Light-Driven Changes in Energy Metabolism Directly Entrain the Cyanobacterial Circadian Oscillator

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Circadian clocks are self-sustained biological oscillators that can be entrained by environmental cues. Although this phenomenon has been studied in many organisms, the molecular mechanisms of entrainment remain unclear. Three cyanobacterial proteins and adenosine triphosphate (ATP) are sufficient to generate oscillations in phosphorylation in vitro. We show that changes in illumination that induce a phase shift in cultured cyanobacteria also cause changes in the ratio of ATP to adenosine diphosphate (ADP). When these nucleotide changes are simulated in the in vitro oscillator, they cause phase shifts similar to those observed in vivo. Physiological concentrations of ADP inhibit kinase activity in the oscillator, and a mathematical model constrained by data shows that this effect is sufficient to quantitatively explain entrainment of the cyanobacterial circadian clock.

Circadian clocks, based on self-sustained biological oscillators with a ~24-hour period, allow organisms to anticipate regular variations in the environment caused by Earth's rotation (*1*). For a circadian system to be most useful to the organism, it must function robustly in the face of fluctuations in the environment but also accept appropriate input signals to stay entrained with the true diurnal cycle. Therefore, a fundamental problem is to elucidate the molecular mechanisms that allow faithful transduction of timing information into the oscillator.

The cyanobacterium *Synechococcus elongatus* PCC 7942 has a core circadian oscillator that can be reconstituted in vitro from three purified proteins: KaiA, KaiB, and KaiC (*2*). KaiC is an enzyme that catalyzes the phosphorylation and dephosphorylation of two of its own residues in an ordered pattern (*3*). KaiA and KaiB work together to modulate the activity of KaiC in a phosphorylation-dependent manner, resulting in stable oscillations in the amount of phosphorylated KaiC (*4*–*6*). We sought to identify light-dependent clock input pathways in living cyanobacteria and to study their mechanism in the reconstituted in vitro oscillator.

Causal links between metabolic activity and circadian clocks have previously been described. The circadian clock in the rat liver can be entrained by feeding (*7*), and adenosine monophosphate–activated protein kinase has been implicated in clock function in liver tissue (*8*). A classical result connecting clocks and metabolism is Aschoff's Rule—the observation that varying the light intensity tends to have opposite effects on the circadian clocks of nocturnal versus diurnal organisms (*9*).

We reasoned that the link to metabolism might be especially important in photoautotrophic cyanobacteria, as these microorganisms are entirely reliant on photosynthesis to extract energy from the environment (*10*). Darkness elicits profound changes in the physiology of *S. elongatus*, beyond the direct effects on photosynthesis. When cultures are removed from the light, global transcription and translation rates drop and large changes in the superhelicity of endogenous DNA are observed (*11*, *12*).

Cyanobacteria growing in the light primarily synthesize adenosine triphosphate (ATP) by photophosphorylating adenosine diphosphate (ADP) at the thylakoid membrane (*13*), and changes in culture illumination have been reported to affect the relative abundance of adenine nucleotides (*14*–*16*). We therefore tested whether incubating synchronized cultures in the dark under conditions sufficient to cause a phase shift in the circadian clock would result in changes in the concentrations of ATP and ADP. We monitored changes induced by a dark pulse in the circadian rhythm of an engineered strain of *S. elongatus* with a bioluminescent reporter of clock-driven transcription (*17*). In agreement with previous studies, an 8-hour dark pulse applied during the subjective day (Fig. 1A) induced a phase shift in the circadian rhythm, and the clock was refractory to a dark pulse applied during the subjective night (Fig. 1B) (*18*–*20*). These phase shifts in the transcriptional reporter were mirrored by shifts in the rhythm of KaiC phosphorylation, as measured by immunoblotting (fig. S1). Irrespective of when the dark pulse was applied, these cultures experienced large changes in their adenine nucleotide pool (Fig. 1C). After 2 to 3 hours in the dark, the ATP/(ADP + ATP) ratio had fallen to nearly 50% and remained low for the duration of the dark pulse; when illumination was restored, this ratio returned to ~85% within 1 hour.

