Modulation of transcriptional burst frequency by histone acetylation

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Many mammalian genes are transcribed during short bursts of variable frequencies and sizes that substantially contribute to cell-to-cell variability. However, which molecular mechanisms determine bursting properties remains unclear. To probe putative mechanisms, we combined temporal analysis of transcription along the circadian cycle with multiple genomic reporter integrations, using both short-lived luciferase live microscopy and single-molecule RNA-FISH. Using the Bmal1 circadian promoter as our model, we observed that rhythmic transcription resulted predominantly from variations in burst frequency, while the genomic position changed the burst size. Thus, burst frequency and size independently modulated Bmal1 transcription. We then found that promoter histone-acetylation level covaried with burst frequency, being greatest at peak expression and lowest at trough expression, while remaining unaffected by the genomic location. In addition, specific deletions of ROR-responsive elements led to constitutively elevated histone acetylation and burst frequency. We then investigated the suggested link between histone acetylation and burst frequency by dCas9p300-targeted modulation of histone acetylation, revealing that acetylation levels influence burst frequency more than burst size. The correlation between acetylation levels at the promoter and burst frequency was also observed in endogenous circadian genes and in embryonic stem cell fate genes. Thus, our data suggest that histone acetylation-mediated control of transcription burst frequency is a common mechanism to control mammalian gene expression.

transcriptional bursting | stochastic gene expression | histone acetylation | circadian oscillator | Bmal1

In higher eukaryotes, gene transcription in individual cells is intrinsically stochastic (1, 2). In particular, in many genes, RNA synthesis is subject to a pulsatile pattern and occurs mainly during short, often intense periods known as transcriptional bursts, followed by longer periods of transcription inactivity (3–5). The transcriptional bursting behavior of a gene is typically described by its burst frequency (i.e., the number of bursts in time units) and burst size (i.e., the mean number of transcripts produced per burst episode). Interestingly, these bursting kinetics are highly gene-specific (5–8) and likely reflect the complexity of regulatory mechanisms underlying gene expression and the diversity of molecular events participating in tuning transcription. Recent studies aimed at understanding how burst frequencies and sizes are controlled (9). In particular, burst frequency is able to tune gene expression and is sensitive to concentration of transcription factors (10–13). Possibly linked to transcription factor binding, DNA loops between distal regulatory elements and the promoter also predominantly influence burst frequency (14–16). Furthermore, nucleosome clearance around transcription start sites (TSSs) modulate burst frequency, which is anticorrelated with nucleosome occupancy in both yeast cells (17, 18) and mammalian cells (19). In contrast, other variables, such as the number and affinity of DNA regulatory elements on gene promoters, influence the burst size (5, 11). Finally, how the chromatin context modulates transcriptional bursting remains controversial, as both genomic position and enrichment of specific histone marks have been shown to influence burst frequency, burst size, or both (5, 10, 19–21).

In the present study, we further dissected how the control of gene expression is implemented on the level of transcriptional bursting parameters, particularly burst frequency and size. To address this, we exploited two opportunities: the possibility of monitoring periodically changing gene expression levels during the endogenously ticking circadian cycle, and using genome engineering to insert reporters at different genomic locations. By focusing on transcriptional bursting of the Bmal1 promoter driving a short-lived luciferase reporter, we found that burst frequency was accompanied by and directly influenced by promoter acetylation. This link between histone acetylation and burst frequency was also observed in endogenous circadian genes expressed at different times and in stem cell genes. Thus, we have found that histone acetylation increases transcription burst frequency, a mechanism that appears to modulate transcription in various mammalian systems.

