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Chick pineal clock regulates serotonin *N*-acetyltransferase mRNA rhythm in culture

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ABSTRACT **Melatonin production in the chick pineal gland is high at night and low during the day. This rhythm reflects circadian changes in the activity of serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AA-NAT; EC 2.3.1.87), the penultimate enzyme in melatonin synthesis. In contrast to the external regulation of pineal rhythms in mammals by the suprachiasmatic nucleus, rhythmic changes in AA-NAT activity in cultured chick pineal cells are controlled by an oscillator located in the pineal cells themselves. Here we present evidence that the chick pineal clock generates a rhythm in the abundance of AA-NAT mRNA in cultured cells that parallels the rhythm in AA-NAT activity. In contrast, elevating cAMP by forskolin treatment markedly increases AA-NAT activity without producing strong changes in AA-NAT mRNA levels, and lowering cAMP by norepinephrine treatment decreases enzyme activity without markedly decreasing mRNA. These results suggest that clock-controlled changes in AA-NAT activity occur primarily through changes at the mRNA level, whereas cAMP-controlled changes occur primarily through changes at the protein level. Related studies indicate that the clock-dependent nocturnal increase in AA-NAT mRNA requires gene expression but not *de novo* protein synthesis, and that AA-NAT mRNA levels are suppressed at all times of the day by a rapidly turning over protein. Further analysis of the regulation of chick pineal AA-NAT mRNA is likely to enhance our understanding of the molecular basis of vertebrate circadian rhythms.**

The daily rhythm in melatonin is important because it feeds forward and backward to regulate many circadian functions (1, 2). The daily effects of melatonin are especially striking in some birds, in which the rhythm in melatonin controls the activity/rest cycle in locomotor activity; it can also contribute to entrainment of the central circadian clock in mammals (3). In addition to these short-term influences of melatonin, annual changes in the robust daily rhythm of circulating melatonin drive many seasonal changes in physiology (4).

The rhythm in circulating melatonin in vertebrates is generated primarily, if not exclusively, by the rate at which melatonin is produced by the pineal gland. Melatonin production is controlled by systems that include endogenous circadian oscillators and light detectors. Although a rhythm in pineal melatonin production is a constant feature of vertebrate physiology, the anatomical organization of melatonin rhythm generating systems differs markedly among vertebrate classes.

In the rat and other mammals, a single oscillator controls the pineal gland. This oscillator is located in the suprachiasmatic nuclei (SCN) and is entrained to the light/dark cycle by light acting through the eyes and projections which terminate in the SCN. Information from the SCN is transmitted to the pineal gland by a multisynaptic neural pathway terminating in adrenergic fibers that release norepinephrine at night, stimulating melatonin synthesis. Exposure to light at night rapidly blocks adrenergic stimulation of the mammalian pineal gland, which results in a rapid decrease in melatonin synthesis (5, 6).

The melatonin rhythm generating system in the chicken and presumably other birds, is distinctly different from that in mammals. First, the chicken pineal rhythm in melatonin synthesis is regulated by two oscillators, one in the pineal gland itself (7–9) and another in the avian homolog of the SCN (10). Although two clocks are involved *in vivo*, the clock in the pinealocyte is sufficient to generate a rhythm in serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase; AA-NAT; EC 2.3.1.87) activity *in vitro*. A second difference is that chick pineal cells are directly photosensitive, which makes it possible for light to act on these cells *in vitro* to acutely suppress nocturnal melatonin synthesis and also to entrain the pineal circadian clock. The acute inhibitory effect of light appears to be mediated by cAMP, whereas the entraining effect of light is not (11, 12). A third difference is that in chick, norepinephrine is released during the day and inhibits melatonin synthesis (10, 13, 14).

Although differences exist in the anatomical organization of vertebrate melatonin rhythm generating systems, a constant biochemical feature is that the rhythm in melatonin synthesis is regulated by the activity of AA-NAT, the penultimate enzyme in melatonin synthesis (15, 16). In the present report, we studied the regulation of AA-NAT mRNA in cultured chick pinealocytes using the chicken AA-NAT cDNA that was recently cloned (17). The purpose of this study was to learn more about how AA-NAT activity is controlled by the circadian pineal oscillator, cAMP, norepinephrine, and light in these clock-containing photosensitive cells.