The mechanism of the KaiABC protein oscillator relies on ordered multisite phosphorylation of KaiC (*4*, *5*). Because ADP cannot serve as the phosphoryl donor in the kinase reaction, physiological changes in the relative amounts of ATP and ADP caused by changes in light have the potential to modulate the activity of enzymes that consume ATP, and might directly affect the function of the circadian clock (*21*). To test this, we developed an experimental system to mimic in vitro the changes in nucleotide ratio we had observed in living cyanobacteria. We initiated oscillator reactions with purified KaiA, KaiB,

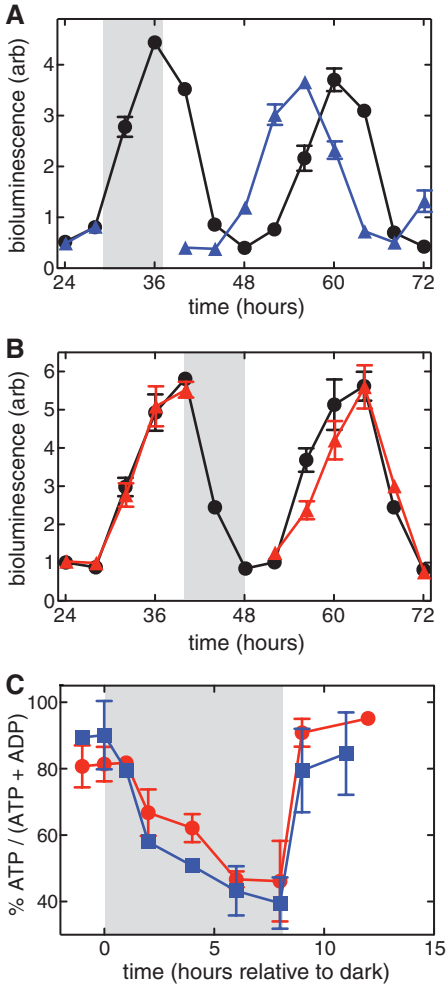

Fig. 1. Sustained drop in the ATP/ADP ratio and a phase shift in the circadian clock in response to a pulse of darkness. (**A**) Bioluminescence rhythms of a reporter under circadian control (P*kaiBC*::*luxAB*) in synchronized cultures either grown in constant light (black symbols) or subjected to an 8-hour pulse of darkness (shaded region) at t = 29 hours (blue symbols). Bars show the range of duplicate measurements from the same experiment. Time is measured from the release of cultures into constant light; arb, arbitrary units. (**B**) Same as (A) except that the dark pulse was applied at t = 40 hours. (**C**) Relative levels of ATP and ADP extracted from cultures before, during (shaded region), and after a dark pulse in the conditions shown in (A) (blue symbols) and in (B) (red symbols). Bars indicate the range from duplicate cultures.

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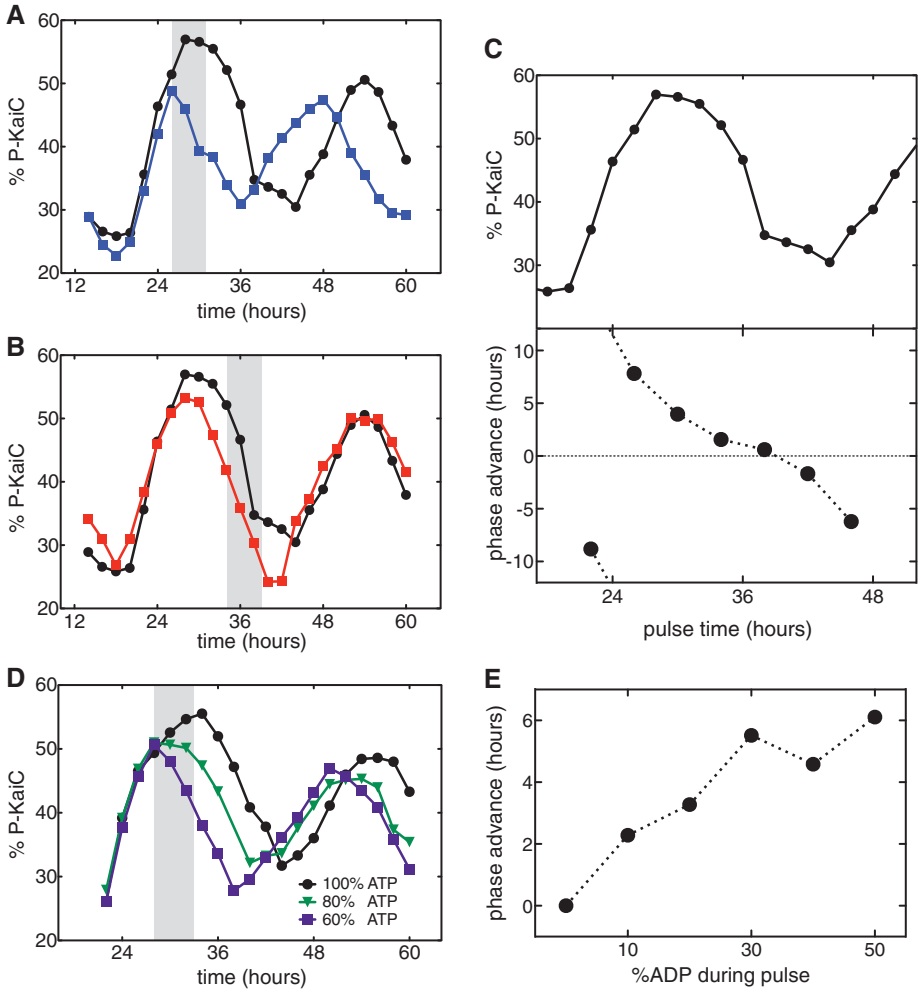

