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Constitutive Promoter Occupancy by the MBF-1 Activator and Chromatin Modification of the Developmental Regulated Sea Urchin α-H2A Histone Gene

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The tandemly repeated sea urchin α-histone genes are developmentally regulated. These genes are transcribed up to the early blastula stage and permanently silenced as the embryos approach gastrulation. As previously described, expression of the α-*H2A* gene depends on the binding of the MBF-1 activator to the 5′ enhancer, while down-regulation relies on the functional interaction between the 3′ *sns* 5 insulator and the GA repeats located upstream of the enhancer. As persistent MBF-1 binding and enhancer activity are detected in gastrula embryos, we have studied the molecular mechanisms that prevent the bound MBF-1 from *trans*-activating the *H2A* promoter at this stage of development. Here we used chromatin immunoprecipitation to demonstrate that MBF-1 occupies its site regardless of the transcriptional state of the *H2A* gene. In addition, we have mapped two nucleosomes specifically positioned on the enhancer and promoter regions of the repressed *H2A* gene. Interestingly, insertion of a 26 bp oligonucleotide between the enhancer and the TATA box, led to up-regulation of the *H2A* gene at gastrula stage, possibly by changing the position of the TATA nucleosome. Finally, we found association of histone de-acetylase and de-acetylation and methylation of K9 of histone H3 on the promoter and insulator of the repressed *H2A* chromatin. These data argue for a role of a defined positioned nucleosome in the promoter and histone tail post-translational modifications, in the 3′ insulator and 5′ regulatory regions, in the repression of the α-*H2A* gene despite the presence of the MBF-1 activator bound to the enhancer.

Keywords: sea urchin histone genes; chromatin immunoprecipitation; MBF-1 activator; nucleosome phasing; histone modifications

Introduction

Packaging of the eukaryotic genome into the nuclei involves the left-hand toroidal wrapping of 147 bp of DNA around the histone octamer to form the nucleosome, the fundamental unit of chromatin.[1,2] Incorporation of DNA into chromatin has a profound impact on gene expression (and other DNA transactions), as it can severely restrict the accessibility to the transcription machinery.[3,4] As a consequence, activation of gene expression strictly depends on the dynamic change of chromatin configuration.[5,6] Cells utilize two enzymatic mechanisms to modify the structure of chromatin. A family of protein complexes relies on ATP-dependant remodelling machineries that use the energy derived from the hydrolysis of the ATP to alter the structure and topology of nucleosomes.[7–9] Another family includes enzymes that chemically modify specific amino acids of the core histones. Generally, specific amino acids of the histone N-terminal tails are targeted by these enzymes,[10] but residues belonging to the accessible surface of

Abbreviations used: HDAC, histone de-acetylase; ChIP, chromatin immunoprecipitation; MNase, micrococcal nuclease.

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the globular nucleosome core are also modified.[11–13] The modification of specific histone residues is mostly associated with activation, while the modification of others is generally associated with repression. For example, acetylation of lysine residues by histone acetyltransferases (HATs) is a mark of transcriptional activation.[14,15] Conversely, de-acetylation carried out by histone de-acetylases (HDACs) mediates transcriptional repression. Methylation of K9 of H3 signals repression, whereas H3K4 methylation signals activation. Post-translational modifications are functional and flag the histones for further modification. It is generally accepted that the combination of specific histone modifications constitutes a "histone code" that defines the transcriptional state of a given gene.[16,17]