EXPERIMENTAL PROCEDURES

Pineal Cell Culture. *Cell preparation.* White leghorn chicks were received 0–2 days after hatch from Clay's Hatchery (Blackstone, VA). Pineal cells were dispersed in trypsin and plated in modified McCoy's 5A medium (no. 12330-023; GIBCO) containing 25 mM Hepes buffer, L-glutamine, penicillin, streptomycin, 10% heat-inactivated fetal bovine serum, and 10% chicken serum essentially as described (18). Each 6-well plate had cells in groups of four wells. Experiments used

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Abbreviations: AA-NAT, arylalkylamine *N*-acetyltransferase, serotonin *N*-acetyltransferase; SCN, suprachiasmatic nuclei; L, white light; R, red light; ZT, zeitgeber time; RR, constant red light; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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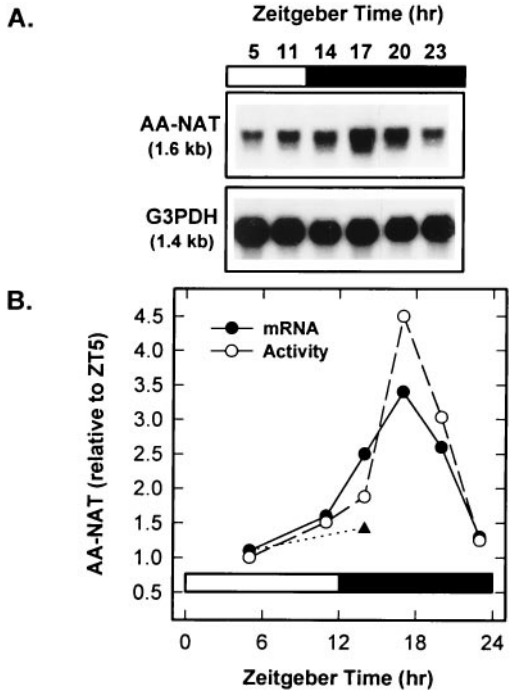

FIG. 1. Daily rhythm in AA-NAT mRNA and activity. Dissociated pineal cells from 1-day-old chicks were cultured for 5 days in a light cycle (LR 12:12, white light on at ZT 0) and were harvested at the indicated times. The filled bar indicates when white light was off. (*A*) Representative Northern blot analysis of AA-NAT and GAPDH (G3PDH) mRNAs (20 μg total RNA per lane). For each time point, total RNA was prepared from two groups of $\approx 6 \times 10^6$ cells. (*B*) Quantitative analysis of the Northern blot, after normalization to the GAPDH signal (●). The symbol ▲ shows the levels of AA-NAT mRNA in cells cultured with 5 μg/ml actinomycin D between ZT 11 and ZT 14. AA-NAT activity was measured at each time point (○). All values (mRNA and activity) represent the mean of duplicate determinations and are expressed relative to the ZT 5 time point. Similar results were obtained in four separate experiments.

cells from up to 250 glands in 48 wells containing $\approx 3 \times 10^6$ cells/well. Days in culture are numbered successively from the day of plating (day 1). Cultures were fed by exchange of medium at least daily. Serum-containing media were used through day 3, and serum-free media with 10 mM KCl added were used from day 4 onward. The effects of feeding schedule, media, sera, and potassium have been described (18).

Light cycles and drugs. Cells were maintained at 37°C under 5% CO_2 in air in tissue culture incubators containing red lights, white lights, and timers as described (18). They were all exposed to a cycle of 12-hr white light (L) and 12-hr red light (R) (LR 12:12) through day 4. In this schedule, L acts as "day" and, by convention, starts at zeitgeber time (ZT) 0; R acts as "night" and starts at ZT 12. Entrainment schedules varied in their relationship to "solar" time; groups of cells within each experiment were often entrained to opposite schedules to permit "midday" and "midnight" harvests to be performed at essentially the same time. In some experiments, groups of cells were switched from LR 12:12 to constant red light (RR) before expected onset of L at the start of day 5. Media were exchanged in L or R so as not to disturb the lighting regimen. Actinomycin D, forskolin, norepinephrine, cycloheximide, and puromycin were obtained from Sigma.