Fig. 2. Phase shifts in the in vitro KaiABC oscillator caused by changing the ATP/ADP ratio. (**A**) Phase shift induced by adding an amount of ADP equal to the amount of ATP in the reaction buffer ~6 hours before peak phosphorylation (blue symbols) relative to a control (black symbols). The shaded region indicates the time after addition of ADP but before the addition of pyruvate kinase to convert ADP to ATP. (**B**) Same as (A) except that ADP was added ~6 hours after peak phosphorylation (red symbols). (**C**) Phase shifts induced by a pulse of ADP in the in vitro oscillator reaction (lower panel) shown adjacent to the rhythm of KaiC phosphorylation in the control (upper panel) to indicate where in the cycle the pulses were applied. Differences in phase were measured by fitting the data to a sinusoidal function. (**D**) Effect of addition of mixtures of nucleotides to lower the ATP/ADP ratio (colored symbols) to various levels relative to a control that received only ATP (black symbols). Total adenine nucleotide concentration was the same across all reactions. The shaded region indicates the time before addition of pyruvate kinase. (**E**) Quantification of phase shifts from the experiment shown in (D). The phase shift when only ATP is added is zero by definition. Differences in phase were measured by fitting the data to a sinusoidal function.

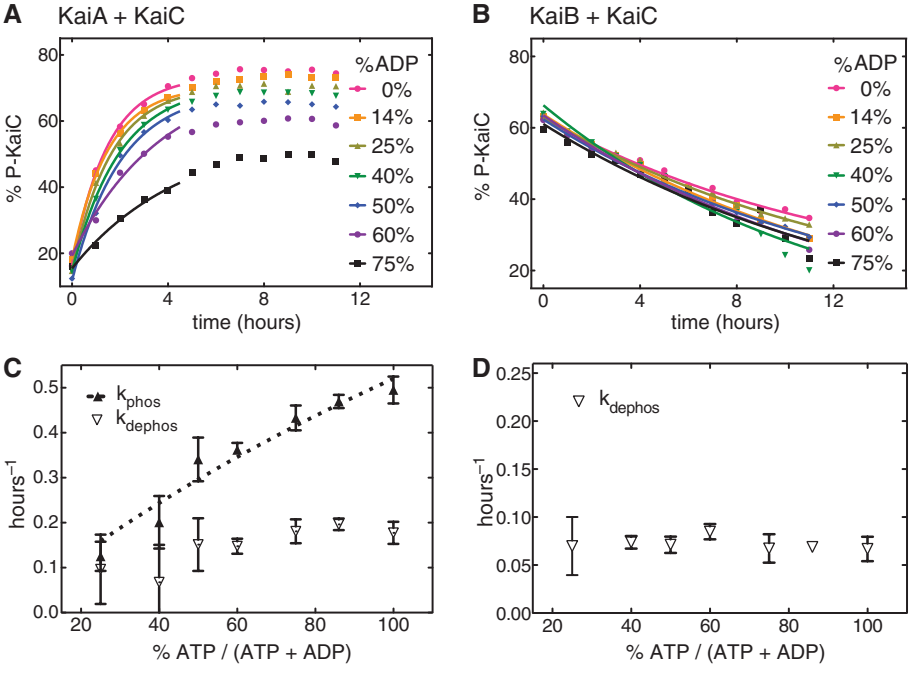

Fig. 3. Inhibition of KaiC phosphorylation by ADP. (**A**) KaiC phosphorylation in a KaiA-KaiC reaction with different ADP/(ATP + ADP) ratios. Solid lines are fits to the first 4 hours of data used to isolate the first phosphorylation step (*28*). (**B**) KaiC dephosphorylation in a KaiB-KaiC reaction with different ADP/(ATP + ADP) ratios. Solid lines are fit to a first-order reversible reaction. (**C**) Kinase (k_{phos}) and phosphatase (k_{dephos}) rate constants are obtained from the data in (A). The dashed line is a fit to the competitive inhibition model $k\{[ATP]/([ATP] + K_{rel}[ADP])\}$, where K_{rel} is an effective relative affinity for ADP versus ATP, fit to 0.76 ± 0.15. Error bars represent SE in the fit. (**D**) Phosphatase rate constants obtained from the data in (B). Error bars represent SE in the fit.

and KaiC in a buffer containing an excess of phosphoenolpyruvate and 4 mM ATP, similar to estimates of adenine nucleotide concentration in cyanobacterial cells in the millimolar range (*22*). After the oscillation had been established, we simulated the metabolic effects of darkness by adding ADP to bring the ATP/(ADP + ATP) ratio to ~50%. To then simulate the effects of a return to growth in light, we added pyruvate kinase to convert the ADP to ATP, a reaction that went to completion in ~1 hour (fig. S2). Because the ATP/ADP ratio in vivo initially falls gradually when cells are incubated in the dark for 8 hours in our liquid culture conditions (Fig. 1C), we used a 5-hour step-like pulse of ADP in vitro to approximate the amount of time that the in vivo cultures experience the lowest ATP/ADP ratio.

