Entrainment of the Circadian Clock in the Liver by Feeding

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For the results of SUB1 expression and transgenic rescue of the *sub1* mutant, supplementary material is available on *Science* Online at www.sciencemag.org/cgi/content/full/291/5503/487/DC1. The *SUB1* coding region corresponding to residues 267 to 552 of the SUB1 translation product (SUB1c) was amplified by PCR, expressed, purified from *Escherichia coli*, and used to raise polyclonal antibodies against SUB1. The $^{45}Ca^{2+}$ overlay calcium-binding assay was as described (*31*, *34*). Light sources, hypocotyl measurement, genetic and transgenic analyses, RNA blot, immunoblot, and GUS fusion protein cellular localization analyses were as described (*12*, *14*, *21*, *22*, *35*).

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Entrainment of the Circadian Clock in the Liver by Feeding

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Circadian rhythms of behavior are driven by oscillators in the brain that are coupled to the environmental light cycle. Circadian rhythms of gene expression occur widely in peripheral organs. It is unclear how these multiple rhythms are coupled together to form a coherent system. To study such coupling, we investigated the effects of cycles of food availability (which exert powerful entraining effects on behavior) on the rhythms of gene expression in the liver, lung, and suprachiasmatic nucleus (SCN). We used a transgenic rat model whose tissues express luciferase in vitro. Although rhythmicity in the SCN remained phase-locked to the light-dark cycle, restricted feeding rapidly entrained the liver, shifting its rhythm by 10 hours within 2 days. Our results demonstrate that feeding cycles can entrain the liver independently of the SCN and the light cycle, and they suggest the need to reexamine the mammalian circadian hierarchy. They also raise the possibility that peripheral circadian oscillators like those in the liver may be coupled to the SCN primarily through rhythmic behavior, such as feeding.

The light-dark (LD) cycle is the most reliable and strongest external signal that synchronizes (entrains) biological rhythms with the environment. In mammals, LD information is perceived by specialized retinal photoreceptors and conveyed directly to the SCN of the hypothalamus, where it entrains circadian oscillators in what is regarded as the master clock of the organism (*1, 2*). In addition, other cyclic inputs, such as temperature, noise, social cues, or rhythmic access to food, may also act as entraining agents, although the effects of these rhythmic signals on behavior are often weak.

When food is available only for a limited time each day, rats increase their locomotor activity 2 to 4 hours before the onset of food availability (*3*). Such anticipatory behavior also occurs in other mammals and in birds and is often paralleled by increases in body temperature, adrenal secretion of corticosterone, gastrointestinal motility, and activity of digestive enzymes (*4–6*). Entrainment of anticipatory locomotion by restricted feeding (RF) occurs independently of the LD cycle, in constant light, and in SCN-lesioned animals (*7, 8*), suggesting that the circadian oscillators entrained by RF are distinct from those entrained by light.

Using a transgenic rat model in which the mouse *Per1* gene promoter has been linked to a luciferase reporter, we continuously monitored the rhythmic expression of this "clock gene" by recording light emission from tissues in vitro (*9*). We used this model to investigate the effects of RF on rhythmicity in the liver, an organ that is directly involved with food processing, as well as in the SCN and lung.

We first exposed young rats to an RF regimen, in which food was available only for 4 hours during the light portion of a 12-hour:12-hour LD cycle, and recorded their locomotor activity (*10*). Within 3 days, the rats began to increase their wheel-running several hours before food became available, and there was an increase in the amount of nighttime running and also a change in the pattern (Fig. 1A), as has been observed previously (*7, 8*). After 2, 7, or 19 days of RF, we killed the animals; explanted the liver, lung, and SCN; and measured luciferase from each tissue in vitro (*11*) (Fig. 2). Despite the marked effects of this regimen on locomotor behavior, the phase of the SCN rhythm was unaffected and remained phase-locked to the light cycle, even after 19 days of RF (Fig. 3A). This result is consistent with reports that RF does not entrain multi-unit neuronal activity in the SCN (*12*) and supports the general notion that entrainment to cycles of food availability does not directly involve the SCN.

