that had not been exposed to EMS (*12*). We examined six mutant strains (SP16, SP20, SP22, LP27, LP40, and LP60) and found that the rhythmic phenotype of each mutant was unchanged by the replacement of the luciferase reporter construct (*10*). This demonstrates that the mutation in each of these strains was not in the reporter gene locus.

An important goal is to clone the mutant genes by complementation rescue. For this strategy to be successful, it was important that the distribution of rhythms from single colonies of mutants be well separated from those of wild-type cells, so that it would be possible to distinguish a rescued clone by bioluminescence screening. Typical histograms of the periods of all colonies on individual agar plates for wild type and some period mutants are depicted in Fig. 4.

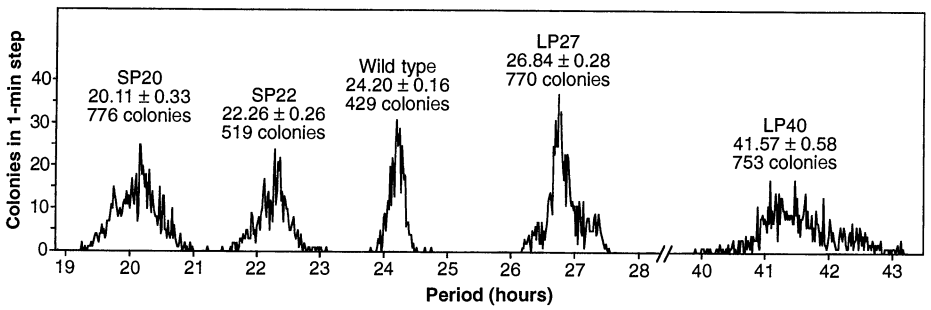

Fig. 4. Distributions of periods for the rhythms of wild-type and mutant colonies assayed in LL. Periods of the rhythms of individual colonies on agar plates were subjected to regression analysis and displayed in 1-min steps. The strain name, the means of the periods in hours with standard deviations, and the number of colonies measured are shown above each peak.

Table 1. Circadian clock mutants of *Synechococcus* sp. PCC 7942. Period mutants are labeled SP or LP for short period or long period, respectively. The number following this label represents the mutant's approximate period length in hours. The period was measured under standard assay conditions described in (*7*); each value is the mean of periods from more than 50 clones ($n > 50$). The doubling time was measured as the amount of time for the cell density to double, as obtained from growth curves of liquid culture under LL of 46 $\mu E\ m^{-2}\ s^{-1}$ (*17*). The value for Q_{10} is calculated from the circadian periods, each of which was the mean of periods from more than 50 clones at 30° compared with 35°C in LL under standard assay conditions. Dashes indicate that the variable was not measured.

Clone	Period (hours)	Doubling time (hours)	Q_{10}	Remarks
SP16	15.8	7.0	1.29	Damps in 3 days in LL
SP20	20.1	6.8	1.11	Persists for many days under various light intensities
SP22	22.3	7.3	0.95	Low amplitude in dim LL
SP23	23.2	—	1.02	Waveform is a sine curve, damps in dim LL
SP23a	22.8	—	0.94	Waveform appears "sawtooth," damps in bright LL
AMC149	24.2	6.6	1.04	Wild type
LP25	25.8	—	1.04	Stable rhythm
LP26	26.0	—	—	Low amplitude
LP27	26.8	6.6	1.01	Stable period under various light intensities
LP34	34.8	—	—	Damps in bright LL
LP40	41.6	8.4	1.05	Persists for many days under various light intensities
LP50	50.3	—	—	Damps in three cycles, in dim LL
LP60	58.5	7.9	—	Damps in three cycles
LA1	—	—	—	Arrhythmic after second cycle
LA2	—	—	—	Arrhythmic
LA3	24.5	—	—	Phase angle advanced by 8 hours, symmetrical waveform
LA4	—	—	—	Irregular ultradian rhythm, yellow colony
LA5	24.2	—	—	Phase angle advanced by 8 hours, bimodal waveform

The histograms of wild type and mutants show a normal distribution with standard deviations of ±0.16 to ±0.58 hour. Therefore, more than 95% of the colonies have a circadian period within a range of 1 hour, and screening for rescue will be practical for most of the mutants reported here.

Efficient complementation in *Synechococcus* is possible by conjugation with *Escherichia coli* cells that carry libraries of wild-type *Synechococcus* DNA (*13*). We used this strategy to recombine tagged segments of wild-type *Synechococcus* DNA into the genome of SP22 and obtained a clone that showed complementation of the mutant phenotype (SP22C in Fig. 5). The tagged DNA fragment recovered from the cured clone was able to again complement SP22 (SP22D in Fig. 5). These results demonstrate that the screening and complementation strategy to identify genes of the clock is effective. The segment of DNA which complements SP22 did not rescue the phenotypes of other mutants tested, suggesting that more than one locus is represented by the array of mutants.

