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Down-regulation of Early Sea Urchin Histone *H2A* Gene Relies on *cis* Regulative Sequences Located in the 5′ and 3′ Regions and Including the Enhancer Blocker *sns*

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The tandem repeated sea urchin α-histone genes are developmentally regulated by gene-specific promoter elements. Coordinate transcription of the five genes begins after meiotic maturation of the oocyte, continues through cleavage, and reaches its maximum at morula stage, after which these genes are shut off and maintained in a silenced state for the life cycle of the animal. Although *cis* regulative sequences affecting the timing and the level of expression of these genes have been characterized, much less is known about the mechanism of their repression. Here we report the results of a functional analysis that allowed the identification of the sequence elements needed for the silencing of the *α-H2A* gene at gastrula stage. We found that important negative regulative sequences are located in the 462 bp *sns* 5 fragment located in the 3′ region. Remarkably, *sns* 5 contains the *sns* enhancer blocking element and the most 3′ *H2A* codons. In addition, we made the striking observation that inhibition of the anti-enhancer activity of *sns*, by titration of the binding proteins in microinjected embryos, also affected the capability of *sns* 5 to down-regulate transgene expression at gastrula stage. A further sequence element essential for repression of the *H2A* gene was identified upstream of the enhancer, in the 5′ region, and contains four *GAGA* repeats. Altogether these findings suggest that down-regulation of the *α-H2A* gene occurs by the functional interaction of the 5′ and 3′ *cis* sequence elements. These results demonstrate the involvement of a genomic insulator in the silencing of gene expression.

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Keywords: histone genes; transcriptional repression; enhancer blocker; genomic insulator

Introduction

Embryonic development and cell differentiation relies on the expression of genes that are precisely controlled in space and time. Gene expression in eukaryotes is largely controlled at the transcription initiation level and depends on the specific interaction between transcription factors and their cognate recognition sequences of enhancer and promoter elements. Binding of transcription factors is accompanied by local changes in the chromatin structure brought about by enzymes that mobilize or change the structure of nucleosomes and post-translationally modify histones.[1]

The sea urchin early (or α) histone gene family represents a model system for the study of transcription regulation during development.[2] The sea urchin genome contains four distinct histone gene families, which are sequentially expressed during ontogeny and which code for a repertoire of histone variants.[3–5] The α-subtype histone genes are organized as tandem arrays of a repeating unit containing the five genes.[6] Their transcription begins after meiotic maturation of the egg, continues through early cleavage, and reaches a peak at

† D.D.C. and R.M. contributed equally to this work.
Abbreviations used: CAT, chloramphenicol-acetyl transferase; MBF-1, modulator binding factor-1; M30, MBF-1 binding site; *sns*, silencing nucleoprotein structure.

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morula stage. After hatching blastula they are already silenced. By the gastrula stage, expression of the early histone genes is undetectable, and the late set of histone genes becomes the most transcriptionally active form.[4,7–9]

Sea urchin α-histone genes were cloned almost three decades ago.[10,11] The *cis*-acting elements required for temporal and maximal expression for each of the five genes have been extensively investigated by gene transfer methodology and promoter binding in nuclear extracts. However, despite years of intense studies, the molecular basis for coordinate timing of expression and the mechanisms underlying their repression at late stages of development are still elusive.

Previously, we reported the identification of the *cis* and *trans*-acting promoter elements required for the timing of transcription of the α-*H2A* gene during embryogenesis of the sea urchin *Paracentrotus lividus*. An enhancer element, termed modulator, is located in the 5′ region.[12–15] The *H2A* enhancer, which has a bipartite structure[13,14] (and unpublished observations), contains a binding site for the MBF-1 transcription factor, a Krüppel-like zinc finger protein.[15,16] MBF-1 is not a stage-specific transcription factor, in that its expression does not parallel the timing of expression of the α-*H2A* histone gene. MBF-1 transcripts are in fact present at roughly constant levels in eggs and at both early and late developmental stages.[16] In addition, persistent MBF-1 binding activity and expression of a transgene driven by the enhancer can be demonstrated after silencing of the α-*H2A* gene at gastrula stage.[17] *cis*-Acting regulative sequence elements have also been characterized in the promoter of the other four genes of the early histone repeating unit of *Strongylocentrotus purpuratus.* Maximum expression of α-H2B relies only on the octamer element,[18] while for α-*H3*, the CCAAT box and a gene-specific element are all that is required for high level ontogenic expression.[19] Similarly, maximal and proper temporal expression of α-H1 gene depends on sequences that lie within the region −65 and +39 that include Inr and internal elements, while two other upstream binding sites have no apparent effect on transcription.[20] By *in vitro* transcription studies, the expression of α-H4 seems also to depend on an Inr, internal, and upstream DNA elements.[21]

The mechanism of down-regulation of the sea urchin early histone genes is unknown and the *cis*-regulative sequences involved are poorly characterized. As mentioned above, when the α-*H2A* gene is silenced as the sea urchin embryo enters the gastrula stage, the enhancer remains constitutively active and the binding of the MBF-1 transcription factor persists.[17] So, the enhancer does not play a predominant role in the down-regulation of the α-*H2A* gene expression. As the embryo approaches gastrulation, the only major change that is detected is the structural change of the early histone genes' chromatin configuration. Down-regulation is accompanied in fact, by a transition from a non-nucleosomal to a nucleosomal pattern[22] and by the appearance of three micrococcal nuclease cutting sites near the 3′ terminus of the α-*H2A* gene, which, because of their spacing, probably flank two positioned nucleosomes.[23,24] The DNA sequence underlying these structures is termed *sns* 2 (silencing nucleoprotein structure 2). As described, repression of the α-*H2A* gene at gastrula stage depends most probably on regulative sequences located in the *sns* 2 DNA fragment.[17] Such evidence is in contrast with the presence of negative sequence elements in the promoter region of α-*H1* and α-*H3* genes.[19,20]