Analytical Techniques. In all experiments, AA-NAT mRNA levels and enzyme activity were measured using cells cultured in separate dishes from the same experimental treatment group.

RNA preparation and Northern blot analysis. Typically, the cells from two wells of a 6-well plate were pooled for each analytical sample and two samples were used for each experimental point. Total RNA was extracted using a guanidine HCl/phenol procedure and separated on a 1.5% agarose/0.7 M formaldehyde gel (19). RNA was transferred to nylon membrane (Nytran; Schleicher & Schuell) and probed using a random-primed ^{32}P-labeled probe generated from the chicken AA-NAT clone 9A (17). Northern blot analysis has revealed that this probe detects a single transcript of $\approx$1.6 kb in the pineal gland and the retina, but in no other chicken tissues (17). Blots were hybridized at 68°C in QuikHyb (Stratagene), and the final wash was at 60°C in 0.1× saline sodium citrate (SSC) containing 0.1% SDS for 15 min. Hybridized blots were imaged and analyzed using a PhosphorImager (Molecular Dynamics). Each AA-NAT mRNA value was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in that sample, to correct for variations in RNA loading and transfer efficiency. All data points are given as either the average of the results obtained from duplicates in one experiment, in which individual values were within 30% of the given average, or as the mean (±SEM) of the results from three experiments.

Enzyme assay. Cells from each of two wells of a 12-well plate were used to determine AA-NAT enzyme activity. AA-NAT activity was measured with 5 mM tryptamine (Research Biochemicals, Natick, MA) and 0.5 mM [^{3}H]AcCoA (specific activity, 4 Ci/mol; 1 Ci = 37 GBq) (New England Nuclear) as described (20).

RESULTS

The abundance of AA-NAT mRNA in chick pineal cells cultured in LR 12:12 increased at night (Fig. 1); the nocturnal

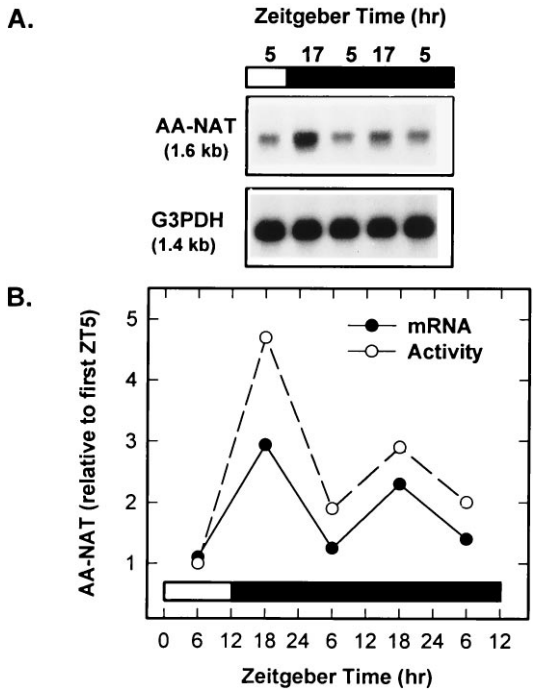

FIG. 2. Circadian rhythm in AA-NAT mRNA and activity. Dissociated pineal cells from 1-day-old chicks were cultured for 4 days in LR 12:12 (white light on at ZT 0) and were transferred to constant red (RR). The filled bar indicates when white light was off. Cells were harvested at the indicated times (zeitgeber times are indicated as a reference). (*A*) Representative Northern blot analysis of AA-NAT and GAPDH (G3PDH) mRNAs (20 μg total RNA per lane). For each time point, total RNA was prepared from 2 groups of $\approx 6 \times 10^6$ cells. (*B*) Quantitative analysis of the Northern blot, after normalization to the GAPDH signal (●). AA-NAT activity was measured at each time point (○). All values (mRNA and activity) represent the mean of duplicate determinations and are expressed relative to the first ZT 5 time point. Similar results were obtained in two other experiments.