Cyanobacteria are perhaps the only organisms in which the question of how many

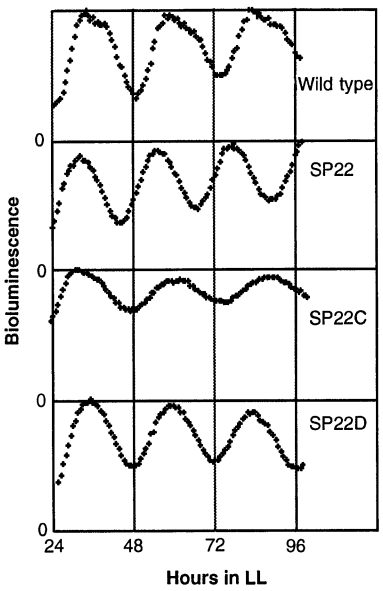

Fig. 5. Complementation of the SP22 short-period phenotype to the wild-type phenotype. Segments of wild-type *Synechococcus* DNA (2 to 4 kb), linked to a kanamycin resistance gene and origin of replication for *E. coli*, were transferred into SP22 cells by means of conjugation with *E. coli* and integrated into the mutant genome by homologous recombination (*13*). Cured clones that showed a wild-type period (SP22C) were identified by the screening system. Genomic DNA was extracted from SP22C cells, digested with Kpn I, circularized with T4 DNA ligase, and electroporated into *E. coli*. Plasmid DNA from kanamycin-resistant clones was used to transform SP22 cells (SP22D). Bioluminescence rhythms of wild-type cells, SP22 mutant cells, a complemented clone (SP22C), and SP22 cells transformed with the recovered complementing DNA segment (SP22D) are shown. Vertical scales for bioluminescence intensity are approximately equivalent; the baseline (dark) level for each trace is shown by the "0" label on each ordinate scale.

genes are involved in building a circadian clockwork can be answered in the near future. The small size of the genome of *Synechococcus* sp. PCC 7942 (2690 kb) (*14*) and the efficiency of our screening method enables a comprehensive search for mutants which have been rescued to the wild-type phenotype by complementation. We have identified a large ensemble of clock mutants. We anticipate that we have identified several different loci, although some of the mutant phenotypes may result from different mutations in the same locus. Sequence analysis of the DNA that complements each of the mutants will answer this question and may provide insight into the biochemical nature of the circadian clockwork.

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7. The system is composed of a cooled charge-coupled device (CCD) camera (TE/CCD512BKS, Princeton Instruments, Trenton, NJ), a large custom-built turntable that holds 12 plates, and a computer (Macintosh LCII, Apple Computer) with custom software that controls the turntable and data acquisition. The turntable was programmed to make a complete circuit in 45 min, which subjected each plate to a cycle of 36 min of white light [46 $\mu E\ m^{-2}\ s^{-1}$ (E is the energy of 1 mol of photons)] and 9 min of darkness at 30°C (these were standard assay conditions). As brief, repetitive dark interruptions of continuous light do not cause major interference to circadian clocks (*5*, *15*), we assumed these conditions were equivalent to continuous light (LL). Photosynthesis during the light period maintained the metabolism of the cells. The bioluminescence image was captured by the CCD camera during the middle 3 min of the 9-min dark interval. The image was initially scanned with a border-following algorithm. On average, 500 to 1000 colonies could be recognized on each plate by this algorithm. After this initial scan, bioluminescence signals from each region were integrated and stored. The parameters of the rhythms (period, phase, and amplitude) were computed by regression procedures similar to those used for the rhythms of eukaryotic algae (*15*).
8. AMC149 cells were cultured in BG-11 medium (*16*) which was bubbled with air in LL (46 $\mu E\ m^{-2}\ s^{-1}$). At the middle of the exponential growth phase [optical density at 730 nm (OD_{730}) = 0.5], 5 ml of culture was spun down, and cells were resuspended in a mixture of 0.5 ml of BG-11 medium and 0.5 ml of 30 mM potassium phosphate buffer (pH 7). After adding EMS (400 mM; Sigma) and incubating for 30 to 40 min at 37°C, 10 ml of 5% sodium thiosulphate solution was added to inactivate the EMS. Cells were washed twice with BG-11 medium and incubated under dim LL (23 $\mu E\ m^{-2}\ s^{-1}$) to allow the surviving cells to grow. About 0.2 to 1% of the cells survived this treatment. When cultures reached stationary growth phase, they were subcultured by a 1/1000 dilution into fresh medium.
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12. To be sure that the mutant phenotypes were not a result of mutagenesis of cis elements of the reporter gene, we removed the P*psbAI-luxAB* reporter in several mutants and then introduced it again. This was achieved by transformation of the mutant strains with a plasmid that carries a kanamycin resistance gene in the neutral site region of the *Synechococcus* chromosome. AMC149 and the original mutant strains carry a spectinomycin resistance gene and the P*psbAI-luxAB* reporter at this locus (*5*). Transformation and selection for kanamycin resistance resulted in recombinants that had replaced the spectinomycin resistance gene and the P*psbAI-luxAB* reporter with the kanamycin resistance gene. These strains, purged of the reporter, were transformed again with pAM977, the plasmid used to generate AMC149; selection for spectinomycin resistance yielded clones which again carried the P*psbAI-luxAB* reporter.
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17. Cells were inoculated into BG-11 medium and cultured with air-bubbling under LL (46 $\mu E\ m^{-2}\ s^{-1}$) at 30°C. Cell growth was monitored by measuring OD_{730} of small volumes withdrawn from the culture. Under these conditions, the cultures grew in an exponential fashion to a density of OD_{730} = 0.8 (about 10^9 cells per milliliter). The doubling time of the cells was calculated from the slope of the growth curve.
18. We thank N. Lebedeva and C. A. Strayer for technical assistance with the reporter gene replacement experiment and N. Yamaguchi for assembling the turntable system. This research was supported by grants from Science and Culture, the Ministry of Education (05670079, 06670093, and 06807029), United States–Japan Cooperative Program (BSAR382), the Shimadzu Foundation (Kyoto), and the Yamada Foundation (Osaka) to T.K. and M.I.; the National Institute of Mental Health (MH43836) and NSF (MCB-9219880 and INT-9218744) to C.H.J.; and NSF (DMB-8958089 and MCB-9311352) and NIH (GM37040) to S.S.G.