Results

Live Analysis of Bmal1 Transcription Shows That Genomic Location and Circadian Time Modulate Burst Size and Frequency. To monitor transcriptional bursting of Bmal1 over the circadian cycle, we used a destabilized luciferase reporter with transcript and protein half-lives of 60 and 22 min, respectively (SI Appendix, Fig. SLA), allowing estimation of transcriptional bursting from single-cell luminescence traces (5, 22, 23). This reporter, hereinafter referred as Bmal1-sLuc2, was stably integrated by FRT recombination as a single copy in the genome of NIH 3T3 fibroblasts (Fig. 1A), and three clones differing in their reporter integration site were selected (SI Appendix, Fig. S1B). While after synchronization, all three clones displayed robust oscillations in luciferase expression at

Significance

Single-cell approaches have shown that many mammalian genes are transcribed stochastically in bursts of specific sizes and frequencies; however, molecular mechanisms controlling these bursting parameters have remained largely undetermined. By studying transcriptional bursting of a luciferase reporter controlled by a circadian gene promoter, we found that the gene integration site mainly influenced the burst size, while the circadian time primarily modulated the burst frequency. These daily variations in burst frequency correlated with histone acetylation levels, and CRISPR-Cas9-mediated acetylation of the promoter was sufficient to change the burst frequency. Since this correlation was also observed in other genes and in several cell types, we conclude that the impact of histone acetylation on gene expression is achieved mainly through modulation of burst frequency.

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the population level, the reporter integration site significantly influenced the mean expression levels (Fig. 1B). Namely, the clone with the highest expression (clone H) globally exhibited a 2.5-fold greater signal than the medium expression clone (clone M) and a 3-fold greater signal than the lowest expression clone (clone L).

For the three clones, luminescence signals were also monitored at the single-cell level with a 5-min time resolution, as described previously (5, 22, 23). While individual cells displayed heterogeneous temporal signals (Fig. 1C), the averaged traces accurately reproduced the population luminescence (SI Appendix, Fig. S2A). These single-cell traces were then used to estimate the transcriptional bursting parameters for each clone along the circadian cycle by adapting an inference approach based on the two-state telegraph model (SI Appendix, Fig. S2B) (5, 23). Specifically, we applied a sliding window to independently analyze trace sections of 8 h, sliding every 4 h. From this, the inferred Bmal1-sLuc2 mRNA copy numbers (ranging from 2 to 20) showed rhythmicity in all three clones (Fig. 1D). The underlying burst frequencies showed clear rhythms with a similar phase to mRNA accumulation for all clones (Fig. 1E), which arose mainly from longer promoter off-times during the expression trough (SI Appendix, Fig. S2C). Strikingly, the burst frequencies were comparable among the clones, indicating little influence from the reporter integration site. On the other hand, the burst sizes displayed less variability between the time points and did not exhibit clear circadian variations (significant differences in the H and L clones), while burst size did not change (Fig. 2 D and E). Moreover, the higher mean mRNA expression levels of the H clone compared with the M and L clones arose from the differences in burst sizes, showing similar values in the smRNA-FISH and live approaches (Fig. 2F). Thus, these smRNA-FISH data support the changes in transcriptional bursting parameters across time and insertion sites obtained from real-time luminescence.

**Rhythmic Histone Acetylation at the Bmal1 Promoter Correlates with Variations in Bursting Frequency, but Not with Burst Size.** Both single-cell luminescence and smRNA-FISH showed that while the reporter integration site affected expression levels through variations of the burst size, the circadian time primarily modulated the burst frequency in each clone. Thus, the burst size and frequency are uncoupled and likely involve specific molecular mechanisms to modulate gene expression. We sought to identify mechanisms that could explain the temporal variations in burst frequency of Bmal1-sLuc2. Previous work showed that the circadian expression of Bmal1 is controlled by two ROR-responsive elements (ROREs) at the TSS that rhythmically recruit the ROR family of activators and REV-ERB repressors (24, 25). Due to the phase-specific recruitment of NCoR and HADC5 corepresser complexes (26), the promoter of Bmal1 is rhythmically acetylated over the circadian period (27).

