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 $\alpha$-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 regulatory 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 $\alpha$-$H2A$ 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 $\alpha$-$H2A$ 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.

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 $\alpha$) 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 $\alpha$-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...
Sea urchin α-histone genes were cloned almost three decades ago. 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 using embryogenesis of the sea urchin Paracentrotus lividus. An enhancer element, termed modulator, is located in the 5' region. The H2A enhancer, which has a bipartite structure and (unpublished observations), contains a binding site for the MBF-1 transcription factor, a Krüppel-like zinc finger protein. 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. 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. cis-Acting regulatory 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 on the octamer element, while for α-H3, the CCAAT box and a gene-specific element are all that is required for high level ontogenic expression. 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.

By in vitro transcription studies, the expression of α-H4 seems also to depend on an Inr, internal, and upstream DNA elements. 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. 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 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. 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. Such evidence is in contrast with the presence of negative sequence elements in the promoter region of α-H1 and α-H3 genes.

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. 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, displays directional enhancer blocking activity in either orientation, both at early and late developmental stages. 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. Interestingly, sns maintains the enhancer blocking function in human cells and deletion of any of the cis-acting sequences abolishes insulator activity (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 vitro 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.
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. Sns 2 extends further upstream and further downstream than the sns enhancer blocking element. It contains the last 90 bp of the protein-coding

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.

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 32P-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.
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, 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 regulatory 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, 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 transcript 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 5 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 5 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 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 (see drawing in Figure 3 for their relative location). The results of the deletion
molar ratio of 50 to 1. Because the enhancer blocking function of \textit{sns} is not dependent on the developmental stage,\textsuperscript{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 \textit{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\textsuperscript{39} that titration of one of the \textit{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 \textit{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 \textit{sns} element. For these experiments we used a DNA construct containing the \textit{P. lividus} \textit{\alpha-H3} and \textit{\alpha-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 \textit{\alpha-H3} promoter all but the TATA box and Inr, sequence elements responsible for temporal regulation.\textsuperscript{19,41} We reckoned that expression of the \textit{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 \textit{\alpha-H3} transgene driven by the basal promoter can be used as internal control of the timing of transcription of the \textit{\alpha-H2A} transgene. In addition, since the H2A enhancer is constitutively active,\textsuperscript{17} we can predict that the \textit{\alpha-H3} transgene devoid of the upstream \textit{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 6a, was microinjected into the closely related sea urchin \textit{Spheroechinus granularis}. As previously described, the \textit{P. lividus} H2A modulator maintains the enhancer activity in this sea urchin species.\textsuperscript{15} The results shown in Figure 6 demonstrate that all predictions were fulfilled. As expected, injection of the \textit{H3} transgene driven by the basal promoter did not produce any detectable RNA transcripts (not shown). On the contrary, high expression of the \textit{H3} gene occurred at morula stage upon microinjection of the \textit{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 a-H3 gene, driven by the basal promoter elements, and the wild-type a-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. granulatus 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.12–44 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, 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 fragment17 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 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 block 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.\textsuperscript{28,29,46–48} Cooperation of multiple components confers insulator properties to genomic elements\textsuperscript{31,49} and the cooperation of multiple components confers insulator activity of sns functions of enhancer properties to genomic elements.\textsuperscript{2,29,46–48} 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.\textsuperscript{28,29,46–48} Cooperation of multiple components confers insulator properties to genomic elements\textsuperscript{31,49} and the cooperation of multiple components confers insulator activity of sns functions of enhancer properties to genomic elements.\textsuperscript{2,29,46–48}

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 Rsal H2A DNA subclone. The promoter fragment was cloned upstream of the CAT reporter gene of the pBL3 as reported.\textsuperscript{25} The sns 2 fragment was isolated by HindIII and NcoI double digestion of \textit{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 5; Sp8 and Sp10 for sns 3; 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.\textsuperscript{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 nucleotides 624 (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...
Box C

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