It is of great interest that *sns* 2 includes the *sns* enhancer blocker. Genomic insulators are a general phenomenon in eukaryotes and have now been found in a wide range of organisms. They are genetic regulators of gene expression that modulate enhancer action by preventing the communication between enhancer and promoter in a directional manner.[25–31] In addition, insulators by acting as barriers against the propagation of condensed chromatin may buffer a transgene from chromosomal position effect.[32–36] Because of these properties, it is thought that insulators organize domains of gene expression. As previously described, the sea urchin *sns*, when included in artificial constructs and tested in enhancer blocking assays,[37] displays directional enhancer blocking activity in either orientation, both at early and late developmental stages.[38] In addition, if *sns* intervenes between two enhancers, only the enhancer located distally from the promoter with respect to the site of insertion is attenuated. Furthermore, *sns* does not prevent the blocked enhancer from *trans*-activating a promoter in the other direction, suggesting that *sns* represses enhancer–promoter interaction neither by enhancer inactivation nor by inducing local assembly of a repressive chromatin structure.[39] Interestingly, *sns* maintains the enhancer blocking function in human cells and deletion of any of the *cis*-acting sequences abolishes insulator activity[40] (and unpublished results).

The real function of the *sns* fragment in the normal context of the early histone gene cluster is not known. Recent evidence suggests that *sns* probably interferes with the *H2A* enhancer in the promiscuous interaction with the downstream H1 promoter (unpublished results). In addition, its location in the *sns* 2 fragment (Figure 1) might imply that *sns* is also implicated in down-regulation of the α-*H2A* gene at gastrula stage. To assess the involvement of the *sns* insulator in the mechanism of silencing we undertook an *in vivo* functional analysis of the α-*H2A* transcription unit. Here we report that the important negative regulative sequences correspond to the 5′*GAGA* repeats, located upstream of the enhancer, and to a 3′ DNA region that includes the last codons of the *H2A* gene and the downstream *sns* enhancer blocking element. We propose that down-regulation of the α-*H2A* gene occurs by formation of a DNA loop domain induced by the functional interaction of all of these *cis*-regulative elements.

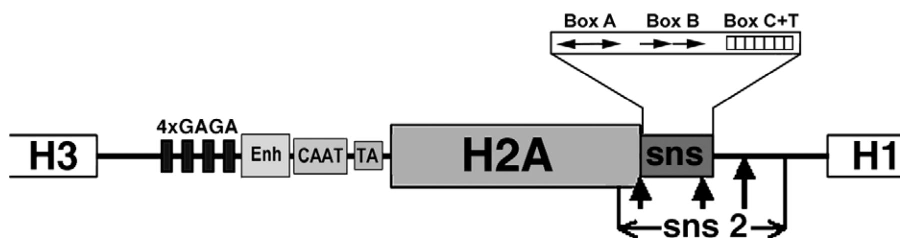

Figure 1. Schematic drawing of *H2A* transcription unit and regulative sequence elements. TA and CAAT represent *TATA* and *CCAAT* boxes, respectively. The enhancer is the *H2A* modulator and upstream of it is located a purine sequence containing four *GAGA* tandem repeats. At the 3′ end *sns* 2, comprised between the coding and spacer regions, down-regulates transgene expression driven by the *H2A* enhancer–promoter region at gastrula stage (see Figure 2). The nuclear protein binding sites, Box A, Box B, and Box C+T (corresponding to 14 GA repeats in the lower strand), which are essential for the enhancer blocking function of the *sns* genomic insulator, are indicated in enlargement. Arrows indicate Micrococcal nuclease cutting sites that appear upon silencing.

Results

sns 2 is a position-dependent silencer that relies on the *sns* insulator for down-regulation at gastrula stage

The molecular arrangement of the *H2A* gene in the α histone repeating unit is shown in Figure 1. The drawing highlights the *cis* regulative elements driving expression of the α-*H2A* gene throughout development. Down-regulation depends on sequence elements present in the *sns* 2 fragment.[17] *sns* 2 extends further upstream and further downstream than the *sns* enhancer blocking element.[38] It contains the last 90 bp of the protein-coding