To assess whether the Bmal1-sLuc2 acetylation state and burst frequency are linked, we quantified the H3K27ac levels of the reporter promoter at the expression peak and trough by chromatin immunoprecipitation (ChIP). In each clone, acetylation levels at Bmal1-sLuc2 promoter were significantly enriched at the expression peak, as for the endogenous Bmal1 locus, but not for the arrhythmic Cyclophilin B gene (Fig. 3A). However, H3K27ac levels remained comparable between the clones. Thus, Bmal1-sLuc2 promoter acetylation covaried with modulation of the burst frequency; both quantities were unaffected by the integration site and varied with circadian time.

We further confirmed the correlation between Bmal1-sLuc2 histone acetylation and burst frequency by generating a mutated Bmal1 reporter lacking the two ROREs and thus unable to recruit RORs and REV-ERBs (SI Appendix, Fig. S4A) (25, 26). After stable integration in the genomic location of the H, the ΔRORE Bmal1-sLuc2 reporter abrogated the rhythmic expression pattern of the WT promoter by maintaining the expression level close to the circadian peak (Fig. 3B), suggesting that REV-ERB–mediated repression dominates the circadian regulation of the stably integrated Bmal1 reporter. In addition, this expression profile resembled that of a drug-mediated hyperacetylated reporter (SI Appendix, Fig. S4B). Indeed, mutations of the ROREs led to constitutively...
elevated levels of H3K27ac specifically at the Bmal1-sLuc2 reporter (Fig. 3A). We then used single-cell luminescence traces to infer the transcriptional bursting parameters of WT or ΔRORE reporters around the times of peak and trough expression. As before in the H clone (Fig. 1E), circadian variations in the expression of the WT Bmal1-sLuc2 could be explained mainly by changes in burst frequency (Fig. 3C), although the burst size was greater in these data (Discussion). In the double-ΔRORE mutant cells, however, the transcriptional bursting parameters remained unchanged between peak and trough despite lower absolute burst size and higher frequency (both by approximately 1.4-fold) compared with the WT measurements at the peak. Thus, the ROREs in the Bmal1-sLuc2 promoter were required for the drop in burst frequency at the expression trough. Taken together, these results suggest that Bmal1-sLuc2 burst frequency is correlated with histone acetylation state. Indeed, the two covary along circadian time while remaining constant between the clones. In addition, the two ROREs responsible for rhythmic acetylation of the Bmal1 promoter modulate bursting frequency, but not burst size.

We next searched for chromatin features that might explain the differences in burst size among the clones. None of the four chromatin marks that we measured by ChIP at the Bmal1-sLuc2 promoter for the three clones H, M, and L was correlated with burst size (SI Appendix, Fig. S5A). Similarly, a set of published chromatin marks quantified at the reporter integration sites did not reveal significant associations with burst size (SI Appendix, Fig. S5B).

Histone Acetylation Levels at the Bmal1-sLuc2 Promoter Determine Its Burst Frequency. To assess the causality of the suggested link between promoter acetylation state and burst frequency, we designed a system to modulate histone acetylation levels of a target promoter. To take advantage of a CRISPR/dCas9- and p300-based epigenome editing system, we used human HEK293T cells (29), into which we introduced a single copy of Bmal1-sLuc2, transcribed in large sporadic bursts (SI Appendix, Fig. S6A). Combined with dCas9 fused to the acetyltransferase domain of p300 (dCas9p300 WT), guide RNAs (gRNAs) specifically targeting the Bmal1-sLuc2 reporter led to luciferase induction of up to threefold in a bulk-transfected population (SI Appendix, Fig. S6B). In contrast, gRNAs of scrambled sequences or dCas9 fused to an inactive p300 catalytic domain (dCas9p300 D1399Y) did not impact luciferase expression. To estimate the amount of dCas9p300 in each cell, we used a GFP marker of transfection efficiency and sorted cells displaying low (GFP−), high (GFP+), or very high (GFP+++) levels of GFP (SI Appendix, Fig. S6C). In agreement with increased dCas9p300, cells with higher GFP levels showed increased histone acetylation of the Bmal1-sLuc2 locus (Fig. 4A). We inferred the transcriptional bursting parameters corresponding to each condition from smRNA-FISH distributions (SI Appendix, Fig. S7 A and B). While cells in the least transfected dCas9p300 WT population and with inactive dCas9p300 contained an average of five transcripts per cell, cells in the active dCas9p300 GFP+ and GFP++ populations contained an average of 13 and 20 transcripts per cell, respectively (Fig. 4B). Interestingly, even though the bursting parameters of Bmal1-sLuc2 were markedly different in the HEK293T cells compared with NIH 3T3 cells, this dCas9p300-mediated increase in mRNA expression arose principally from increased burst frequency.
(Fig. 4C), since the burst size did not change significantly across all conditions (Fig. 4D). Thus, these data suggest that histone acetylation at the Bmal1-sLuc2 promoter modulates transcriptional bursting by increasing the burst frequency.