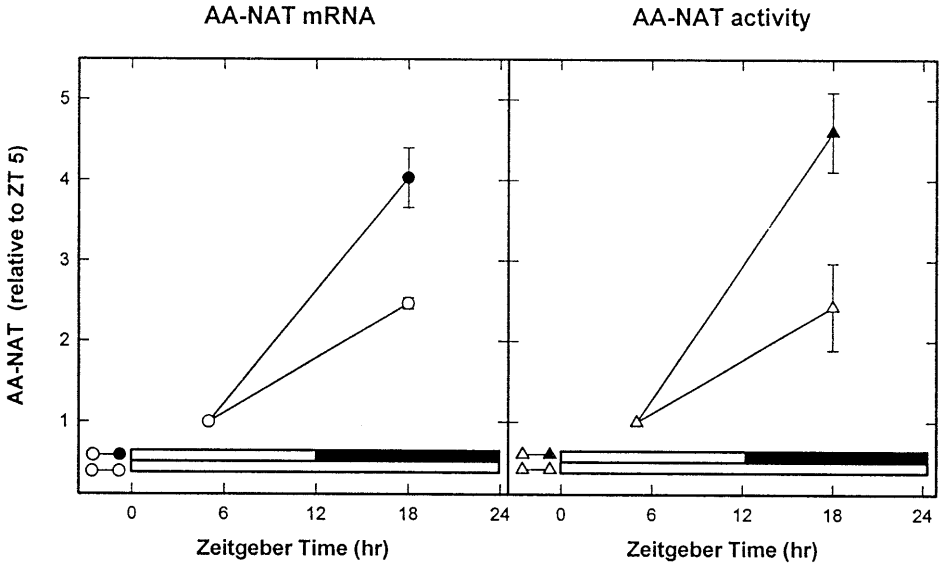

FIG. 3. Effect of light on the nocturnal increase in AA-NAT mRNA and activity. Dissociated pineal cells from 1-day-old chicks were cultured for 4 days in LR 12:12 (white light on at ZT 0). On the fifth night, some cells were exposed to unexpected light (= white light was not turned off at ZT 12). In each experimental group, total RNA was prepared from two groups of $\approx 6 \times 10^6$ cells and analyzed by Northern blot (20 μg total RNA per lane). AA-NAT mRNA values have been normalized to the GAPDH signal. AA-NAT activity was also measured in each group. All values (mRNA and activity) represent the mean ± SEM of three separate experiments and are expressed relative to ZT 5.

increase ranged from ≈2- to ≈5-fold in 14 independent experiments. In all cases the rhythms in AA-NAT mRNA and activity were similar.

The rhythm in AA-NAT mRNA persisted in RR, although the amplitude was damped relative to that seen in LR; this was also true for the AA-NAT activity rhythm (Fig. 2). It should be noted that the phase of the rhythms in RR was set by the initial entraining schedules, which were 180° out of phase in some cases (see details in *Experimental Procedures*; results not shown). Exposure to light during the first 6 hr of night inhibited the increase in AA-NAT mRNA levels by ≈40% and that in AA-NAT enzyme activity by ≈50% (Fig. 3).

Treatment with the cAMP elevating agent forskolin strongly increased AA-NAT enzyme activity (Fig. 4). This response occurred during both the light and dark period (Fig. 4*B*). Treatment with forskolin during daytime increased activity to a level considerably greater than that seen spontaneously at night. In contrast, forskolin treatment had relatively little influence on AA-NAT mRNA, resulting in levels below those seen spontaneously at night (Fig. 4*A*). In general, the forskolin-induced increase in AA-NAT activity was at least 3-fold greater than that in AA-NAT mRNA. Similar results were seen in eight independent experiments. We extended analysis of the effects of cAMP on AA-NAT mRNA by using norepinephrine, which reduces AA-NAT activity by reducing cAMP levels (13, 21). Norepinephrine treatment during the first 6 hr of night reduced the nocturnal increase in AA-NAT enzyme activity by 80%, but decreased AA-NAT mRNA levels by only 15% (Fig. 4*C*).