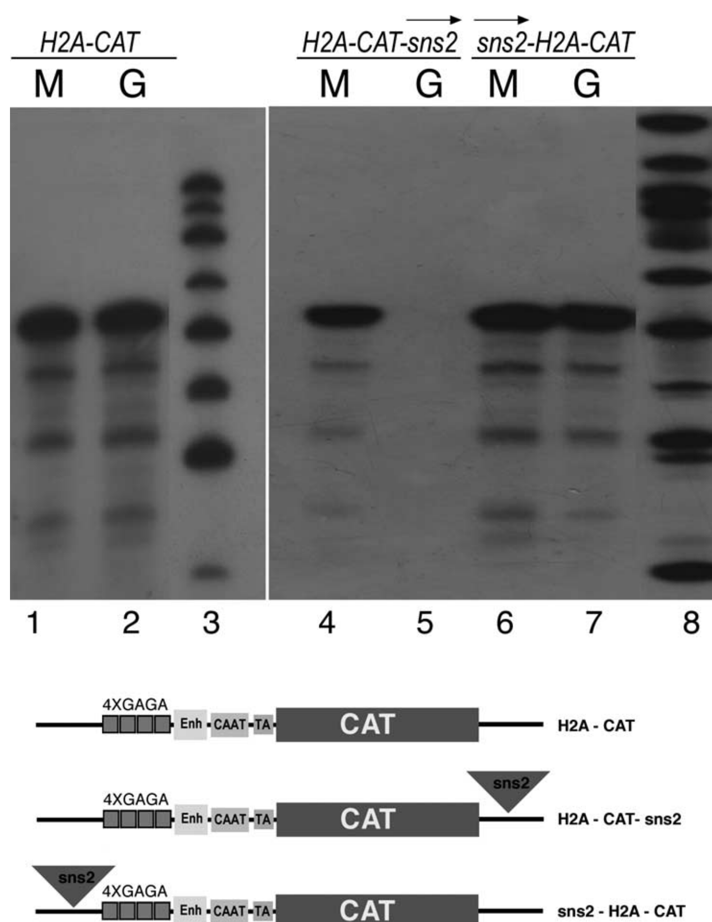

Figure 2. Determination of positional silencing capability of *sns* 2 in transgenic embryos. Total RNA from 30 *P. lividus* embryos at morula (M) and gastrula (G) stages microinjected with the indicated transgenes were hybridized with a ^{32}P-labelled CAT antisense probe and processed for the RNase protection assay described in Materials and Methods. Lanes 3 and 8, end-labelled DNA markers. Drawings highlight the position of the *sns* 2 element relative to the *H2A* promoter. Down-regulation of the *H2A*-CAT transgene at gastrula stage occurs only when *sns* 2 is located downstream of the coding region.

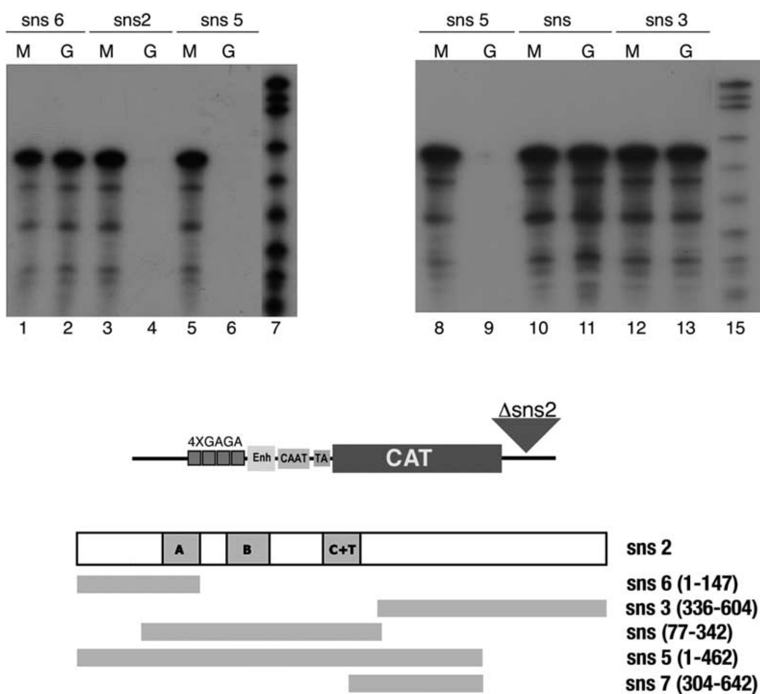

Figure 3. Functional activity of *sns* 2 deletion fragments. RNase protection assays performed with RNA extracted from 30 or 40 transgenic embryos injected with the CAT construct driven by the *H2A* enhancer–promoter region and containing *sns* 2 deletion fragments, schematically drawn below, downstream of the CAT gene. Protein binding sites A, B and C+T, required for the anti-enhancer activity of *sns* are boxed. Only *sns* 5 down-regulates reporter gene expression at gastrula stage. The enhancer blocking element *sns* and the other deletion mutants allow expression from the *H2A* promoter at both early and late developmental stages.

sequence of the *H2A* gene, the three nuclear protein binding sites (Box A palindrome, Box B direct repeat, and Box C+T), essential for the enhancer blocking activity of *sns*,[39] and spacer sequences. We used an *in vivo* transgene expression analysis to study the mechanism of function of *sns* 2 and to identify the *cis* negative regulative elements. Firstly, we microinjected *H2A*-CAT DNA plasmids schematically drawn in Figure 2. In two of the constructs *sns* 2 was placed at the 3′, downstream of the coding region, and at the 5′, upstream of the *H2A* enhancer promoter fragment, respectively. Following fertilization, embryos were raised and transcription of the reporter gene was determined at morula and gastrula stages. The results of the RNase protection assays carried out with RNA samples from the same number of microinjected embryos are shown in Figure 2. In agreement with previous observations,[17] the reporter transgene driven by the α-*H2A* enhancer/promoter fragment was not silenced at gastrula stage and it was constitutively expressed. By contrast, it followed the timing of expression of the α-*H2A* endogenous gene when the *sns* 2 sequence was included in the transgene transcripton unit. However, the *sns* 2 fragment did not inhibit promoter *trans*-activation at early and late developmental stages when placed 5′ to the enhancer. Hence, the presence of *sns* 2 is not sufficient for repression of transcription. Down-regulation occurs only when *sns* 2, as in the natural context, is placed downstream of the coding region of the CAT reporter gene.