**H3K27ac and Burst Frequency Are Correlated for Other Clock-Dependent and -Independent Genes in Several Systems.** We next assessed whether other rhythmically expressed genes similarly modulated their burst frequency rather than burst size, and if this phenomenon was also correlated with variation in histone acetylation. For this, we focused on the endogenous Bmal1 and Dbp genes. While Bmal1 is expected to behave like the luminescence reporter, Dbp is expressed antiphysically (SI Appendix, Fig. S8) and regulated by other factors (30). Here we used two-color intronic and exonic smRNA-FISH probes to quantify the number and intensity of active transcription sites (TSSs), as well as the total number of cellular mRNAs (SI Appendix, Fig. S9A).

We first estimated the fraction of extrinsic transcriptional variability in our conditions, since the unbiased inference of transcriptional bursting parameters from smRNA-FISH via the negative binomial distribution requires low levels of extrinsic noise (4, 31). By computing the covariance of all pairs of TSS intensities within the tetraploid NIH 3T3 cells (SI Appendix), we showed that the extrinsic noise accounted for 10–20% of the total noise in our smRNA-FISH system (SI Appendix, Fig. S9B). We then estimated transcriptional bursting parameters from the count distributions of mature transcripts as for Bmal1-sLuc2 (SI Appendix, Fig. S9C). For both Bmal1 and Dbp, circadian time significantly changed the burst frequency, which was higher at the respective peak expression times, while the burst size did not change significantly (Fig. S4).

To substantiate these results, we also inferred the bursting parameters of the endogenous Bmal1 and Dbp from the nascent transcript signals (SI Appendix, Fig. S9D). These analyses confirmed that burst frequency (in units of transcription elongation time) is higher at peak transcription for both genes, although the changes were greater than for mature transcripts (Fig. 5B). While the inferred burst sizes were similar and constant for cellular and nascent mRNAs for Dbp, Bmal1 burst sizes were smaller for nascent transcripts and showed a slight temporal variation. Thus, while quantitatively the mature and nascent transcript approaches differ slightly, the consistent finding that changes in bursting frequency according to circadian time were more significant than changes in burst size was very robust.

We next assessed the acetylation states of Bmal1 and Dbp promoters by H3K27ac ChIP-seq at peak and trough circadian expression levels. Although both genes displayed acetylation profiles with signal accumulating mainly at the TSS in Bmal1 and within the gene body in Dbp, most of their H3K27ac peaks were reduced at the expression trough (Fig. 5C). Thus, as for Bmal1-sLuc2, the burst frequency of endogenous circadian genes also changed during the circadian period together with the promoter acetylation state, independent of the phase of expression and promoter cis-regulatory elements.