In the sea urchin embryo, the correlation between modification of nucleosomes and transcriptional competence has been poorly investigated. The few reports that in this embryonic system have dealt with chromatin architecture and transcriptional state, concern mainly the early histone genes. Sea urchin early (or α) histone genes are organized as tandem arrays of five independent transcription units (in the order 5′*H1-H4-H2B-H3-H2A*-3′), repeated several hundred fold. Co-ordinate transcription of the α-histone genes is limited to the rapid cleavage stages, reaching a peak at morula/early blastula stage. Thereafter these genes are repressed and never expressed again in the life cycle of the animal.[18–21] The heritable repressed state of the α-histone genes correlates with changes of chromatin organization. During the period of maximum transcription the chromatin of these genes, probed with the coding or spacer regions, shows a highly irregular nucleosomal package, with a randomized nucleosome spacing, and hypersensitivity to nuclease digestion. After cessation of the developmentally programmed transcription of the α-histone genes, a defined regular micrococcal nuclease pattern reappears.[22–24]

We have previously described the *cis*-regulatory sequences and the necessary MBF-1 transcription factor involved in the timing of expression α-*H2A* gene.[25–29] The MBF-1 binding site is located in the modulator element.[29–31] Of some interest, the 30 bp MBF-1 recognition sequence *trans*-activates a viral promoter in sea urchin embryos from remote location, in either orientation and to a similar extent as a tandem array containing several copies of the MBF-1 binding site (unpublished results and Palla *et al.*,[32]). We have also identified the important negative regulatory sequences needed for the silencing of the α-*H2A* gene at gastrula stage. A sequence element, containing four GAGA tandem repeats is located upstream of the enhancer, in the 5′ region. At least four negative *cis* regulatory sequences are found in the 462 bp *sns* 5 fragment, which is comprised between the last *H2A* codons and 3′ spacer sequences.[33] Three micrococcal nuclease sites specifically appear at this position at gastrula stage.[34] Remarkably, *sns* 5 contains an enhancer blocking element, termed *sns*, that as the best characterized insulators displays the capability to block enhancer-activated transcription in a polar and directional manner, in both sea urchin and human cells.[32,35,36] In addition, *sns* interferes with the interaction between the human β-globin LCR and the γ-globin promoter in stable transfected erythroid cells.[37] Both *sns* and *sns* 5 are capable of reducing the influence of the mammalian chromatin environment on an integrated retroviral transgene (unpublished observations). In the normal context of the histone gene cluster the *sns* 5 genomic insulator seems to restrain the action of the H2A enhancer on the downstream H1 promoter (unpublished observations). All *cis*-acting sequences needed for insulator function as well as the 5′ *GAGA* repeats are required for the down-regulation of the *H2A* gene at gastrula stage.[33]

Several lines of evidence suggest that down-regulation of the *H2A* gene occurs by an active repression mechanism, namely, in the presence of the necessary MBF-1 activator. Firstly, the *MBF-1* gene is constitutively transcribed[29] and persistent MBF-1 binding activity can be detected at early and late blastula/gastrula stages.[28] In addition, expression of a transgene driven by multiple MBF-1 binding sites or by the *H2A* promoter-enhancer can be demonstrated after repression of the α-*H2A* gene.[28,32] Consistently, at both early and late developmental stages, the MBF-1 transcription factor bound to the modulator can *trans*-activate the basal *H3* promoter in the opposite direction.[33] Altogether these studies strongly suggest that MBF-1 is not inactivated by chemical modifications and that the *H2A* modulator is accessible to the MBF-1 regulator even under conditions of transcriptional repression. However, association of MBF-1 to the endogenous *H2A* chromatin has yet to be demonstrated. The molecular mechanism that blocks the MBF-1 *trans*-activation of the *H2A* promoter at gastrula stage is unknown. Furthermore, the role of nucleosomes, and the possible chemical modifications of histone tails in the repression of the α-*H2A* gene are unclear.