To identify the minimal sequence responsible for repression we made nested 5′ and 3′ deletions of the *sns* 2 fragment. To assess the effect of deletions on the regulation of transgene expression, the *sns* 2 subfragments were cloned, in the same orientation as the endogenous *sns* 2 element, downstream of the coding region of the CAT reporter gene driven by the α-*H2A* enhancer/promoter region. Following microinjection into sea urchin embryos, CAT transcripts were detected at morula and gastrula stages by RNase protection assays. The results are shown in Figure 3. The DNA fragments *sns* 6 (lanes 1 and 2) and *sns* 3 (lanes 12 and 13), respectively, corresponding to the most 5′ and 3′ regions of *sns* 2, did not repress transgene expression at gastrula stage. Neither did the *sns* enhancer blocking element (lanes 10 and 11) nor *sns* 7 (not shown). Only the *H2A*-CAT DNA construct containing at the 3′ end *sns* 5 (lanes 5, 6, 8 and 9), which includes the protein coding region, the genomic insulator *sns*, and downstream sequences, followed the temporal expression profile of the α-*H2A* endogenous gene.

The anti-enhancer function of *sns*[38] is independent of orientation and *sns* is essential for transcriptional repression. Therefore, we assessed the capability of *sns* 5 to silence transgene expression when placed 3′ to the coding region in the other direction. Indeed, as shown in Figure 4, inversion of orientation of *sns* 5 did not influence its capability to repress gene expression.

In summary, the mutational analysis of *sns* 2 suggests that the silencing activity requires sequences spanning a large region defined by *sns* 5.

In vivo competition of protein binding to the *sns* insulator and deletion of the 5′*GAGA* repeats abolish down-regulation function of *sns* 5

As previously described *sns* contains three protein binding sites, termed Box A, Box B, and Box C+T[39] (see drawing in Figure 3 for their relative location). The results of the deletion

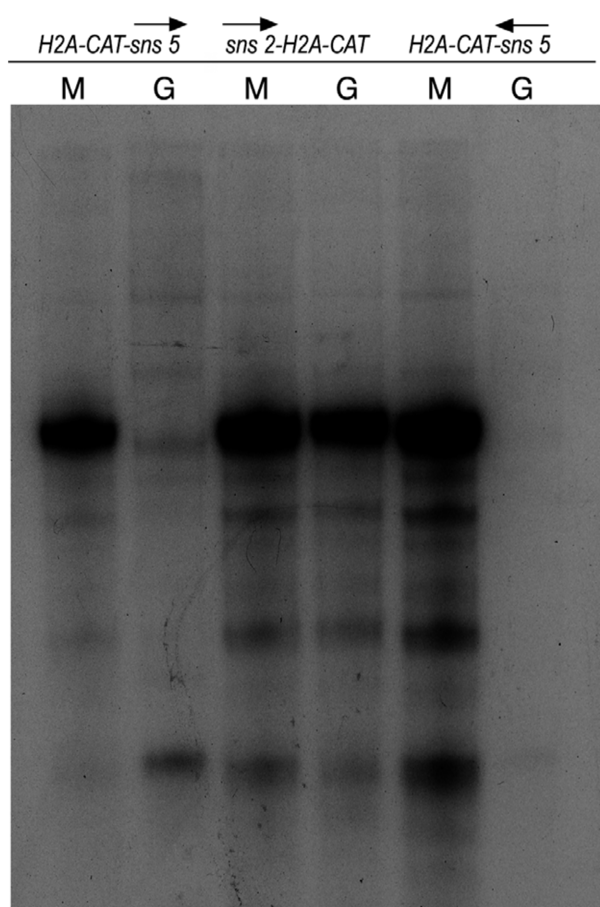

Figure 4. Assessing directionality of transcriptional repression function of *sns* 5 in microinjected embryos. RNase protection assays on 50 injected embryos were carried out as described in Figures 2 and 3. Microinjected *H2A*-CAT DNA constructs were those schematically drawn in Figures 2 and 3, except that *sns* 5 was cloned in either orientation. *sns* 5 silences transgene expression at gastrula stage in both directions.

analysis presented in the previous section suggest that binding of proteins to *sns* 5 is essential for down-regulation of the α-*H2A* histone gene at gastrula stage. To definitively prove the involvement of DNA protein binding sites in the silencing process, we performed *in vivo* competition assays. Previously, we showed that microinjecting excess of either Box A or Box B binding sites inhibited the directional anti-enhancer function of the *sns* element and relieved enhancer activity in an enhancer blocking assay.[39] We used the same approach to define the function of the Box C+T binding site in both enhancer blocking and down-regulation.