In an attempt to determine if the nocturnal increase in AA-NAT mRNA levels requires new mRNA and protein synthesis, cells were treated with selected inhibitors. Treatment with actinomycin D blocked the nocturnal increase in the 1.6-kb AA-NAT mRNA transcript (Fig. 1), suggesting that the increase does not merely reflect conversion from immature AA-NAT mRNA and that ongoing gene expression is required for the increase in AA-NAT mRNA to occur. In contrast, inhibition of protein synthesis by treatment with cycloheximide or puromycin (Figs. 5 and 6) did not block the nocturnal increase in AA-NAT mRNA levels but instead markedly enhanced it (Fig. 5). The concentrations of inhibitors used inhibit protein synthesis by >90% and did block the nocturnal or forskolin-induced increase in AA-NAT activity (Fig. 6). It should be noted that the stimulatory effect of cycloheximide on AA-NAT mRNA was observed at all phases of the circadian cycle (Fig. 5).

Cycloheximide treatment also elevated AA-NAT mRNA levels in cells treated with forskolin, which elevates cAMP, and in cells treated with norepinephrine or light, which both depress cAMP levels (Fig. 6). The magnitude of the influence of cycloheximide varied from treatment to treatment (Fig. 6). It is difficult to meaningfully compare possible effects of these treatments on the size of cycloheximide's effects, either as incremental or relative changes, because the treatments themselves changed AA-NAT mRNA levels in cells not treated with cycloheximide. However, marked and reliable reductions of cycloheximide's effect were not seen.

DISCUSSION

The results of these experiments demonstrate that there is a daily rhythm in the levels of AA-NAT mRNA in cultured chick pineal cells. Its persistence in RR indicates that this *in vitro* rhythm is truly circadian. Accordingly, it can be concluded that it is driven by the circadian oscillator endogenous to these cells. From this it is clear that the link between a clock and AA-NAT mRNA levels in the chick pinealocyte is more immediate and direct than it is in mammals, where the clock in the SCN is linked to the pinealocyte by a multisynaptic neural system (5, 22, 23).

The temporal patterns of the rhythms in cultured chick pineal AA-NAT mRNA levels and enzyme activity were found to be similar. No attempt was made to determine or measure whether there is a phase difference of a few hours between the rhythms in mRNA levels and in enzyme activity. Results suggest, but do not prove, that the clock-driven rhythm in AA-NAT mRNA is responsible for generating the rhythm in AA-NAT activity and melatonin production. This is also consistent with the observation that the nocturnal increase in AA-NAT activity is blocked by actinomycin D (8).

The finding that the pineal oscillator directly controls AA-NAT mRNA raises two questions. One is the molecular basis of the mechanism involved in regulating the AA-NAT mRNA