Transient manipulation of the ratio of ATP to ADP in the KaiABC oscillator created large phase shifts in the phosphorylation rhythm (Fig. 2A). Further, the phase response curve obtained by altering the ATP/ADP ratio in vitro was similar to that observed in live cells treated with pulses of darkness: The oscillator was most sensitive during the middle of subjective day (when KaiC phosphorylation was increasing) and nearly insensitive during subjective night (when KaiC phosphorylation was decreasing) (Fig. 2, B and C) (*18*, *19*, *23*). During the pulse of increased ADP, KaiC phosphorylation was decreased relative to that in the control reaction, similar to changes in KaiC phosphorylation in vivo when cells were subjected to a dark pulse (fig. S1) (*24*).

To determine the increase in the relative amount of ADP needed to produce this effect, we added varying amounts of ADP to several identical KaiABC reactions during a fixed portion of the oscillator cycle. The induced phase shift was a graded function of the amount of ADP added (Fig. 2, D and E). Similarly, the magnitude of the phase shift decreased when the length of the pulse of ADP was decreased (fig. S3). Thus, even small changes in the ATP/ADP ratio, smaller or shorter in duration than those induced by a rapid transition to prolonged darkness, adjusted the time specified by the circadian clock.

To investigate the molecular mechanism by which changes in the relative fraction of ADP are able to alter the oscillator phase, we studied the effect of the ATP/ADP ratio on nonoscillatory partial reactions in which one of the Kai proteins was absent. In the KaiA-KaiC reaction, KaiA chronically stimulates KaiC's autophosphorylation activity, resulting in a steady state in which KaiC is highly phosphorylated (*5*, *25*). Increasing the relative fraction of ADP present in the reaction resulted in both reduced steady-state phosphorylation and a slower approach to that steady state (Fig. 3A). In the KaiB-KaiC reaction, KaiC has very weak kinase activity and the fraction of phosphorylated KaiC slowly decreases, allowing us to isolate the dephosphorylation reaction (*5*). We did not detect any effect of varying the ATP/ADP ratio on KaiC's phosphatase activity (Fig. 3B), nor on the stability of KaiB-KaiC complexes (fig. S4).

Quantitative analysis of the kinetics of the partial reactions confirmed the above observations and was consistent with a model in which ADP acts as a competitive inhibitor of KaiC's kinase activity. In fitting the phosphorylation kinetics to an initial velocity approximation, we estimated an effective relative affinity for ATP and ADP that is close to unity ($K_{rel} = 0.76 \pm 0.15$) (Fig. 3C). This value is compatible with two simple mechanistic hypotheses: Either the true binding affinities for ATP and ADP are comparable in the CII lobe [the seat of KaiC autophosphorylation (*26*)], or the rate of ATP hydrolysis in CII is much faster than the rate of nucleotide dissociation, so that any difference in affinity is negligible. In either case, ADP acts in proportion to its relative abundance to decrease the KaiC phosphorylation rate.