Ligated Box C+T oligonucleotides were microinjected into sea urchin embryos together with the insulated *6XM30-sns-CAT* DNA (6XM30 is the enhancer cassette, used for the enhancer blocking assays,[38] which contains six copies of the MBF-1 binding site) and the developmentally regulated *H2A-CAT-sns5* (see drawing in Figure 5) at the molar ratio of 50 to 1. Because the enhancer blocking function of *sns* is not dependent on the developmental stage,[38] transgene expression was determined by RNase protection only at gastrula stage for the insulated and non-insulated constructs. The results of Figure 5 clearly indicate that co-injection of excess of the Box C+T binding site impaired the *sns* capability to block enhancer–promoter interaction (lanes 1 and 2). Because the level of CAT transcripts per embryo was similar to that obtained with the uninsulated *6XM30–CAT* DNA construct (lane 3), these results confirmed[39] that titration of one of the *sns* binding proteins is sufficient to inhibit insulator function. Next, we assessed the involvement of the Box C+T in the silencing of gene expression, and hence of the *sns* fragment in this process. Again, to compare the abundance of transgene transcripts among RNA samples, the same number of microinjected embryos was processed for the RNase protection assays. As indicated by the results shown in Figure 5, titration of the Box C+T binding protein by excess of recognition site had no influence on the transcriptional activity of the *H2A* promoter at morula stage (lane 4) and allowed the expression of the transgene also at gastrula stage (lane 5), when the non-competed construct is down-regulated.

Identical results were obtained when competition was directed towards protein binding to the Box A site of the *sns* element. For these experiments we used a DNA construct containing the *P. lividus* α-*H* 3 and α-*H2A* histone genes. As it will be described elsewhere, we have compelling evidence demonstrating that the *H2A* enhancer prefers to interact with the cognate promoter. We took advantage of this finding, and deleted from the α-*H3* promoter all but the TATA box and Inr, sequence elements responsible for temporal regulation.[19,41] We reckoned that expression of the *H3* transgene in this two gene construct, would only occur if the basal promoter received a transcription signal by the *H2A* enhancer. In this way the expression of the α-*H3* transgene driven by the basal promoter can be used as internal control of the timing of transcription of the α-*H2A* transgene. In addition, since the *H2A* enhancer is constitutively active,[17] we can predict that the α-*H3* transgene devoid of the upstream *H3* promoter regulative elements should be expressed at early and late developmental stages. To distinguish between endogenous and transgene histone transcripts, the two gene construct, schematically drawn in Figure 6, was microinjected into the closely related sea urchin *Spherechinus granularis.* As previously described, the *P. lividus H2A* modulator maintains the enhancer activity in this sea urchin species.[15] The results shown in Figure 6 demonstrate that all predictions were fulfilled. As expected, injection of the *H3* transgene driven by the basal promoter did not produce any detectable RNA transcripts (not shown). On the contrary, high expression of the *H3* gene occurred at morula stage upon microinjection of the *H3-H2A* plasmid (construct A).

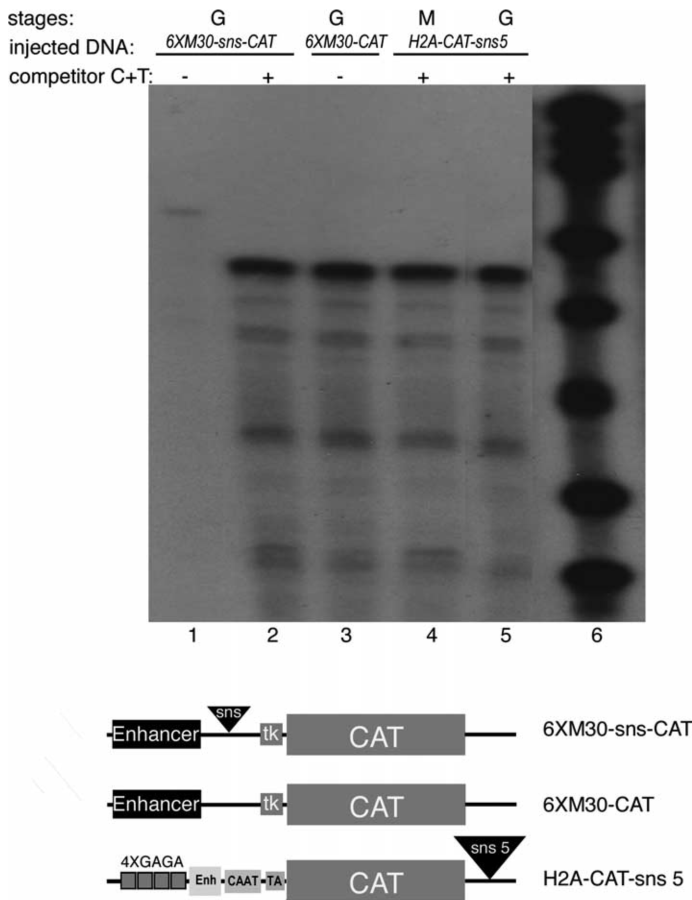

Figure 5. Excess of CT repeats competes with both *sns* enhancer blocking function and *sns* 5 down-regulation capability. The enhancer cassette contains an array of six copies of the binding site (M30) for the MBF-1 transcription factor for the *H2A* enhancer. CAT transcripts were detected by RNase protection assays in embryos at gastrula (G) stage for the insulated and non-insulated constructs and at morula (M) and gastrula stages for the regulated *H2A*-CAT-sns5 plasmid.