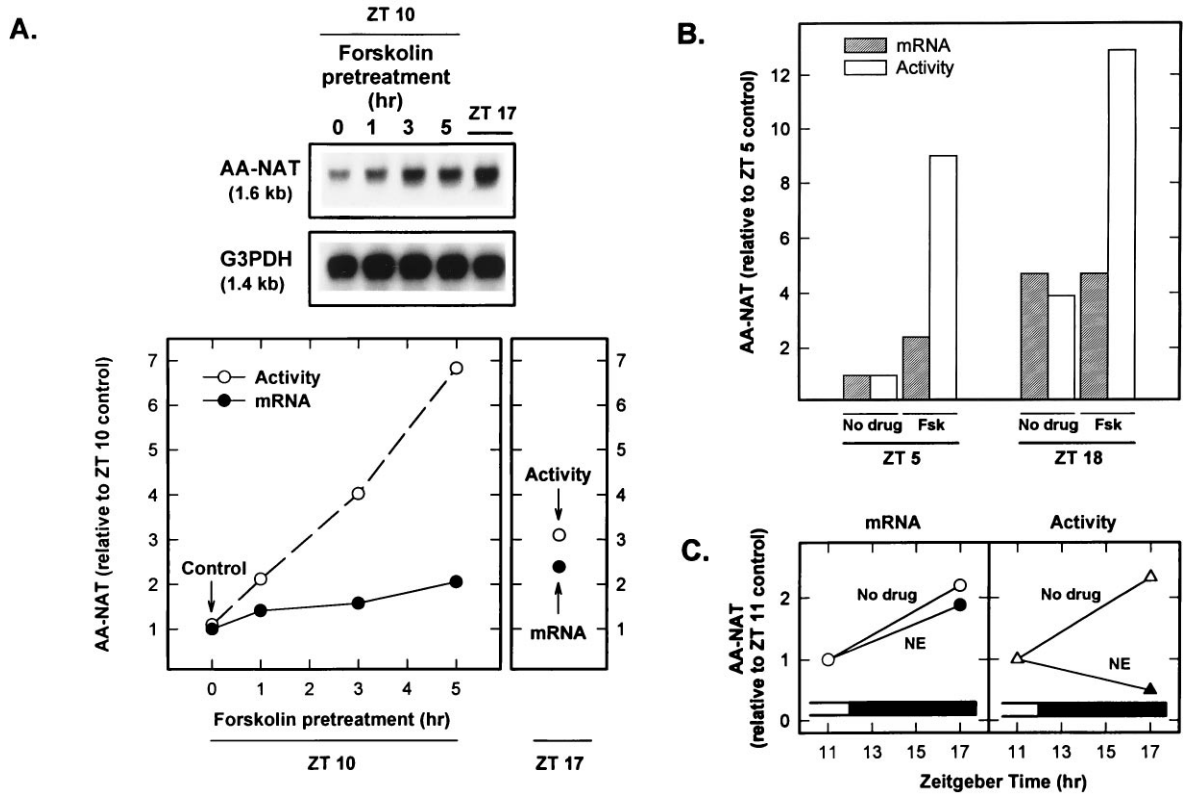

FIG. 4. Effects of forskolin and norepinephrine treatments on the levels of AA-NAT mRNA and activity. Dissociated pineal cells from 1-day-old chicks were cultured for 4 days in LR 12:12 (white light on at ZT 0). (*A*) On the fourth day, groups of cells were incubated for the indicated times with 1 μM forskolin (Fsk). All treatments were ended at ZT 10. In each experimental group, total RNA was prepared from 2 groups of $\approx 6 \times 10^6$ cells. (*Upper*) Representative Northern blot analysis of AA-NAT and GAPDH (G3PDH) mRNAs (20 μg total RNA per lane). (*Lower*) Quantitative analysis of the Northern blot, after normalization to the GAPDH signal (●). AA-NAT activity was measured at each time point (○). The symbols (● and ○) on the *Right* represent, respectively, the levels of AA-NAT mRNA and activity in untreated cells harvested at ZT 17. All values (mRNA and activity) represent the mean of duplicate determinations and are expressed relative to the ZT 10 control time point (= end of treatment). (*B*) On the fourth day of culture, cells were incubated for 6 hr with 1 μM forskolin either during the day (ZT 23 to ZT 5) or during the night (ZT 12 to ZT 18). Analysis of AA-NAT mRNA and activity were as described in *A*. All values represent the mean of duplicate determinations and are expressed relative to the ZT 5 control time point. Similar results were obtained in four other experiments. (*C*) On the fourth day of culture, cells were incubated for 6 hr with 10 μM norepinephrine (NE) between ZT 11 and ZT 17. Analysis of AA-NAT mRNA and activity were as described in *A*. All values represent the mean of duplicate determinations and are expressed relative to the ZT 11 control time point. Similar results were obtained in two other experiments

rhythm, and the second is how light acts on this system. These will be addressed sequentially below.

Mechanisms Involved in Generating the AA-NAT mRNA Rhythm. Our study indicates that the nocturnal increase in AA-NAT mRNA may not be driven by the *de novo* synthesis of a protein, such as a transcription factor, because the increase in AA-NAT mRNA levels is not blocked by protein synthesis inhibitors. Rather, we find that inhibition of protein synthesis enhances the increase in AA-NAT mRNA at all phases of the circadian cycle, suggesting that the abundance of this transcript is tonically suppressed by a protein with a rapid turnover. Possible candidates for such factors are an RNase or an inhibitory transcription factor.

Several possible mechanisms exist that might generate the rhythmic changes in AA-NAT mRNA. These include translocation or association of stable proteins that regulate the abundance of AA-NAT mRNA by controlling either its transcription or its degradation rate, or rhythmic posttranslational modifications (e.g., phosphorylation) of preexisting transcription factors.