Finally, we tested whether histone acetylation correlated with burst frequency in other cell systems. We focused on a dataset of 38 mouse embryonic stem cell (mESC) genes with transcript counts per cell measured by smRNA-FISH (32). For each gene, the burst size and frequency were inferred from the mRNA distributions and transcript half-lives (33). The bursting parameters were then correlated with the enrichment of seven genomic marks in a 5-kb or 500-kb window around the gene body (34). For both the 5-kb window and the gene body, among all genomic marks assessed, histone acetylation (H3K27ac and H3K9ac) was highly correlated with burst frequency (Fig. 5D and SI Appendix, Fig. S10A), indicating that hyperacetylated genes tend to have higher burst frequencies. This correlation was no longer present in the larger 500-kb window. However, no correlation could be detected between burst size and histone acetylation at any assessed genomic scale. Thus, while the correlation between active transcription and acetylation state is well known (35), our results suggest that this is caused by variations in burst frequency rather than by variations in burst size (SI Appendix, Fig. S10B).

**Discussion**

**Combining Approaches to Monitor Transcriptional Bursting.** Measuring transcriptional bursting properties of a promoter is technically challenging, requiring quantitative measurements of expression product at single-gene resolution. Here we estimated the bursting parameters of the short-lived Bmal1-sLuc2 reporter from protein levels in real time, from the distribution of mature transcripts in fixed cells and from the nascent transcripts at TSSs. Each approach has pros and cons. Notably, in the live approach, transcription states are mathematically inferred from measured protein levels (23), while for smRNA-FISH distributions, the negative binomial fit assumes short bursts and negligible cell-to-cell variability (4, 19). Here we found that these strategies converged to similar results: the burst frequency was modulated over the circadian cycle, while the integration site mainly affected the burst size. This supports previous reports that the burst frequency and size are uncoupled and can be separately controlled to regulate expression levels (5, 11, 19, 20).

However, we also noticed quantitative differences between bursting parameters inferred using these various approaches. For example, smRNA-FISH tended to display lower amplitudes between circadian peaks and troughs compared with real-time luminescence or RNA time course analyses. Whether this resulted from imprecise estimations of the circadian times or technical limitations in the detection of transcripts remains unclear. Independent of the approach, the telegraph model only explains expression inhomogeneous to the gene activity (intrinsic noise) and may provide inaccurate estimations of the bursting parameters in the presence of significant extrinsic noise (36, 37). By using nondividing cells and controlling the circadian cycle, we minimized the levels of extrinsic noise in both real-time luminescence (23) and smRNA-FISH (SI Appendix, Fig. S9B) analyses. Additional efforts to decrease extrinsic noise levels could include the control of such factors as cell size (38, 39); however, even if the extrinsic noise remains low within an experimental condition, it may vary between biological replicates. Experimental variables, such as cell density or synchronization efficiency, can vary between replicates despite strict protocols and affect sensitive readouts, such as bursting parameters. Thus, while changes in bursting parameters within experiments performed...
Histone Acetylation Determines the Burst Frequency. Here, by analyzing the bursting of a circadian gene, we found that histone acetylation levels modulate transcriptional burst frequency. In NIH 3T3 cells, H3K27ac levels at both WT and ΔRORE Bmal1-sLuc2 promoters and in endogenous circadian genes covaried with the burst frequency. In a previous study, temporally averaged Bmal1 burst frequency increased only marginally on drug-mediated histone hyperacetylation, because we did not stratify the analysis of bursting with respect to time (5). Similar links between H3K27ac levels and burst frequency were also observed in the Cry1 gene in mouse liver, where burst frequency oscillated between the expression peak and trough together with enhancer-promoter contacts and histone acetylation (16). In addition, controlled increase of the Bmal1-sLuc2 promoter acetylation levels in HEK293T cells by targeted p300 activity exclusively increased the burst frequency. Thus, promoter acetylation had a direct role in tuning the burst frequency, consistent with previous findings in yeast where deletion of most components of the acetylation machinery significantly reduced the burst frequency (40). While the bulk of our experiments were performed on Bmal1-sLuc2, the link between histone acetylation and burst frequency was also found in other systems, such as cell fate genes in mESCs. Thus, our correlative and functional analyses suggest that histone acetylation proximal to gene promoters may be a widespread determinant of burst frequency. The chromatin-loosening properties of histone acetylation could provide a possible explanation. Indeed, acetylated chromatin is more easily remodeled (41), and nucleosome density around TSSs has been shown to influence burst frequency (17, 19) as well as expression noise (42, 43), itself largely influenced by the burst frequency (44). Other known regulators of burst frequency, such as transcription factors or DNA looping, could be involved through active recruitment of the acetylation machinery (45), while the histone acetylation context reciprocally affects transcription factor binding and DNA looping formation by altering chromatin permissiveness.