To test whether the competitive inhibition mechanism we had identified was sufficient to explain the large phase shifts in the full oscillating reaction, we turned to a mathematical model of the circadian clock based on experimental measurements of the rates of phosphorylation and dephosphorylation at two sites on KaiC: Ser^{431} and Thr^{432} (*5*). In this model, stable oscillations arise because KaiC phosphorylated only on Ser^{431} interacts strongly with KaiB, trapping KaiA stoichiometrically and globally promoting dephosphorylation. In this sense, KaiC phosphorylated on Ser^{431} is autocatalytic, generating a nonlinearity that drives the oscillator.

We modified this model so that, in accord with our experimental observations, the kinase rates were modulated by the ATP/(ADP + ATP) ratio present in the reaction, as well as by the level of KaiA activity (Fig. 4A) (*5*). Thus, at each point in the oscillation, KaiC integrates clock information (through KaiA's kinase-stimulating activity) with metabolic information (through the adenosine pool). We simulated our in vitro experiments within this model by transiently lowering the ATP/(ADP + ATP) ratio from 100% to 50% at various times in the circadian cycle. In simulation, pulses of competitive inhibition by ADP were sufficient to generate large phase shifts in the oscillator when these pulses occurred during the phase of the cycle when KaiC phosphorylation was rising (Fig. 4B).

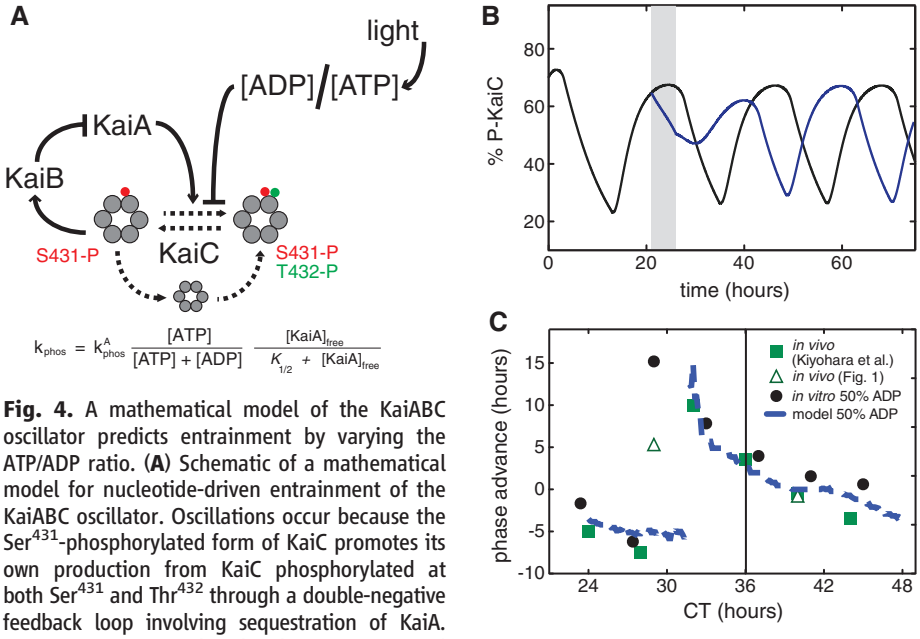

Fig. 4. A mathematical model of the KaiABC oscillator predicts entrainment by varying the ATP/ADP ratio. (**A**) Schematic of a mathematical model for nucleotide-driven entrainment of the KaiABC oscillator. Oscillations occur because the Ser^{431}-phosphorylated form of KaiC promotes its own production from KaiC phosphorylated at both Ser^{431} and Thr^{432} through a double-negative feedback loop involving sequestration of KaiA. Free KaiA promotes phosphorylation of KaiC and inhibits dephosphorylation. Changing illumination conditions in vivo drive changes in the adenosine pool, which in turn modulate phosphorylation rates (k_{phos}). Although the figure shows only the effect on Ser^{431}-phosphorylated KaiC, changes in KaiA activity and in the ATP/ADP ratio affect all phosphorylation steps in the model. K_{rel} is an effective relative affinity for ADP versus ATP. $[KaiA]_{free}$ is the free concentration of [KaiA], and $K_{1/2}$ is the concentration of [KaiA] needed to produce a half-maximal effect on KaiC. k^{A}_{phos} is the maximum phosphorylation rate with saturating KaiA and 100% ATP. See (*5*, *28*) for complete details. (**B**) Simulation of the effect of lowering the ATP/(ADP + ATP) ratio from 100% to 50% for 5 hours during the subjective day (shaded region) using equal affinities for ATP and ADP (blue curve) compared to a control in constant 100% ATP (black curve). (**C**) Phase response curve predicted by the model using equal affinities for ATP and ADP (blue dashed line) as in (B) compared with in vitro data (black circles) and in vivo data (green squares) from (*19*) (4-hour dark pulse, solid media) and from this study (open triangles) (8-hour dark pulse, liquid media). All data sets were aligned so that peak phosphorylation occurs at 36 hours (grid line), and the modeling results were scaled on both axes to make the period equal to 24 hours.