Previous studies have shown that agents which elevate intracellular cAMP levels potentiate the clock-induced increase in AA-NAT activity (12, 13, 24). This suggested that cAMP might play a role in influencing or expressing the clock mechanism. However, a role for cAMP in clock function was counterindicated by the observations that changes in cAMP levels were not able to phase-shift the rhythm in AA-NAT activity (11, 12) and that melatonin rhythms persisted in the presence of very high concentrations of cAMP (25). Further evidence against a role for cAMP in clock-driven changes in AA-NAT activity comes from the present study, which shows that agents which either elevate (forskolin) or decrease (norepinephrine) cAMP levels and AA-NAT activity, do not produce dramatic changes in AA-NAT mRNA levels, whereas clock-dependent changes in AA-NAT activity are associated with changes in AA-NAT mRNA. Accordingly, it would appear that effects of the clock on AA-NAT activity may not be mediated by cAMP-induced changes in AA-NAT mRNA.

Although cAMP does not markedly alter AA-NAT mRNA, it is clear from previous studies and from the results of the present work that cAMP strongly stimulates AA-NAT activity. This suggests that cAMP causes large changes in AA-NAT activity by acting primarily at the translational or posttranslational level. Posttranscriptional modifications of AA-NAT protein could involve the two putative regulatory regions that combine protease cleavage sites and cyclic nucleotide protein kinase sites in the chicken AA-NAT amino acid sequence (17, 23). It is reasonable to suspect that cAMP may play a role in determining the degree to which changes in AA-NAT mRNA are translated into changes in AA-NAT activity.

Although it is evident that cAMP does not have a strong influence on AA-NAT mRNA levels similar to those on

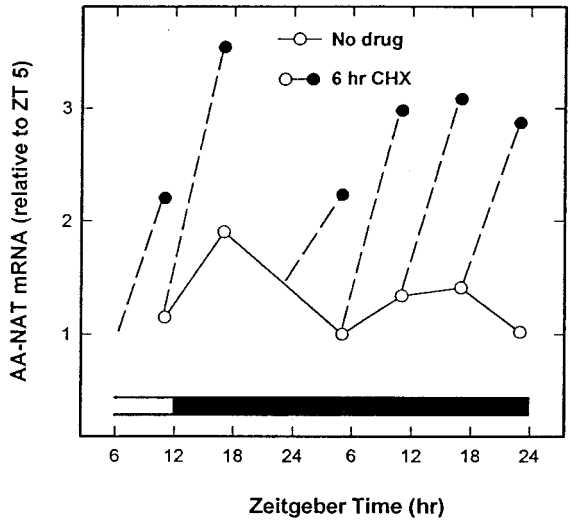

FIG. 5. Effect of protein synthesis inhibition on AA-NAT mRNA levels. Dissociated pineal cells from 1-day-old chicks were cultured for 5 days in LR 12:12 (white light on at ZT 0) and were then transferred to constant red. At different times of the subjective cycle (zeitgeber times are indicated as a reference) cells were incubated with 10 μM cycloheximide (CHX) for 6 hr. Total RNA was prepared from two groups of $\approx 6 \times 10^6$ cells and analyzed by Northern blot (20 μg total RNA per lane). AA-NAT mRNA values have been normalized to the GAPDH signal. All values represent the mean of duplicate determinations and are expressed relative to the ZT 5 control in RR.

AA-NAT activity, a weak influence on AA-NAT mRNA was consistently observed and should not be ignored. This could reflect the presence of a cAMP response element in the chicken AA-NAT gene, one that is weaker than the one which is thought to mediate the cAMP-dependent expression of the rat AA-NAT gene (22).

Effects of Light. As stated in the Introduction, light acts on chick pineal AA-NAT activity in two ways: to acutely suppress its nocturnal levels and to entrain its rhythm. Previous studies have indicated that the acute suppressive effect is correlated with a decrease in cAMP (24). It has recently been found in *in vivo* studies that light can acutely suppress AA-NAT activity without changing AA-NAT mRNA levels (17), indicating that the acute suppressive effects of light on AA-NAT activity could take place through posttranscriptional influences. Our results here, suggesting that cAMP alters AA-NAT activity by posttranscriptional mechanisms, make it seem probable that the acute suppressive effects of light on AA-NAT activity *in vivo* are mediated by changes in cAMP levels and involve posttranscriptional events.