In contrast, the circadian clock in the liver was entrained by the 4-hour RF regimen (Fig. 3A). By the second day of RF, the four liver samples that were measured had already shifted an average of 10 hours, a slightly smaller and somewhat more variable response than the 12-hour shift achieved by liver cultures from rats exposed to RF for 7 or 19 days (Fig. 3A). The large phase shift after only 2 days suggests that the liver may have a unique ability to adapt temporally to changes in the feeding pattern.

Rhythmicity in the lung was also affected by the 4-hour RF regimen. Explants taken from four animals on the second day of RF showed a range of responses: two were arrhythmic, one was rhythmic but with such low amplitude that phase could not be reliably measured, and the fourth was rhythmic but unshifted relative to ad lib–fed controls. By the seventh day of the 4-hour RF regimen, the lung explants were shifted by 6 hours and were not shifted further after 19 days of RF (Fig. 3A).

Because the lung is not directly involved in the response to food and yet was shifted by the 4-hour regimen, we considered the possibility that RF might be acting through a global signal(s) such as the hormonal changes accompanying the stress that this treatment is known to produce [e.g., increases in blood levels of corticosterone (*13*)]. We tested the

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possibility that a general phase-shifting signal was provided by the stress of RF in two ways. We increased the duration of the period of food availability from 4 to 8 hours. There is evidence to suggest that this regimen allows a normal daily food intake and decreases both the amount of corticosterone secreted (*13*) and the intensity of anticipatory running (*14*). We partially confirmed these observations; daily food consumption after 3 to 4 days in our 8-hour RF animals was similar to that of ad lib–fed rats (*15*), anticipatory running was markedly reduced, and the pattern of running in the dark was changed in comparison to that of rats exposed to 4-hour RF (compare Fig. 1, A and B). We therefore assume that the stress engendered by 4-hour RF was attenuated in the 8-hour RF regime. However, circulating levels of corticosterone measured 30 min before the onset of food availability were markedly increased above basal daytime levels (*16*). After 7 days of 8-hour RF, the phase of liver rhythmicity was shifted by 12 hours, whereas that of the lung was unshifted in relation to ad lib–fed controls (Fig. 3B). In order to confirm that liver rhythmicity had been entrained rather than masked in some way by the 8-hour RF regimen, we repeated this experiment but fasted the animals for 1 or 2 days after 7 days of 8-hour RF. Rhythmicity in livers from the fasted animals retained their phase relation to the previous RF cycle, demonstrating that they had been entrained (Fig. 3B). The phase of liver rhythmicity in ad lib–fed control animals was unaffected by 1 or 2 days of fasting (*15*).

The animals' response to the 8-hour RF regimen suggested that the stress of RF was not responsible for phase-shifting the liver (although it may have been involved in shifting the lung) or that stress might contribute to phase-shifting both organs, with the liver being more sensitive to its effects. We directly tested the effect of the stress hormone corticosterone on the phase of rhythmicity in the liver and lung by injecting 5 mg of corticosterone intraperitoneally into ad lib–fed rats each day for 7 days (*17*), 5 hours after lights were turned on, which is the time when corticosterone levels reach the anticipatory peak in RF animals. Circulating levels of corticosterone measured 30 min after the injection were on average 581 ± 174 (SEM) ng/ml, which is a 15-fold increase over basal levels (*18*). Neither the corticosterone injection nor the vehicle control injections had any effect on the phase of rhythmicity of the liver or lung explants (Fig. 3C), nor did they have any noticeable influence on wheel-running behavior (Fig. 1C). These results do not support a role for corticosterone in mediating the effects of RF on the phase of rhythmicity in either the liver or lung. Balsalobre *et al.* (*19*) have recently reported that they were able to shift the rhythm of *Dbp* gene expression in mouse liver with timed injections of dexamethasone. Although their results may appear to conflict with ours, in fact they do not; our corticosterone injections were made at zeitgeber time 5, a time at which their dexamethasone injections had no effect on phase. Although increases in corticosterone do not account for the phase-shifting effects of RF, adrenal steroids may have other effects on liver rhythmicity.

