

**Fig. 4.** Model for gene regulation within the *Drosophila* circadian oscillator. During the late evening (right), PER-TIM dimers (closed and open squares, respectively) enter the nucleus and bind dCLK-CYC dimers (closed and open circles, respectively), thereby repressing *per-tim* activation. Concurrently, the binding of PER-TIM dimers to dCLK-CYC releases dCLK-CYC-dependent repression of *dClk*, thus enabling *dClk* transcription via a separate activator or activator complex (triangle). By midday (left), high levels of dCLK-CYC (in the absence of PER-TIM) serve to activate *per-tim* transcription and repress *dClk* transcription (either directly or through intermediate factors). As the circadian cycle progresses, PER-TIM dimers accumulate and enter the nucleus during the late evening to start the next cycle. Dashes, maximal repression; plus signs, maximal activation; wavy lines, mRNA.

expression in other tissues. However, the autonomy and synchrony of *per* expression in diverse tissues in the head and body suggest that the circadian feedback loop mechanism is the same in all tissues (23) and argue against fundamental tissue-specific differences in the feedback loop mechanism.

An important aspect of circadian biology is how the clock regulates clock-controlled genes (CCGs). In mammals, it has been shown in vitro that CLOCK and BMAL-1 (the mammalian ortholog of CYC) activate vasopressin gene transcription and that all three mouse PERs and TIM repress this activation, resulting in peak vasopressin mRNA transcripts by midmorning (ZT 6) (24). Although this mode of regulation may be more general for CCGs whose mRNA transcripts peak in phase with *per* (or *mPer*), it does not explain how CCGs that cycle in antiphase are regulated. The results presented here provide a possible mechanism by which the clock regulates CCGs whose mRNAs cycle in antiphase to those of *per*. The similarities between *dClk* and *cry* mRNA profiles in the wild type and in several single and double circadian mutants suggest that PER-TIM release of dCLK-CYC repression may serve a more general role in regulating CCG mRNAs that cycle in antiphase to *per* mRNA.

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25. We thank I. Edery for the *dClk* RNase protection probe; M. Rosbash and J. Hall for *dClk<sup>l<sup>rk</sup></sup>* and *Cyc<sup>0</sup>* single mutants and *per<sup>01</sup>;dClk<sup>l<sup>rk</sup></sup>* and *per<sup>01</sup>;Cyc<sup>0</sup>* double mutants; B. Krishnan for statistical help; G. Cahill for comments on the manuscript; and A. Eskin, S. Dryer, G. Cahill, J. DeBruyne, and B. Krishnan for helpful discussions. Supported by National Institute of Neurological Disorders and Stroke grant NS-31214.

25 May 1999; accepted 6 August 1999

## Light-Independent Role of CRY1 and CRY2 in the Mammalian Circadian Clock

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Cryptochrome (CRY), a photoreceptor for the circadian clock in *Drosophila*, binds to the clock component TIM in a light-dependent fashion and blocks its function. In mammals, genetic evidence suggests a role for CRYs within the clock, distinct from hypothetical photoreceptor functions. Mammalian CRY1 and CRY2 are here shown to act as light-independent inhibitors of CLOCK-BMAL1, the activator driving *Per1* transcription. CRY1 or CRY2 (or both) showed light-independent interactions with CLOCK and BMAL1, as well as with PER1, PER2, and TIM. Thus, mammalian CRYs act as light-independent components of the circadian clock and probably regulate *Per1* transcriptional cycling by contacting both the activator and its feedback inhibitors.

Daily rhythms in physiology and behavior are driven by endogenous oscillators called circadian clocks (1). In all known cases, circadian timekeeping is cell-autonomous, generated at least in part by a feedback loop involving clock proteins that inhibit the transcription of their own genes (2). Regulation of the transcriptional

feedback loop by light is thought to mediate setting of circadian clocks to light-dark cycles (2). In mammals, as in *Drosophila*, a negative feedback loop of *Per* gene transcription involving PER and TIM proteins is probably central to the clock (2-4). A heterodimeric activator consisting of the basic helix-loop-helix (bHLH)-PAS proteins CLOCK (5) and BMAL1 (6, 7) drives mouse *Per1* (*mPer1*) transcription from E-box regulatory sequences (6), and the mPER1 protein in turn acts to inhibit CLOCK-BMAL1 activity (4). In *Dro-*

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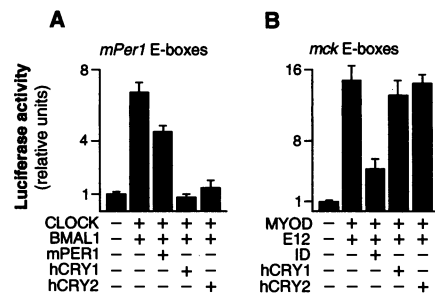
*sophila*, a homologous activator, CLOCK-CYC(dBMAL), plays a similar role (8).