The second action of light on AA-NAT activity, entrainment, is thought not to involve cAMP (11, 12). In the present study it was found that light cycles entrain the rhythm of AA-NAT activity and mRNA *in vitro*, in incubators with lighting schedules that were 180° out of phase. This demonstrates that at least part, if not all, of the phase-shifting/entraining effects of light on the AA-NAT activity rhythm occurs at the mRNA level. It is reasonable to assume that light is acting on AA-NAT mRNA primarily through effects on the clock. However, the nature of the mechanisms involved in this action of light still remains undefined.

The phase-shifting effects of light on the clock may explain why the nocturnal increase in AA-NAT mRNA levels was reduced when light exposure was extended into the night. We suspect that this may reflect an immediate phase delay of the rhythm in AA-NAT mRNA. The same mechanism may explain *in vivo* observations of similar effects of light on AA-NAT mRNA (17).

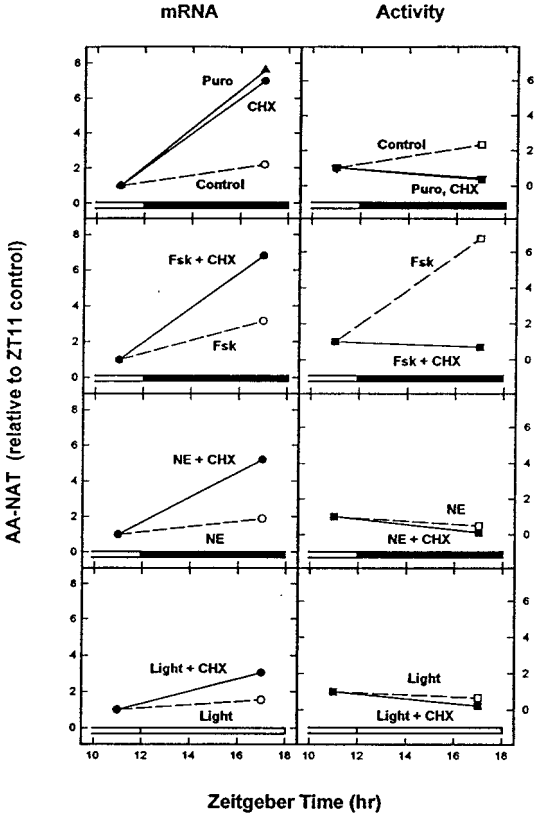

FIG. 6. Effect of cotreatments with protein synthesis inhibitors and forskolin, norepinephrine, or light on AA-NAT mRNA and activity. Cells that had been cultured for 5 days in LR were incubated for 6 hr with 1 μM forskolin (Fsk) or 10 μM norepinephrine (NE), in the presence or absence of 10 μM cycloheximide (CHX) (or 50 μg/ml puromycin). All treatments were performed at the beginning of the night (ZT 11 to ZT 17). For one group of cells the cycloheximide treatment was performed in the presence of light. Total RNA was prepared from two groups of $\approx 6 \times 10^6$ cells and analyzed by Northern blot (20 μg total RNA per lane). AA-NAT mRNA values have been normalized to the GAPDH signal (*Left*). AA-NAT activity was also measured in each group (*Right*). All values (mRNA and activity) represent the mean of duplicate determinations and are expressed relative to the ZT 11 control. Similar results were obtained in another experiment.

In summary, these experiments have established that the circadian oscillator in chick pineal cells drives a rhythm in AA-NAT mRNA in these cells, and that this rhythm is entrained by light through mechanisms that do not appear to involve cAMP or neural input. Changes in cAMP levels markedly influence enzyme activity, primarily through mechanisms that do not involve large changes in AA-NAT mRNA. This posttranscriptional regulation may provide a way to rapidly modulate AA-NAT activity and melatonin production. Continued analysis of this system should provide further insight into how the pineal clock controls the rhythm in AA-NAT mRNA and how cAMP regulates AA-NAT activity.

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