In the RF paradigm, rhythmicity in the liver may respond to one or more of the many signals directly associated with feeding (e.g., the taste of food, the distension of the stomach, increased uptake of metabolites, or the change in insulin levels); the lung might be shifted by its own increased activity associated with the large increase in amount of wheel-running and the change in its temporal pattern, which occurs in the 4-hour RF but not in the 8-hour RF regimen.

Our data invite a reexamination of the current simple model of hierarchical organization in the mammalian circadian system. In this model, the SCN is entrained by the light cycle and, from its position at the top of the circadian hierarchy, drives or entrains peripheral rhythms in adaptive phase relations to the environment and to each other. Food's action as an entraining signal in the absence of the SCN has been seen as a minor exception. Our

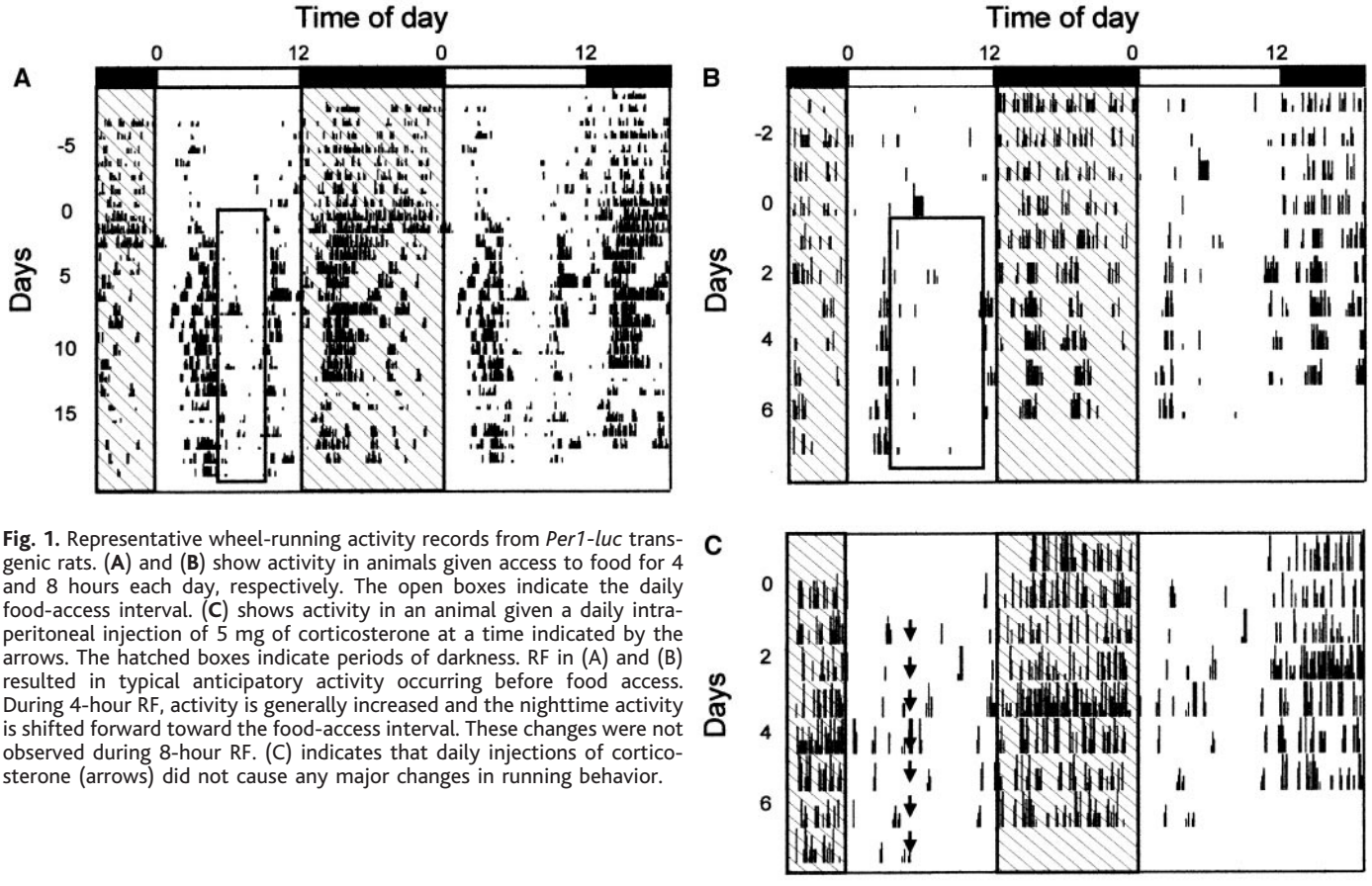

Fig. 1. Representative wheel-running activity records from *Per1-luc* transgenic rats. (**A**) and (**B**) show activity in animals given access to food for 4 and 8 hours each day, respectively. The open boxes indicate the daily food-access interval. (**C**) shows activity in an animal given a daily intraperitoneal injection of 5 mg