Genetic studies have implicated cryptochromes (CRYs) (9) in light-dependent signaling to circadian clocks in plants (10) and *Drosophila* (11). *Drosophila* CRY acts directly as a circadian photoreceptor: it exhibits light-dependent binding to TIM, and it blocks the negative feedback action of the PER-TIM complex on CLOCK-CYC(dBMAL) in a light-dependent manner (12). Targeted disruption of either of the two identified mouse *Cry* genes results in alterations of circadian period (13, 14), and disruption of both together results in arrhythmicity (14). These results suggest that mammalian CRYs could play a central role in the circadian clock independent of light, leaving debatable the question of whether or not they might have additional photoreceptor functions (15).

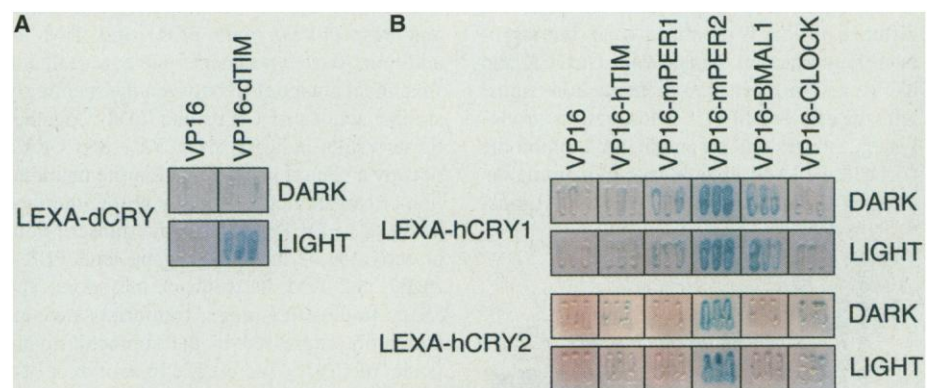
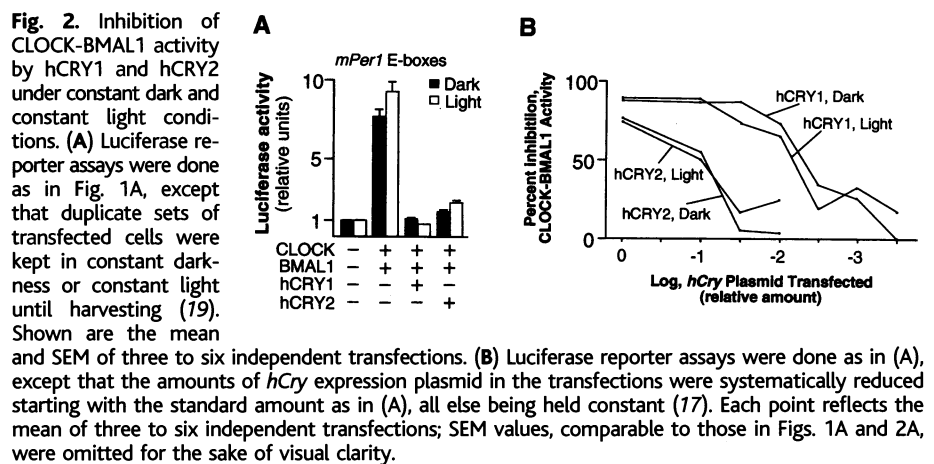
To test the hypothesis that mammalian CRYs have light-independent actions in the circadian clock, we examined the activity of human CRY1 and CRY2 (hCRYs) (16) in our previously described assay in which CLOCK-BMAL1 drives a luciferase reporter gene from *mPer1* gene E boxes in cultured cells (4, 6). Figure 1A shows results from experiments in which the cells were kept in darkness from the time of transfection until harvesting (17). As we previously reported (4), mPER1 produced a partial but significant inhibition of CLOCK-BMAL1 activity, which is indicative of its role in negative feedback. Both hCRY1 and hCRY2 strongly inhibited