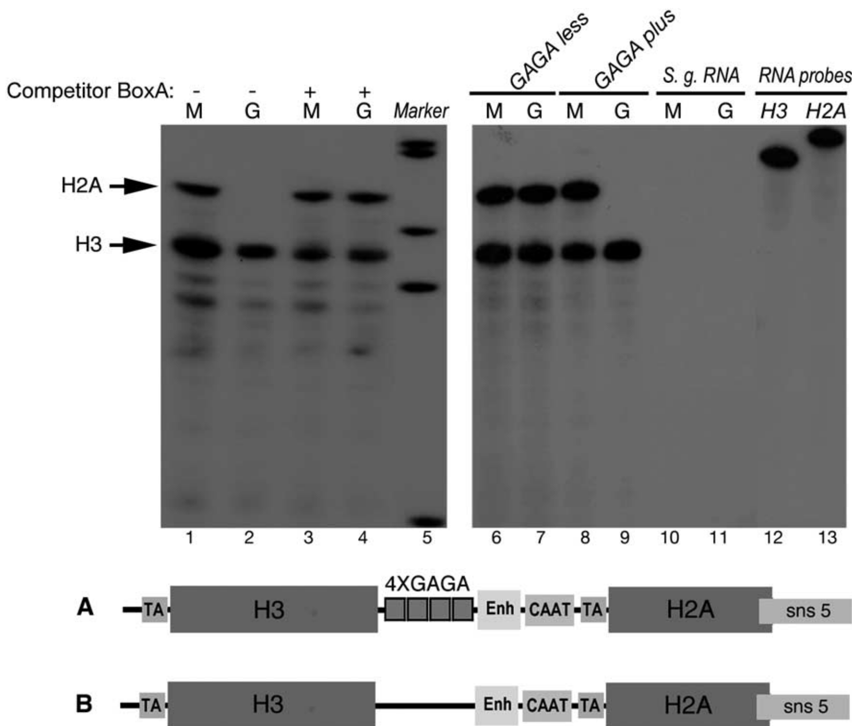

Figure 6. Histone gene expression analysis in transgenic embryos co-injected with excess of the protein, the BoxA binding site and *H3-H2A* gene construct. The *P. lividus* two-gene constructs (A and B), orientated as in the endogenous histone gene repeat, were injected in *S. granularis* embryos. The histone DNA contained the deletion mutant α-*H3* gene, driven by the basal promoter elements, and the wild-type α-*H2A* transcription unit with all regulative sequences. RNase protection was carried out by hybridizing antisense labelled RNA transcribed *in vitro* from *H3* and *H2A* subclones with total RNA from 30 injected embryos or *S. granularis* total RNA from morula (M) and gastrula stages (G). The two *P. lividus* *H2A* and *H3* antisense RNA probes were hybridized together and did not protect any endogenous *S. granularis* RNA band (lanes 10 and 11). Arrows point to the protected 409 nt and 357 nt RNA bands, respectively, for the *H2A* and *H3* transcripts. Impairment of the *sns* function by excess of Box A binding sequences affects the capability of *sns* 5 to down-regulate the *H2A* gene at gastrula stage. Constitutive expression of the *H2A* gene occurs also upon deletion of the 5′ GA repeats.

Remarkably, the level of the *H3* expression was slightly higher than that of the *H2A* gene in one experiment (lane 1), whereas the abundance of the *P. lividus H3* and *H2A* mRNAs was very similar in several other microinjected embryos (lanes 3–8). Transactivation of *H3* gene expression occurred also at gastrula stages (lane 2). The striking finding was that co-injection of excess Box A binding site abolished down-regulation of the *α-H2A* histone gene at gastrula stage (lane 4) without affecting its expression at the earlier stage (lane 3). By contrast, *H3* expression occurred as in the non-competed embryos, indicating once more that the regulation of this gene occurs by gene-specific sequences. The results shown in Figure 6 have some interesting implications. Firstly, they demonstrate the capability of the *H2A* modulator to enhance transcription from the basal *H3* promoter placed at a distance of 1.2 kb from the enhancer and in the other direction. This finding confirms previous results obtained with artificial constructs.[15] Second, transactivation of the basal *H3* promoter by the *H2A* enhancer excludes the presence of an enhancer blocking sequence element in the spacer between the *H3* and *H2A* genes.

In summary, the results of the *in vivo* competition experiments shown in Figures 5 and 6 would imply that all the *cis*-regulative sequences of *sns* 5 are essential for the transcriptional repression of the *α-H2A* at late developmental stages, and that at least two of such sequences are needed for both down-regulation and enhancer blocking function of *sns*.

The Box C+T binding site contains 14 *GA* repeats in the bottom strand.[38] Eight *GA* repeats are also present in the upper strand upstream the *H2A* enhancer (*GAGA* repeats in the drawing of Figure 1) and, as previously shown by EMSA experiments in nuclear extracts, they can compete almost as efficiently as the homologous sequence in the binding of proteins to the Box C+T site.[39] To assess whether the GA repetitions localized upstream of the enhancer are also involved in temporal silencing at gastrula stage, we tested the effect of their deletion on *H2A* transgene expression. The two *P. lividus* histone gene constructs depicted in Figure 6A and B, one containing (*GAGA* plus) and the other lacking (*GAGA* less) the four 5′*GAGA* repeats, were microinjected into *S. granularis* eggs. Embryos were allowed to develop and then were processed for the RNase protection assay. As it may be clearly seen, deletion of the GA repeats allowed constitutive expression of the *H2A* gene during embryogenesis (lanes 6 and 7).