of corticosterone at a time indicated by the arrows. The hatched boxes indicate periods of darkness. RF in (A) and (B) resulted in typical anticipatory activity occurring before food access. During 4-hour RF, activity is generally increased and the nighttime activity is shifted forward toward the food-access interval. These changes were not observed during 8-hour RF. (C) indicates that daily injections of corticosterone (arrows) did not cause any major changes in running behavior.

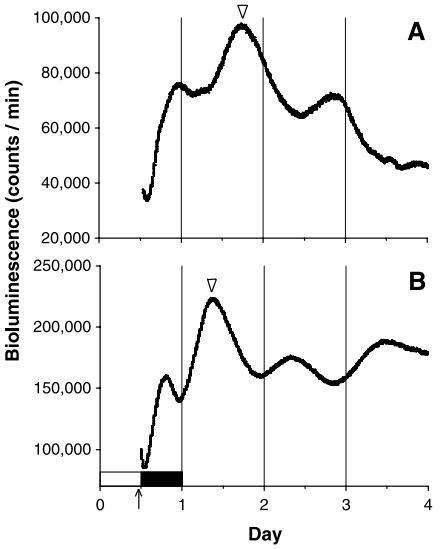

Fig. 2. Rhythms of light emission by liver explants. Shown are raw data from (**A**) an ad lib–fed control animal and (**B**) an animal that had been exposed to a 4-hour RF regimen for 7 days. Both animals had been kept on the light cycle indicated by the white and black bars in (B), and both were killed and the tissues explanted at the time shown by the arrow. Because the pattern of light emission is quite variable during the first 12 to 14 hours after explantation, we consider that the phase of the tissue in vivo is best reflected by the phase of the peak during the first full subjective day (12 to 36 hours after explant). The phase of these peaks is consistent from animal to animal (Fig. 3). Here, the phase chosen is indicated by the inverted triangles.