CLOCK-BMAL1 activity, with hCRY1 consistently showing a somewhat more potent inhibition than hCRY2. No detectable effect on this strong inhibition by hCRY1 or hCRY2 was produced by coexpression of mPER1 (18). Like mPER1 (4), hCRY1 and hCRY2 produced no significant inhibition of MYOD-E12 activity, a heterodimeric bHLH factor also acting from E boxes, whereas the known inhibitor of MYOD-E12, the ID protein, inhibited strongly (Fig. 1B). These results indicate that hCRY1 and hCRY2 potently and specifically inhibit *mPer1* gene transcription by CLOCK-BMAL1 in the absence of light, and they suggest that a role for CRYs in the mammalian circadian clock is to inhibit *Per1* gene expression.

To determine whether light could modulate hCRY activity, we next performed transcriptional reporter assays as above in paired sets of cells that had been kept in constant darkness or constant light (19). No systematic or substantive differences were observed in the ability of hCRY1 or hCRY2 to inhibit CLOCK-BMAL1 in light and dark conditions (Fig. 2A). Because inhibition of CLOCK-BMAL1 activity by hCRYs is so potent in our standard assay, it was possible that we missed a modulatory effect of light. We therefore carried out experiments in which the amount of *hCry* cDNA expression plasmid in each transfection reaction was systematically decreased over several orders of magnitude, all else being held constant (17). The curves for inhibition of CLOCK-BMAL1



**Fig. 1.** Potent and specific inhibition by hCRY1 and hCRY2 of *mPer1* gene activation by the CLOCK-BMAL1 heterodimer in the absence of light. Transcriptional activation in cultured mammalian cells of a luciferase reporter gene from E-box sequences derived from the *mPer1* gene (A) or the muscle creatine kinase (*mck*) gene (B) is shown (4). (A) Effect of mPER1, hCRY1, or hCRY2 on transactivation by the CLOCK-BMAL1 heterodimer. (B) Effect of ID protein, hCRY1, or hCRY2 on transactivation by the MYOD-E12 heterodimer. A plus or minus sign denotes an expression plasmid with or without, respectively, the indicated full-length cDNA insert. Cells were kept in constant darkness from the time of transfection until harvesting, except for a brief exposure to a dim red safelight at the time of medium change (17). Shown are the mean and SEM of three to six independent transfections.



**Fig. 3.** Light-independent interactions of hCRY1 and hCRY2 with mammalian clock components. Yeast two-hybrid filter-lift assays were performed as described (20). Blue precipitate reports cumulative  $\beta$ -galactosidase activity, resulting from transactivation of the *lacZ* reporter gene by protein-protein interaction. Triplicate yeast patches expressing the indicated LEXA hybrid (rows) and the indicated VP16 hybrid (columns) were derived from three independent transformants. All results were verified with at least three additional independent transformants generated with new preparations of each expression plasmid. All hybrid proteins were full length, except for VP16-CLOCK, which was CLOCK(1-389) in this experiment. hTIM, human TIM. "Dark" or "light" indicates yeast grown in constant darkness or light from the time of transformation with plasmid DNA (12). (A) Control assays showing light-dependent interaction of *Drosophila* CRY (dCRY) with *Drosophila* TIM (dTIM), as reported (12). (B) Light-independent interactions of hCRY1 and hCRY2 with other clock components. Yeast patches showing hCRY1 interactions were grown on the same plate and assayed on the same filter as the *Drosophila* controls shown in (A). Pink discoloration of some patches in bottom panels is due to the *Ade 2* mutation in the L40 strain.

transactivation as a function of the amount of *hCRY* transfected were superimposable for dark and light conditions (Fig. 2B). The slightly greater potency of *hCRY1* than *hCRY2* observed under our standard conditions (Figs. 1A and 2A) was easily discernable as a large displacement on the *x* axis between the *hCRY1* and *hCRY2* curves (Fig. 2B). Thus, not only is light unnecessary for *hCRY1* and *hCRY2* inhibition of CLOCK-BMAL1 transcriptional activity, it has no detectable modulatory effect over the full dynamic range of *hCRY1* and *hCRY2* action.