In conclusion, altogether these evidences indicate a direct involvement of the *sns* fragment in the assembly of a silencing complex that represses *α-H2A* histone gene expression at gastrula stage without interfering with the interaction between the *H2A* enhancer and the upstream basal promoter of the *H3* gene. Furthermore, they suggest a functional interaction between the *GAGA* regulative elements positioned 5′ to the enhancer and those located in the 3′ coding and spacer region.

Discussion

Most changes in gene expression are brought about by either the activation or the repression of gene transcription. In principle, transcription of genes could be repressed and their silenced state be maintained by the lack of the necessary activators. However, in most cases transcriptional repression occurs by the action of *trans*-acting protein molecules which by association with the target genes affect gene expression. Bound repressors can prevent transcription by interfering with activators or with the transcriptional machinery.[42–44] In addition, developmentally regulated genes are repressed by epigenetic mechanisms that maintain their silenced state by modulating the structure of chromatin.[45]

The sea urchin α-histone genes are developmentally regulated. The silencing of the *α-H2A* gene has peculiar features. In the first place, down-regulation occurs by an active repression mechanism,[44] in that, transcriptional repression at late blastula stage takes place with the necessary MBF-1 activator still present in the embryo.[16,17] In addition, the negative *cis*-regulative sequences are not clustered in the previously described *sns* 2 fragment[17] located at the 3′ end of the *H2A* gene, as one would had expected. Instead, as we report here, at least one important sequence, corresponding to the eight *GA* repeats, is found upstream of the enhancer, in the 5′ flanking region. Additional negative regulative sequences are included in the *sns* enhancer blocking element. Previously, we have used deletion and *in vivo* competition analysis in transgenic embryos to identify the protein binding sites in the *H2A* enhancer and in the *sns* fragment.[15,17,39] By the same approach we report that some of the important regulative sequences involved in *H2A* repression correspond to the Box A, and Box C+T. Remarkably, the sequence elements Box A, and Box C+T, located within *sns* are important for both enhancer blocking and down-regulation functions. Although not directly proven, we hypothesize that also the Box B, the third protein binding site essential for the enhancer blocking function of *sns*,[39] participates in down-regulation. This is to say that the enhancer blocker *sns* is directly involved in the silencing of the *α-H2A* gene after hatching. However, as reported here, the presence of *sns* in a normal location failed to silence the expression of the transgene driven by the *H2A* enhancer–promoter region that includes the GA repeats.[17] For silencing the *H2A* gene expression, other important sequences are needed. These might be located in the last codons of the *H2A* gene and downstream of *sns*. The DNA fragment containing all these 3′ sequence elements is termed *sns* 5. As for the enhancer blocking activity of *sns*,[38] repression of transgene expression by *sns* 5 is independent of orientation.

The results described here highlight the function of a genomic insulator in its normal chromosomal location. Insulators are genetic regulative elements capable of attenuating the activity of enhancers or other regulative elements. Although the mechanism is poorly understood, insulators maintain transcription fidelity by interfering with the interaction between enhancer and promoter only when interposed between the two. This function, which is defined as enhancer blocking or anti-enhancer is a feature present in most metazoan insulators. Insulators, by restricting enhancer and silencer function may impart functional independence to transcription units in the eukaryotic genome.[28,29,46–48] Cooperation of multiple components confers insulator properties to genomic elements[31,49] and the anti-enhancer and barrier functions are for some insulators, such as the chicken *HS4*, separable entities, each containing different sequence elements.[50] We have already described the characterization of the enhancer blocker *sns*. Because *sns* 5 includes both *sns* and additional *cis*-acting sequences we believe that the insulator activity of the 3′ region of the *H2A* gene lies in the *sns* 5 fragment. In agreement to this, *sns* 5 maintains the capability to directionally restrict enhancer activity in an enhancer blocking assay and protects a transgene from a position effect when integrated in mammalian chromatin (unpublished results). Insulators, such as *sns*/*sns* 5, are not generally repressors of transcription, in that a blocked enhancer is not prevented from eliciting transcription from a promoter of a divergent transcription unit.[49,51,52] Indeed, recent unpublished evidence suggests that the sea urchin insulator in the natural environment of the histone gene sequences, at early blastula stage, decoys the *H2A* enhancer away from the downstream H1 promoter while allowing activation of transcription from the upstream *H3* promoter (unpublished observation). The results of the experiments described here demonstrate an additional role of insulators, i.e. their involvement in the mechanism of repression. Such evidence is not surprising because the *Drosophila gypsy* insulator can also function as a promoter-specific silencer if the activity of mod(mdg4), the second insulator protein that interacts with the suppressor of Hairy wing [su(Hw)], is reduced by mutations.[53] It remains to be established whether the two functions of *sns* 5, i.e. attenuation of enhancer activity and promoter-specific silencer, depend on the interaction of developmental stage-specific proteins.