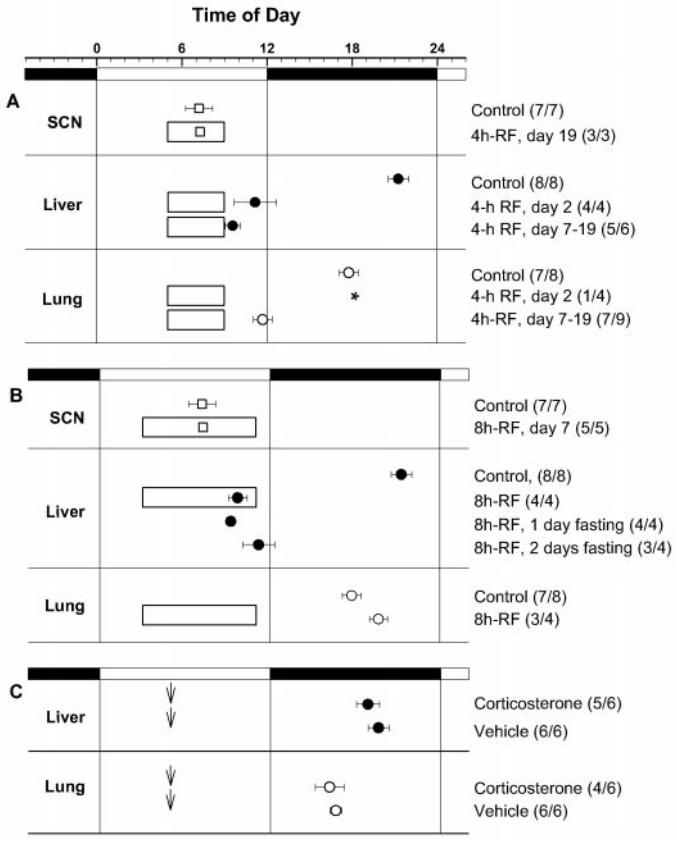

Fig. 3. Effects of restricted feeding [(**A**) 4-hour RF and (**B**) 8-hour RF] and (**C**) corticosterone treatment on tissue luciferase rhythmicity. The average times (±SEM, shown by error bars) of peaks from the different tissues are plotted against the LD cycle shown at the top of each panel. The timing and duration of food availability is indicated by open boxes in each section. Arrows indicate the times of corticosterone or vehicle (DMSO) injection. The sample size is shown in parentheses, denoting the number of explants (each from a different animal) from which phase could be accurately measured/the number of explants tested. The asterisk indicates the peak of oscillation in the one lung explant on the 4-hour RF regimen in which phase could be determined. Control values from ad lib–fed animals are repeated in (A) and (B). In these experiments, the phases of the liver and lung in control animals occurred later in the night than in our previous study (*9*). This may be due to the fact that animals in the present study were older and were weaned and caged individually, whereas those in the earlier study were still with their mothers. Daily injections of corticosterone, with DMSO as the vehicle (C), had no effect on rhythmicity in either the liver or lung. The phase of liver rhythmicity was significantly different from control values in all groups of RF rats; the phase of lung rhythmicity was significantly different from control values only after 7 days of 4-hour RF [analysis of variance, followed by Dunnett's test ($P < 0.05$)]. All other phase differences (within structures) were not significantly different.

data now show that the phase of circadian rhythmicity in the liver is independent of both the SCN and the light cycle under conditions that are no less "normal" than those that usually prevail in the laboratory (*20*). In light of this result, one can ask whether there are, as predicted by the hierarchical model, neural or hormonal signals generated by the SCN that entrain peripheral oscillators. If so, why do they not act on the liver in the food entrainment paradigm? Alternatively, does food intake itself generate entraining signals for the liver and perhaps other digestive organs as well, with the role of the SCN limited to the entrainment of feeding behavior—a view that places at least one link in the causal chain completely outside the animal. Certainly the hierarchical model is still at least partially valid: the SCN does generate rhythmic neural and hormonal signals that influence rhythms in other brain areas (*21, 22*), in peripheral endocrine organs (e.g., pineal) (*23*), and in behavior (*24*). Nevertheless, circadian oscillations in the liver (and perhaps in other peripheral organs) may respond more directly to the environment. Thus, the liver of mammals may be similar to some peripheral organs in *Drosophila* and zebra fish, whose rhythms respond directly to environmental signals (*25, 26*). This revised view of mammalian circadian organization emphasizes the possible role of behavior in maintaining internal temporal structure and underlines the importance of analyzing circadian organization in environments in which behavior is carefully monitored and controlled with more sophistication than has been customary.

Our results have potential practical importance. Major complaints of shift workers and time zone travelers often involve gastrointestinal symptoms, which might be alleviated by careful attention to meal timing. Furthermore, entrainment of the liver (and perhaps other peripheral organs) to a specific phase without disrupting SCN-driven rhythms, such as sleep and body temperature, might increase both the practicality and the therapeutic efficacy of timed administration of drugs or radiation.