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Activation of the Myogenic Lineage by MEF2A, a Factor That Induces and Cooperates with MyoD

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Muscle enhancer factor–2A (MEF2A), a member of the MADS family, induced myogenic development when ectopically expressed in clones of nonmuscle cells of human clones, a function previously limited to the muscle basic helix-loop-helix (bHLH) proteins. During myogenesis, MEF2A and bHLH proteins cooperatively activate skeletal muscle genes and physically interact through the MADS domain of MEF2A and the three myogenic amino acids of the muscle bHLH proteins. Thus, skeletal myogenesis is mediated by two distinct families of mutually inducible and interactive muscle transcription factors, either of which can initiate the developmental cascade.

When ectopically expressed, the family of muscle bHLH transcription factors—MyoD, myogenin, MRF4, and myf5—independently activates the cascade of myogenic development (*1*). The myogenic activity of muscle bHLH factors was mapped to three amino acid residues located within the basic junction domain, which is predicted to reside within the major groove during DNA binding (*2*, *3*). In addition to binding DNA as heterodimers with bHLH E-proteins, the muscle bHLH proteins associate with several non-HLH proteins, including c-Jun, the retinoblastoma protein (Rb), and p107 (*4*–*6*). Despite this functional diversity, gene transfer and gene knockout studies have shown that certain features of the genetic regulation of myogenesis cannot be attributed to the bHLH proteins (*1*, *7*).

The muscle enhancer factor–2 or related to serum response factor (MEF2-RSRF) family, a second class of muscle transcription factors that belong to the MADS superfamily (MCM1, agamous, deficiens, and human serum response factor), also plays an important role in myogenic differentiation. Four distinct genes encoding the MEF2 family (*MEF2A*, *B*, *C*, and *D*) have been cloned in mammals, two in *Xenopus* (*SL1* and *SL2*), and a single gene in *Drosophila* (*D-MEF2*) (*8*, *9*). Whereas the muscle bHLH factors are expressed only in skeletal muscle cells, the MEF2 factors are predominantly, though not exclusively, expressed in skeletal, cardiac, and smooth muscle and in neuronal cells (*8*, *9*). Like the

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Table 1. Comparison of the efficiency of myogenic conversion after stable transfection of MyoD, myogenin, or MEF2A in mouse fibroblasts. 10T1/2, 3T3-L1, and 3T3-C2 fibroblasts were cotransfected as described (Fig. 2) and immunostained with the MHC mAb MF-20 and the ABC-Vectastain kit (Vector Laboratories). Myogenic conversion represents the number of myogenic colonies divided by the total number of *neo*^{r} colonies. Control, pMSV.

Muscle regulatory gene	Myogenic colonies (*n*)	Total colonies (*n*)	Myogenic conversion (%)
	10T1/2 cells		
Control	0	50	0
MyoD	17	65	26
Myogenin	10	35	28
MEF2A	35	250	14
	3T3-L1 cells		
Control	0	56	0
MyoD	8	20	40
Myogenin	3	20	10
MEF2A	5	35	14
	3T3-C2 cells		
Control	0	54	0
MyoD	5	15	33
Myogenin	7	19	37
MEF2A	3	35	8