In order to obtain a strong resetting effect, KaiC must have sufficiently high sensitivity to ADP, at the upper end of the range of effective affinities we estimated experimentally (fig. S5). We used this model to predict a full phase-response curve based on this competitive inhibition mechanism and compared it to our experimental data and to measurements of the phase shift induced by darkness in vivo (Fig. 4C) (*19*). The agreement between these data and the model indicates that varying the relative nucleotide concentrations in the reconstituted oscillator approximates the response of the circadian clock in living cyanobacteria to changes in illumination, and an increase in the amount of ADP appears to alter the phase of the oscillator through inhibition of KaiC's kinase activity.

In the model, the ATP/ADP ratio describes a family of limit cycles that differ in the amplitude of the phosphorylation rhythm. Indeed, KaiABC reactions in buffers with tonically lowered ATP/ADP ratios continue to oscillate with a circadian period, but KaiC cycles through distinct patterns of phosphorylated states (fig. S6). If the ATP/ADP ratio is lowered abruptly, phosphorylation is inhibited, and the system must adjust to a new limit cycle. If this transition produces amounts of Ser^{431}-phosphorylated KaiC sufficient to block KaiA activity, a large phase shift can result.

We have described a simple entrainment mechanism for a circadian clock in which enzymatic activity is directly tied to the availability of biochemical energy in the cell. In this view, no signaling pathway that specifically targets the oscillator is required to couple the clock to the environment, and the core oscillator proteins interact directly with metabolites, as has been reported for KaiA in vitro (*27*). Although factors outside the Kai proteins have been implicated in light-driven input to the cyanobacterial circadian system, including LpdA and the histidine kinase CikA (*18*, *20*), strains lacking these proteins can be effectively entrained with repeated light-dark cycles, supporting the hypothesis that there exist basic synchronization mechanisms intrinsic to the KaiABC core oscillator itself. Because unexpected darkness will unavoidably lead to changes in the production and consumption of ATP in an obligate phototroph, KaiC's sensitivity to ADP represents a robust intrinsic mechanism for maintaining synchrony with the environment.

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Supporting Online Material

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Suppression of Avian Influenza Transmission in Genetically Modified Chickens

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Infection of chickens with avian influenza virus poses a global threat to both poultry production and human health that is not adequately controlled by vaccination or by biosecurity measures. A novel alternative strategy is to develop chickens that are genetically resistant to infection. We generated transgenic chickens expressing a short-hairpin RNA designed to function as a decoy that inhibits and blocks influenza virus polymerase and hence interferes with virus propagation. Susceptibility to primary challenge with highly pathogenic avian influenza virus and onward transmission dynamics were determined. Although the transgenic birds succumbed to the initial experimental challenge, onward transmission to both transgenic and nontransgenic birds was prevented.

The diversity of avian influenza viruses (AIVs) and their propensity for interspecies transmission make them a global threat to animal and public health communities. Cross-species transmission of influenza viruses may occur directly or be facilitated by intermediate host species that amplify and diversify virus populations, notably domestic chickens, ducks, and pigs (*1*). Although control of AIV infection in its wild aquatic bird reservoir is impractical, control of AIV in domesticated hosts is possible (*2*). The diversity of viral antigenic subtypes and their potential for evolutionary shift and drift are a challenge, particularly because current vaccines do not generally achieve sterile immunity even against antigenically well-matched viruses (*3*). One potential route to control AIVs in commercial poultry is to use genetic modification to introduce novel genes that confer resistance to infection (*4*, *5*). Here we evaluate transgenic expression of an RNA hairpin molecule capable of inhibiting influenza viral polymerase activity (*6*).

An RNA expression cassette (Fig. 1A) was designed to use a chicken U6 promoter (*7*) to express the short hairpin RNA molecule, decoy 5 (D5, Fig. 1B) (*8*). This decoy contains the conserved 3′- and 5′-terminal sequences of influenza virus genome segments that encompass the complementary RNA (cRNA) binding site for

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