Note added in proof: While this report was being prepared for publication, a paper on a similar subject was published (*27*).

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10. Newly weaned, 3- to 4-week-old male and female heterozygous transgenic rats were caged individually with access to a running wheel and exposed to 12-hour: 12-hour LD cycles. Food-restricted rats were given access to food either 4 or 8 hours each day, beginning 5 or 3 hours after lights were turned on, respectively, whereas controls had ad lib access. Food was given and taken away by hand, and at the time that food was given to restricted rats, controls received an amount of food similar to what they had consumed during the previous 24 hours.
11. Within 1 hour before lights were turned off, rats were anesthetized by CO_2 and decapitated. Explants of the SCN, liver, and lung were cultured as described previously (*9*). Original data (1-min bins) were smoothed by an adjacent-averaging method with 2-hour running means as described [P. Meerlo *et al.*, *J. Biol. Rhythms* **12**, 80 (1997)]. "Peak" was defined as the highest point of smoothed data and was used as a phase marker. Data from males and females were pooled because no sex differences were observed.

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16. Serum concentrations of corticosterone were measured with a commercial radioimmunoassay kit (Coat-A-Count, Diagnostic Products, Los Angeles). One rat showed 207.9 and 41.0 ng/ml and another showed 105.8 and 68.9 ng/ml at 3 hours after lights were turned on ("prefeeding") and 9.5 hours after lights were turned on ("basal"), respectively. The difference between our results and those reported in (*13*) may be due to the fact that our animals were just weaned and growing rapidly, so that any restrictions in food access may be stressful. Aging markedly reduces the prefeeding corticosterone secretion in rats exposed to RF [S. Honma *et al.*, *Am. J. Physiol.* **271**, R1514 (1996)].
17. Five milligrams of corticosterone (Sigma) dissolved in 0.2 ml of dimethyl sulfoxide (DMSO) was given daily as intraperitoneal injections for 7 days. Control animals received 0.2 ml of DMSO.
18. On the seventh day of treatment, the serum level of corticosterone, 30 min after injection, was 581 ± 174 (SEM) ng/ml ($n = 6$) and 39 ± 17 ng/ml ($n = 6$) in animals receiving corticosterone and DMSO injections, respectively.
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28. We thank M. Quigg for measuring corticosterone concentrations and K. M. Greene and S. C. Miller for technical assistance. This work was supported in part by the NSF Center for Biological Timing, NIH grant MH 56647 (to M.M.); by travel grant 130173/410 from the Norwegian Research Council (to K.-A.S.); and by a research grant from the Japanese Ministry of Education, Science, Sports and Culture and the Japanese Ministry of Health and Welfare (to H.T.).

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Effects of *cis* Arrangement of Chromatin Insulators on Enhancer-Blocking Activity

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Chromatin boundary elements or insulators are believed to regulate gene activity in complex genetic loci by organizing specialized chromatin structures. Here, we report that the enhancer-blocking activity of the *Drosophila* suHw insulator is sensitive to insulator copy number and position. Two tandem copies of suHw were ineffective in blocking various enhancers from a downstream promoter. Moreover, an enhancer was blocked more effectively from a promoter by two flanking suHw insulators than by a single intervening one. Thus, insulators may modulate enhancer-promoter interactions by interacting with each other and facilitating the formation of chromatin loop domains.

Insulators regulate gene activity in diverse organisms (*1–8*). The defining feature of insulators as a class of regulatory elements is their ability to block enhancer-promoter interactions when positioned interveningly. One of the best characterized insulators is suHw, a 340–base pair (bp) element from the *Drosophila* gypsy retrotransposon. It protects transgenes from chromosomal position effects and blocks various enhancer-promoter interactions (*9–13*). SUHW, a zinc-finger DNA binding protein, and MOD(MDG4), a BTB domain protein, are essential for suHw function (*13–16*). Using divergently transcribed reporter genes in transgenic *Drosophila* embryos, we have shown that an enhancer blocked from the downstream promoter by suHw is fully competent to activate an upstream promoter (*12*).