Taken together the evidence presented here strongly indicates that at late stage of sea urchin development multiple *cis*-acting sequences cooperatively interact to assemble a silencing domain on the *H2A* transcription unit. However, the *H2A* enhancer is not inactivated, in that it can interact in the opposite direction with the basal promoter of the *H3* gene. Hence, any model of silencing must take into account that the *H2A* enhancer is accessible to the MBF-1[16] and to other transcription factors even in the condition of transcriptional repression of the *H2A* gene. How can the bound activators be impeded from interacting with the transcription factors bound to the downstream *H2A* promoter and at the same time be free to transactivate the *H3* gene in the other direction? Down-regulation of the sea urchin α-histone genes entails the assembly of a silenced state that is inherited by the cell progenies during late embryogenesis. This heritable silencing correlates with the transition from a relaxed to a condensed chromatin structure. In fact, during the period of maximum transcriptional activity, at early blastula stage, the chromatin of these genes does not present the regular nucleosomal package and is more accessible to digestion by endonucleases. Concurrent with transcription inactivation of α-histone genes at blastula stage, a defined regular micrococcal nuclease pattern reappears[22,54–56] and two positioned nucleosomes probably assemble in the *sns* 5 fragment.[23,24] Transition from randomized to regular nucleosome spacing occurs also in the enhancer and TATA box-containing promoter region of the *H2A* gene (not shown). It is tempting to speculate that at gastrula stage the assembly of a repressive domain of transcription on the *H2A* gene allows the positioning of a nucleosome in the basal promoter, whereby the binding of general transcription factors and the formation of the transcriptional pre-initiation complex are inhibited without interfering with the binding of the transcription factors, such as MBF-1, to the enhancer. Experiments are in progress to verify this possibility.

Materials and Methods

Construction of plasmids

The *H2A*-CAT plasmids were constructed as follows. A DNA fragment, spanning nucleotides −226 to 40, relative to the *H2A* transcription start site, was generated by restriction enzyme digestion and PCR amplification of a RsaI *H2A* DNA subclone. The promoter fragment was cloned upstream of the CAT reporter gene of the pBL3 as reported.[41] The *sns* 2 fragment was isolated by HindIII and NcoI double digestion of *P. lividus* PH70 histone DNA and cloned at the 3′ or 5′ of the reporter gene of the *H2A*-CAT plasmid. *sns* 2 deletion fragments were obtained by PCR or restriction enzyme digestion and cloned downstream of the reporter coding region. Primers were the following: Sp3 and Sp4 for *sns* 3; Sp8 and Sp6 for *sns* 6; Sp8 and Sp10 for *sns* 5; Sp11 and Sp10 for *sns* 7. The orientation of the DNA inserts was determined by sequence analysis. Construction of the insulated *6XM30-sns-CAT* and non-insulated *6XM30-CAT* plasmids containing the enhancer cassette *6XM30* was described.[38]

P. lividus H3-H2A histone DNA plasmid, containing the *H3* basal promoter and a wild-type *H2A* transcription unit, was constructed in the following way. A histone DNA fragment, comprised between nucleotide −62 (from the *H3* start site) and nucleotide 811 (from the *H2A* start site) was obtained by PCR amplification using the primers H3L1 and Sp17 and cloned in the pBluescript

KS (+) vector. The *GAGA*-less *H3-H2A* plasmid was generated in a similar way. The *H3* gene, spanning nucleotides −62 to 1062, from the *H3* transcription start site, was generated by PCR by using the H3L1 and H3L2 primers. The GA deletion mutant of the *H2A* gene comprised between residues −60 and 811, from the *H2A* start site, was amplified with the H2AP1 and Sp17 primers. The two amplified DNA fragments were cloned in the same orientation and in the order 5′*H3-H2A*3′ as in the sea urchin genome. All DNA clones were checked by sequencing.

Oligonucleotides used in this study

For the complementary oligonucleotides only the sense strand is reported. The underlined sequence refers to the Sau3AI restriction site present also in the complementary oligonucleotide to create 5′ protruding ends after annealing.

BoxA:	GATCCAAACCTCAACACCTCAACG GCCCTTATCAGGGCCACCA;
Box C+T:	GATCCCTCTCTCTCTCTCTCTC TCTCTCTCTCTCTGTCT;
H3L1:	GCTGCGAAGGCGGTGCGA;
H3L2:	GCTCTGTTGA TCTCATGAG;
H2AP1:	TGTGCACACCGTGTCGCT;
Sp3:	GTCGATCATCCGATCTAATAT;
Sp4:	CCATGGATCTTATGTGTATAAATC;
Sp6:	TTGGTGGCCCTGATAAGGGCCGTTGAG;
Sp8:	CAAGCTTCTTGGAGGTGTGA;
Sp10:	ACTGAGCGACACAGAGTA;
Sp11:	TCTGTCTTCCCCATTCCAGT;
Sp17:	CAGGTTGATTACTGTGCGACA.

Microinjection and transgene expression

One picoliter of linearized plasmids, obtained by restriction enzyme digestion, at the concentration of 80–200 ng/μl in 25% (v/v) glycerol, was microinjected into unfertilized eggs from a mature female as described.[40] In the competition experiments, double-stranded Box A or Box C+T oligonucleotides were ligated with T4 DNA ligase and fractionated onto polyacrylamide gel. DNA fragments containing four to six tandem copies were eluted from the gel and mixed with the plasmid solution to be microinjected, at the ratio of 50 to 1. Microinjected embryos were raised up to morula (five hours of development) and gastrula stages (20 hours of development) and processed to monitor transgene expression by RNase protection experiments. Total RNA was extracted from the embryos by one hour incubation at 55 °C in a solution containing 200 μg/ml of Proteinase K and phenol-extraction. Nucleic acid samples were digested with RNase-free DNaseI, and RNA from 30 or 50 microinjected embryos hybridized with antisense ^{32}P-labelled RNA probes. Hybridization conditions, RNase digestion and gel fractionation of the RNase-resistant hybrids were as described.[38]

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