Could *hCRY* inhibition of CLOCK-BMAL1 activity in cultured cells reflect a direct interaction with CLOCK or BMAL1 (or both) or an interaction with endogenous TIM or PER proteins (or both), which are known feedback inhibitors of CLOCK-BMAL1 activity? To test whether such interactions occur, we used a yeast two-hybrid assay (20) in which yeast transformants were grown in duplicate sets under constant light or darkness (12). As we recently reported (12), *Drosophila* CRY interacts with TIM in a light-dependent manner (Fig. 3A), whereas no detectable interaction was observed between CRY and dCLOCK, CYC(dBMAL), or PER under dark or light conditions (12, 18). In contrast, *hCRY1* and *hCRY2* showed specific interactions with mammalian clock components that were independent of light (Fig. 3B). *hCRY1* produced strong interaction signals with both mPER2 and BMAL1, a modest but reproducible interaction signal with mPER1, and no interaction signal above background with CLOCK or hTIM. *hCRY2* was similar to *hCRY1* in that it produced a strong interaction signal with mPER2, but different in that it produced weak but reproducible interaction signals with CLOCK and hTIM and no detectable interaction signal with mPER1 or BMAL1. These results strongly suggest that *hCRY1* and *hCRY2* inhibit the CLOCK-BMAL1 heterodimer in mammalian cells by forming direct contacts with it, pos-

sibly within a multiprotein complex including PER and TIM proteins.

In our functional assays, mammalian CRYs, PERs, and TIM all inhibit *mPer1* promoter activation by CLOCK-BMAL1. The ability of CRYs to bind both to the activator (CLOCK and BMAL1) and to other inhibitors (PER2, PER1, and TIM) suggests that CRYs could regulate the actions of the other inhibitors. To test this possibility, we carried out transcriptional reporter assays designed so that CLOCK-BMAL1 activity would be inhibited only partially by *hCRY1* or *hCRY2*. We then looked for synergistic or antagonistic effects of *hCRYs* on the inhibitory actions of hTIM or mPER2. The typical partial inhibition we observe with hTIM was significantly antagonized by either *hCRY1* or *hCRY2*, whereas the comparable partial inhibition produced by mPER2 was not significantly affected by *hCRY1* or *hCRY2* (Fig. 4). Furthermore, coexpression of hTIM together with mPER2 produced the same partial inhibition as either alone (18), as we previously reported for hTIM together with mPER1 (4). These results indicate a specific functional antagonism between CRYs and TIM and suggest cross-regulation among the proteins inhibiting CLOCK-BMAL1 activity within the circadian clock feedback loop.

We conclude that CRY1 and CRY2 are circadian clock components that negatively regulate *Per1* transcription independently of light. Our work indicates that they inhibit CLOCK-BMAL1 transactivation activity, very likely by means of direct contacts between CRY1 and BMAL1 and between CRY2 and CLOCK. We have established that CRY1 and CRY2 can interact with PER2 and, respectively, with PER1 and TIM. In addition, our experiments have revealed a functional antagonism between the seemingly similar actions of CRYs and TIM. Together these results indicate that CRY1 and CRY2 occupy a central position within the mammalian circadian clock, virtually at the interface between CLOCK-BMAL1, the transcriptional activator of the *mPer1* gene, and PER1, PER2, and TIM, its feedback inhibitors. The recent finding that *mPer1* transcripts are constitutively expressed in the suprachiasmatic nuclei of *Cry1*, *Cry2* double mutant mice at a high level, comparable to the peak in wild-type mice, provides strong *in vivo* support for our conclusions (21).

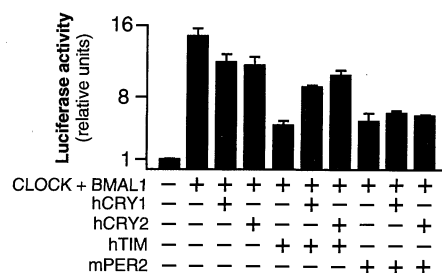
A role for CRY1 and CRY2 in mammalian circadian clock negative feedback has been reported independently (22), although in that study there was no examination of the effect of light or dark on CRY1 or CRY2 function. The distinct patterns of interaction of CRY1 and CRY2 with PER1, PER2, and TIM revealed in our two-hybrid assays are consistent with the reported ability of CRY1 or CRY2 to coimmunoprecipitate with PER1, PER2, PER3, or TIM

from transfected mammalian cells (22). Because of its high sensitivity, coimmunoprecipitation from cellular extracts might detect very low-affinity interactions not detected in two-hybrid assays. Alternatively, coimmunoprecipitation could reflect the presence of two noninteracting proteins within a multiprotein complex containing additional endogenous PER or TIM proteins, which are known to be expressed in various mammalian cell lines (18, 22, 23). As shown here, the distinct protein interaction profiles of CRY1 and CRY2 could underlie the different circadian phenotypes observed in *Cry1* and *Cry2* mutant mice.