To probe the insulator mechanism, we tested the effect of suHw copy number on its insulator strength in *Drosophila* embryos. The *zerknullt* enhancer VRE (ventral repression element) has been shown to be partially blocked by suHw (*12*). In blastoderm embryos, the *V2* transgene containing VRE and E2, an *even-skipped* stripe 2 enhancer, directs reporter expression in a composite pattern of broad dorsal activation and dominant ventral repression of the E2 stripe (Fig. 1, A and D) (*13, 17, 18*). A single 340-bp suHw insulator element in the *VS2* transgene partially blocked the upstream VRE enhancer (Fig. 1, B and D). Two tandem suHw elements (arranged as direct repeats) were inserted between VRE and E2, resulting in *VSS2*. Instead of enhanced blockage, *VSS2* embryos exhibited a loss of suHw insulator activity (Fig. 1, C and D). This was observed in most *VSS2* embryos (Fig. 1D) and in all 10 independent *VSS2* lines, indicating that it is unlikely to be caused by chromosomal position effects. Genomic polymerase chain reaction (PCR) analysis of independent *VS2* and *VSS2* lines further verified the structural integrity of the transgenes in vivo (Fig. 1E) (*19*).

To determine whether the loss of insulator function in *VSS2* embryos is enhancer-specific, we constructed transgenes using a *rhomboid* neuroectodermal enhancer (NEE) and a *hairy* stripe 1 enhancer (H1) (*13*). The *NLH* embryos containing NEE and H1 enhancers separated by a 1.4-kb neutral spacer (L) exhibited a composite *lacZ* pattern directed by both enhancers (Fig. 2, A and H). A single suHw element in the *NSH* transgene blocked the upstream NEE enhancer (Fig. 2, B and H), whereas two tandem suHw elements (*NSSH*) did not block the NEE enhancer (Fig. 2, C and H). A second group of transgenes uses a *twist* mesoderm enhancer (PE) and an *evenskipped* stripe 3 enhancer (E3) (*13*). Both enhancers are active when separated by the L spacer (*PL3*) (Fig. 2, D and H). Insertion of a suHw element in the *PS3* transgene blocked the upstream PE enhancer (Fig. 2, E and H), whereas two tandem suHw elements (*PSS3*) did not block the PE enhancer (Fig. 2, F and H). Replacing one of the two suHw elements in *PSS3* with a spacer of comparable size (A) restored the enhancer-blocking activity of the remaining suHw in *PSA3* embryos (Fig. 2G), indicating that loss of insulator activity with two suHw elements is not due to the spacing change but to the presence of the additional insulator. Genomic PCR with individual *NSH*, *NSSH*, *PS3*, and *PSS3* lines indicated that the transgenes were structurally intact (Fig. 2I). These results suggest that the loss of insulator activity with tandemly arranged suHw is independent of the enhancer tested.

The enhancer-blocking activity of suHw may require its interaction with other sites (or insulators) within the nucleus. A second suHw nearby may compete dominantly for the existing suHw and affect the neighboring enhancer-promoter interactions, depending on the *cis* arrangement of these elements. To test this hypothesis, we constructed the *SVS2* transgene in which the VRE enhancer is flanked by two suHw elements. In contrast to the loss of insulator function seen in *VSS2* embryos, the VRE enhancer is more effectively blocked in *SVS2* embryos than in *VS2* embryos (Fig. 3, A, B, and D). Thus, it is the tandem arrangement rather than physical proximity that causes the loss of insulator activity. VRE-mediated dorsal activation of the divergently transcribed *miniwhite* is also diminished in *SVS2* embryos (*19*), indicating that VRE is blocked from promoters on either side. suHw-mediated blockage of VRE is significantly reduced in *SVS2/mod(mdg4)^{u1}* embryos (Fig. 3C), indicating that a MOD(MDG4)-mediated complex is required for the enhanced insulator activity (*13, 16, 20*). *VSS2*,

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