Here we used chromatin immunoprecipitation (ChIP) assays, on formaldehyde fixed embryos (X-ChIP), and restriction enzyme cleavage of chromatin, as tools to address these issues. We detected binding of the MBF-1 transcription factor to the endogenous chromatin template at both early and late developmental stages. In addition, we mapped, at gastrula stage, two nucleosomes positioned on the *H2A* modulator and promoter regions. Finally, we found association of the histone de-acelytase (HDAC-I) and de-acetylation and methylation of the K9 residue of histone H3 on the promoter and insulator of the repressed H2A chromatin. These data suggest that the assembly of a nucleosome on the basal promoter, and histone tail post-translational modifications in the 3′ insulator and 5′ regulatory region, trigger repression of the α-*H2A* gene despite the presence on the modulator of the MBF-1 activator.

Results

The MBF-1 activator is bound to the enhancer in the repressed α-*H2A* gene at gastrula stage

In order to elucidate the molecular details of the correlates of constitutive enhancer binding activity and down-regulation of α-*H2A* histone gene expression in sea urchin embryos, we determined the effective binding of the MBF-1 activator to the enhancer in the endogenous chromatin template. To this end, we expressed different portions of the MBF-1 protein in *Escherichia coli*. As the activation domain, corresponding to the N-terminal 256 amino acid residues[29] gave the maximum yield of the protein in a soluble form, we have generated polyclonal antibodies against this peptide. As shown in the Western blot of Figure 1(a), the antibodies recognized a single protein band of very similar molecular mass (lanes 2 and 3) in both sea urchin nuclear extracts and *in vitro* MBF-1 mRNA translation products. No reaction occurred with the pre-immune serum (lanes 5 and 6) or with the MBF-1 protein lacking the N terminus region synthesized in the reticulocyte system (lanes 1 and 4). Given the specificity of the anti-MBF-1 antibodies we performed ChIP assays with the aim to determining the MBF-1 activator occupying the H2A enhancer in the transcribed and in the silenced histone gene cluster. Sea urchin embryos at morula and gastrula stages were treated with formaldehyde, the nuclei were purified, and the cross-linked chromatin was sonicated to obtain short DNA fragments ranging between 200 and 1000 bp. The same amount of soluble chromatin from both developmental stages was immune-precipitated with pre-immune and anti-MBF-1 serum. After reversion of the cross-links, DNA