The light-independent role of mammalian CRYs in circadian clock negative feedback contrasts sharply with that of *Drosophila* CRY, which has been demonstrated by us and our collaborators to function directly as a photoreceptor that regulates the action of the PER-TIM complex (12). We suggest that *Drosophila* CRY exemplifies the ancestral role of a photoreceptor acting as a light-dependent regulator of the circadian feedback loop, whereas mammalian CRYs have preserved a role within the circadian feedback loop but shed their direct photoreceptor function. We cannot, however, exclude the possibility that mammalian CRYs act as photoreceptors for other possible functions, circadian or otherwise, not detected in our assays.

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17. Full-length *hCry1* and *hCry2* cDNA expression plasmids were prepared, respectively, from pDH1996-1 and pDH1996-2 (16) by digestion with Sac I and Xba I (*hCry1*) or Nde I and Xba I (*hCry2*) and blunt-ligation inserts into the Eco RV site of pCDNA3 (Invitrogen). Transfections of NIH 3T3 cells and dual luciferase reporter assays were performed as described (4), with 200 ng of each pCDNA3 expression plasmid under standard conditions. The total amount of expression plasmid in each transfection reaction was held to 1



**Fig. 4.** Functional antagonism between *hCRY1* or *hCRY2* and hTIM. Luciferase reporter assays in cells kept under dark conditions, as in Fig. 1A, are shown. On the basis of the titrations shown in Fig. 2B, the amounts of *hCry1* and *hCry2* expression plasmids transfected were chosen to give approximately 20% inhibition of CLOCK-BMAL1 activity.

μg by addition of nonrecombinant pCDNA3, as necessary. Results represent three to six different transfected cultures, with the experiments performed on at least two different days. For dark conditions, plates of NIH 3T3 cells were wrapped in foil immediately after transfection until the time of harvesting (48 hours), except for a single brief medium change performed under dim red light (Kodak Safelight, Wratten Series 1A) 24 hours before harvesting.

18. E. A. Griffin Jr., D. Staknis, C. J. Weitz, unpublished data.

19. Transfected cells under dark (17) and light conditions were processed in parallel and incubated together. For light conditions, cells were illuminated (Fiber-Lite High Intensity Illuminator, Series 180; set at 10 relative illumination units) immediately after transfection until the time of harvesting. Plates were positioned 15 to 30 cm from the tips of the twin fiber-optic guides.

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24. We thank A. Sancar for *hCry1* and *hCry2* cDNA clones and S. A. Kay for comments on the manuscript. Supported by National Institute of Mental Health grant MH-59943 and the Armenise-Harvard Foundation for Advanced Scientific Research (both to C.J.W.).

22 July 1999; accepted 9 September 1999

# Negative Feedback Regulation of TGF-β Signaling by the SnoN Oncoprotein

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Smad proteins mediate transforming growth factor-β (TGF-β) signaling to regulate cell growth and differentiation. The SnoN oncoprotein was found to interact with Smad2 and Smad4 and to repress their abilities to activate transcription through recruitment of the transcriptional corepressor N-CoR. Immediately after TGF-β stimulation, SnoN is rapidly degraded by the nuclear accumulation of Smad3, allowing the activation of TGF-β target genes. By 2 hours, TGF-β induces a marked increase in SnoN expression, resulting in termination of Smad-mediated transactivation. Thus, SnoN maintains the repressed state of TGF-β-responsive genes in the absence of ligand and participates in negative feedback regulation of TGF-β signaling.

TGF-β signals through the Smad proteins to regulate cell growth and extracellular matrix production (1). In the absence of TGF-β, Smad proteins are distributed in the nucleus and cytoplasm (2–5). Upon stimulation by TGF-β1, Smad2 and Smad3 become phosphorylated by the activated TGF-β receptors and oligomerize with Smad4 (4–7). These Smad complexes then accumulate in the nucleus where they interact with other transcription factors (8), bind to DNA through their NH<sub>2</sub>-terminal Mad homology-1 (MH1) domains (9–11), and activate transcription of TGF-β-responsive genes through the COOH-terminal MH2 domains (3, 5, 7). As the common mediator, Smad4 complexes with various pathway-restricted Smads to transduce signals by TGF-β, bone morphogenic proteins, and activin (5, 7).