Identified Molecular Origins of the Burst Size. The molecular determinants of the burst frequency are becoming clearer, the mechanisms influencing burst size remain largely unknown. In this study, we found that the integration sites of the reporters could change burst sizes, while the frequencies seemed less sensitive. Unfortunately, the limited number of integration sites available did not enable identification of the underlying discrepancies in burst sizes (SI Appendix, Fig. S5). Since none of the assessed histone marks correlated with burst size in a collection of 38 mESC genes (Fig. 5D), burst sizes may be influenced by combinations of factors or molecular states that are not captured by ChIP analysis of histone marks, such as transcription reinitiation. Notably, burst size could be influenced by the transient formation of gene clusters with enhanced transcription (47), as physical contacts with other active genes are good predictors of transcriptional output (48). These transcription domains could influence burst size by notably favoring the sharing of transcriptional machinery, such as RNA polymerase II (Pol II), between actively transcribing genes, as a reduction in Pol II level is sufficient to decrease burst size (39).

Materials and Methods

More detailed information on the materials and methods used in this study is provided in SI Appendix.
Cell Lines and Cell Culture. NIH 3T3-FRT cells were generated by transfecting a pFRT-Neo plasmid, and the presence of a single FRT site was verified by Southern blot analysis. Stable Bmal1-ΔLUC2 NIH 3T3 clones H, M, and L were obtained by Flp/FRT recombination of a Bmal1-ΔLUC2 expression vector. HEK293T cells stably expressing Bmal1-ΔLUC2 were obtained from transduction of lentivirus (5). Cells were maintained at 37 °C in a humid environment with DMEM complemented with 10% FBS.

Luminescence Recordings. The circadian clock was synchronized with desa-methasone before recording with an Actimetrics LumiCycle 32 (population) or LuminoView LV200 microscope (single-cell). Single-cell recordings were analyzed with the CAST platform (49).

Inferring Transcription Parameters from Single-Cell Luminescence Time-Traces. Luciferase protein and mRNA half-lives were estimated as described previously (5) from luminescence decay following actinomycin D or cyclohexi-mide treatment. Likelihoods of individual time traces were calculated using a two-state telegraph model, with \( k_{on}, k_{off} \) and \( k_{b} \) values estimated from Bayesian inference (23).

smRNA-FISH and Transcriptional Bursting Parameters Inference. smRNA-FISH was performed on serum-starved cells using Stellaris probes and imaged with a Leica DM5500B wide-field microscope. Transcripts were detected with CellProfiler on Z-projected stacks. Transcriptional bursting parameters were inferred from mature smRNA-FISH distributions (4, 19) or from nascent smRNA-FISH distributions using a Bayesian approach.

ChIP. ChIP analyses were performed using the MAGnify system. After soni-cation and immunoprecipitation, eluted samples were analyzed by quanti-tative PCR or by sequencing in an Illumina NextSeq 550 sequencing system.

dCas9p300-Mediated Epigenome Editing. HEK293T cells, for which the system was originally developed and optimized (29), were transiently transfected with dCas9p300 together with a GFP transfection efficiency marker and gRNAs. Cells were sorted accordingly to GFP intensity and analyzed by smRNA-FISH or ChIP.

Public Databases of Genomic Markers. Genomic features in 38 mESC genes were determined from public databases (34). Pearson correlation coefficients were calculated between the ChIP signals in different windows and bursting pa-rameters inferred from previously published smRNA-FISH distributions (32).

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