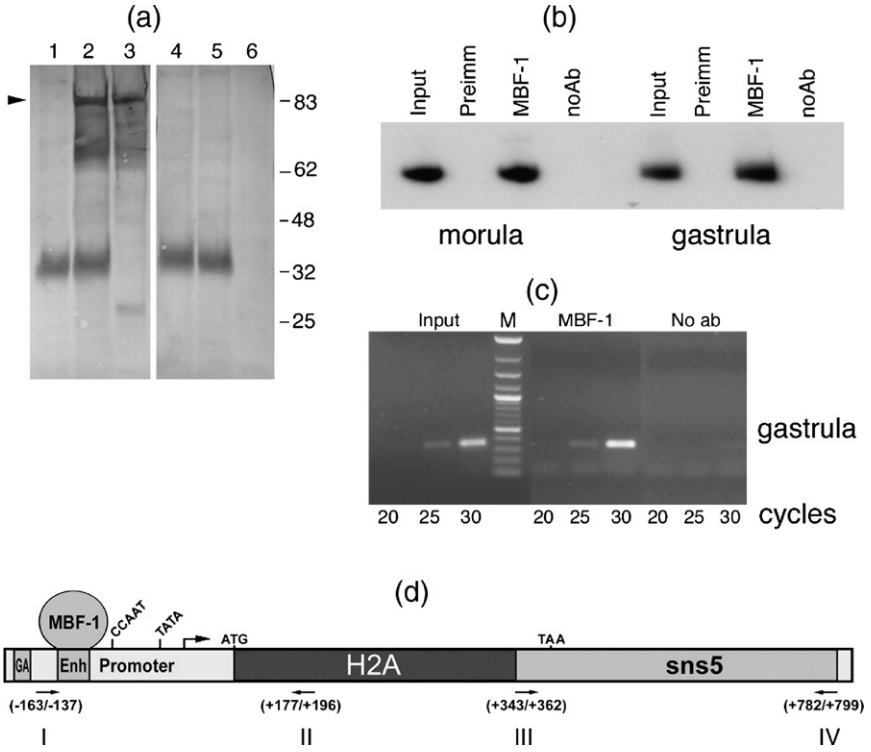

Figure 1. Association of MBF-1 activator to the H2A enhancer. The activation domain of MBF-1 was expressed in *E.coli* and antibodies were raised from the affinity purified protein. (a) Western blot analysis to test the specificity of the anti-MBF-1 antibodies. Nuclear extracts from gastrula embryos (lanes 3 and 6), *in vitro* translation product of MBF-1 (lanes 2 and 5), and *in vitro* translation product of Δ-MBF-1 lacking the activation domain (lanes 1 and 4), were fractionated by SDS-PAGE, blotted on nitrocellulose membrane and incubated with anti-MBF-1 (lanes 1−3) or pre-immune serum (lanes 3−6). The anti-MBF-1 recognized a single band of the expected mass in both nuclear extracts and reticulocyte extracts containing the translation products from the full-length MBF-1 mRNA. (b) Chromatin immunoprecipitation analysis. The same amount of soluble chromatin from crosslinked embryos at morula and gastrula stages was precipitated with anti-MBF-1 polyclonal antibodies (MBF-1), pre-immune serum (Preimm), or incubated without adding anti-MBF-1 or pre-immune serum (noAb). After reversion of the crosslink, DNA from the immunoprecipitates or imput chromatin was extracted and the enhancer promoter region was amplified for 25 cycles with primers I and II, indicated in the drawing in (d), in the presence of labeled d-CTP. The autoradiograph image of the PCR products fractioned on 6% polyacrylamide gel shows MBF-1 occupying its binding site at both morula and gastrula stages. (c) Ethidium bromide staining of an agarose gel of a ChIP experiment carried out with gastrula chromatin as described in (b). M is a 100 bp DNA ladder. The increased intensity of the staining with cycles demonstrates the linear range of the amplification reactions. (d) Schematic representation of the H2A transcription unit. The regulatory sequence elements, GA repeats, enhancer (Enh) with the bound MBF-1 activator, and *sns* 5 insulator, are indicated. Arrows point to the position of the PCR primers used in the ChIP analysis shown in this Figure and in Figure 5.

was purified from the precipitates. Genomic DNA from the precipitated chromatin and input were amplified with two oligonucleotide primers. We found occupancy of the enhancer-promoter region by the MBF-1 transcription factor regardless of the transcriptional state of the *H2A* gene (Figure 1(b)). As shown in Figure 1(c), amplification of the promoter-enhancer region occurred in the linear range, ruling out that in the gastrula stage chromatin, MBF-1 is bound only to a fraction of the *H2A* gene copies. In summary, the results of the ChIP analysis indicate a constitutive association of the MBF-1 regulator with its binding site.

Nucleosome positioning in the enhancer and promoter of the repressed α-*H2A* gene