To identify new components of the Smad pathway, we used epitope tagging coupled with affinity purification to isolate Smad4-associated proteins (12). Flag-tagged full-length or truncated Smad4 was stably transfected into 293T cells. Cellular proteins associated with Smad4 were affinity-purified with anti-Flag agarose followed by elution

with the Flag peptide and visualization by silver staining (13). Two proteins of 80 and 97 kD associated predominantly with the MH2 domain of Smad4 (S4C) (Fig. 1A), less strongly with the full-length Smad4 (14), but not with the MH1 and linker domains (S4NL). Microsequencing analysis (13) identified the 80-kD protein as the human c-SnoN and the 97-kD protein as c-Ski (15, 16). SnoN and Ski are two closely related members of the Ski family of nuclear oncoproteins that were identified on the basis of homology with v-Ski, the transforming protein of the Sloan-Kettering virus (17). Human SnoN is a ubiquitously expressed nuclear protein of 684 amino acids (15, 18). Overexpression of SnoN leads to transformation of chicken and quail embryo fibroblasts as well as muscle differentiation of quail embryo cells (19). High levels of SnoN were also detected in carcinoma cells of the stomach, thyroid, and lung (15). SnoN is a transcriptional repressor and interacts with the nuclear hormone receptor corepressor N-CoR (20). Both oncogenic transformation and transcriptional repression require the NH<sub>2</sub>-terminal portion of SnoN (20, 21), which is highly homologous to c-Ski and v-Ski (15).

In 293T cells cotransfected with various Flag-tagged Smad proteins and hemagglutinin (HA)-tagged SnoN, SnoN coprecipitated with Smad4 or Smad2 (Fig. 1B) but not with Smad1, Smad5, and Fast1 (22). A low level of SnoN associated with Smad3, partly as a

result of reduced SnoN expression in cells cotransfected with Smad3 (Fig. 1B). This Smad3-mediated reduction of SnoN expression is reproducible and important (see below). Purified recombinant Smads and SnoN can also interact in vitro (14), indicating a direct interaction between the two. Associations of SnoN with the Smads were mediated by the MH2, but not by the MH1 domains of the Smads (Fig. 1B). Binding of SnoN to Smad2 (Fig. 1C) and Smad3 (14) required the NH<sub>2</sub>-terminal 96 amino acids of SnoN, whereas residues 138 to 255 in SnoN mediated interaction with Smad4 (Fig. 1C).

SnoN binds to a specific DNA element, GTCTAGAC, but only with the help of an unidentified DNA binding partner (or partners) (21). Because this sequence is identical to the Smad-binding element (SBE) (11), we examined whether Smad4 is the DNA binding partner of SnoN. Whereas purified SnoN proteins failed to bind DNA in an electrophoretic mobility shift assay (EMSA) (10), the Flag-SnoN-HA-Smad4 complex bound to the SBE (Fig. 2A). Furthermore, anti-Flag or anti-HA supershifted the complex, confirming the presence of both SnoN and Smad4. Thus, Smad4 mediates binding of SnoN to SBE.

The effect of SnoN on TGF-β-induced and Smad-mediated transcriptional activation was examined in Hep3B cells. Expression of increasing amounts of SnoN resulted in a stronger inhibition of transcription from the 3TP-lux promoter, induced either by TGF-β (Fig. 2B) or by overexpression of Smad2 and Smad4 (Fig. 2C), but had little or no effect whenever Smad3 was also coexpressed. The NH<sub>2</sub>-terminal 366 amino acids of SnoN are sufficient for binding to the Smads, and this region also mediated transcriptional repression (22). On the other hand, a SnoN fragment containing residues 255 to 684 failed to bind to all three Smads (Fig. 1C) and did not repress TGF-β-induced transactivation (22).

SnoN repressed transactivation of Smads not by blocking the hetero-oligomerization of the Smads (14), but by recruitment of the transcriptional corepressor N-CoR. In nuclear extracts of 293T cells cotransfected with HA-Smad4 and Flag-SnoN, endogenous N-CoR was detected in the anti-HA immunoprecipitate (Fig. 2D). Thus, N-CoR formed a complex with Smad4 and Smad2 (14), but only when SnoN was coexpressed (Fig. 2D). Be-

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*Science*, New Series, Vol. 286, No. 5440. (Oct. 22, 1999), pp. 768-771.

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## References and Notes

### <sup>5</sup> **Mutagenesis and Mapping of a Mouse Gene, Clock, Essential for Circadian Behavior**

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