We analyzed the chromatin configuration and nucleosome positioning in the promoter-enhancer region of the *H2A* gene. Nuclei were isolated from embryos at morula and gastrula stages. To reduce the risk of nucleosome sliding during nuclei handling, the histone–DNA contacts were fixed by formaldehyde crosslinking. Following micrococcal nuclease (MNase) digestions, nucleosomal DNA samples were extracted and analyzed by agarose gel electrophoresis and Southern blot hybridization with a 164 bp fragment encompassing the MBF-1 binding site and the basal promoter. The highly transcribed histone gene chromatin produced a radioactive smear, highly enriched in the mono/di-nucleosome size fragments (Figure 2(a)). Thus the 5′ regulatory region of the *H2A* is not packaged with nucleosomes, or presents a chromatin structure that is highly susceptible to nuclease digestion. By contrast, a regular nucleosomal ladder, similar to the ethidium bromide staining of the bulk chromatin was evident in the repressed α-histone gene chromatin from gastrula embryos. The different chromatin architecture of the *H2A* regulatory region was confirmed by low resolution chromatin indirect end-labeling. Morula and gastrula nucleosomal DNA were digested with the DdeI restriction enzyme and the cleavage products analyzed by blot hybridization with probe C (Figure 2(c)). The double digested morula sample produced a DdeI-DdeI 0.9 kb fragment and a smear of 0.2–0.3 kb, a pattern that is compatible with the lack of a nucleosomal organization. By contrast, the gastrula nucleosomal DNA revealed a discrete banding pattern, generated, most probably, by the MNase cleavage in the linker DNA (Figure 2(b)). Two regions mapping at roughly 150 bp and 500 bp from the DdeI site are not cleaved in chromatin but are in naked DNA, suggesting the presence of nucleosomes at these locations. A third cleavage occurs at about 300 bp from the DdeI restriction sequence, in both, nucleosomal and protein free DNA. This cutting site overlaps with the Sau3AI and HpaII recognition sequences that, as it will be described below, are accessible by those two enzymes in gastrula chromatin. Overall, these results, substantiate the structural change of the whole histone gene repeat chromatin during development[22] and demonstrate that nucleosomes re-organize in the enhancer promoter region of the α-*H2A* gene upon repression. We interpret the MNase pattern as an indication for nucleosome phasing in the chromatin of the repressed histone *H2A* 5′ regulatory region. However, the low resolution of the method and the lack of MNase protected cleavage sites in the basal promoter region, does not allow us to map unambiguously the nucleosome boundaries. Hence, to gain more details on the chromatin architecture of the repressed *H2A* promoter, we determined the accessibility to restriction endonucleases.[38]

The presence of a nucleosome is an effective barrier to restriction enzymes and prevents the cutting of the underlying DNA.[39,40] As described for the micrococcal nuclease, nuclei from formaldehyde fixed gastrula embryos or protein-free DNA were digested with the appropriate restriction endonuclease, DNA was extracted and either analyzed directly or incubated with a second enzyme before being processed for Southern blot hybridization and indirect terminal labeling. The results are shown in Figure 3. The single digestion pattern obtained with ApaL1, Sau3AI, and HpaII restriction enzymes revealed by blot hybridization with probe A (see map of Figure 3) gave different results. In fact, full accessibility was seen only with the HpaII enzyme, suggesting that both HpaII cutting sequences are located in the linker DNA. By contrast, one or both Sau3AI sites are probably protected by a nucleosome. Identical results were obtained with the ApaL1 enzyme (not shown). The specific location of a nucleosome between the two HpaII sites was confirmed by the RsaI digestion. Indirect terminal labeling from the HpaII site with probe A showed full protection to RsaI cutting at position 90 (Figure 3(b)). Furthermore, nucleosome mapping from the ApaL1 sites with probes A and B suggested that the Sau3AI position at −47 lies also in linker DNA (Figure 3(c) and (e)). Next we determined the accessibility to the AccI restriction enzyme located in the enhancer region. Indirect terminal mapping from the ApaL1 and Dde1 sites, respectively with probes A and C, showed protection to AccI digestion (Figure 3(d) and (f)). As no accessibility was detected also to the ApaI and ApaL1 restriction enzymes (not shown), we conclude that at gastrula stage a nucleosome is most probably positioned in the enhancer of the α-*H2A* gene. In summary, these results strongly suggest that silencing of the α-*H2A* gene at gastrula stage correlates with the positioning of two nucleosomes in the enhancer and basal promoter, and that down-regulation occurs despite the MBF-1 activator being bound to the enhancer.

Insertion of a 26 bp fragment between enhancer and promoter causes constitutive expression of α-*H2A trans*-gene

Next we investigated whether the positioning of a nucleosome on the basal promoter is the mechanism

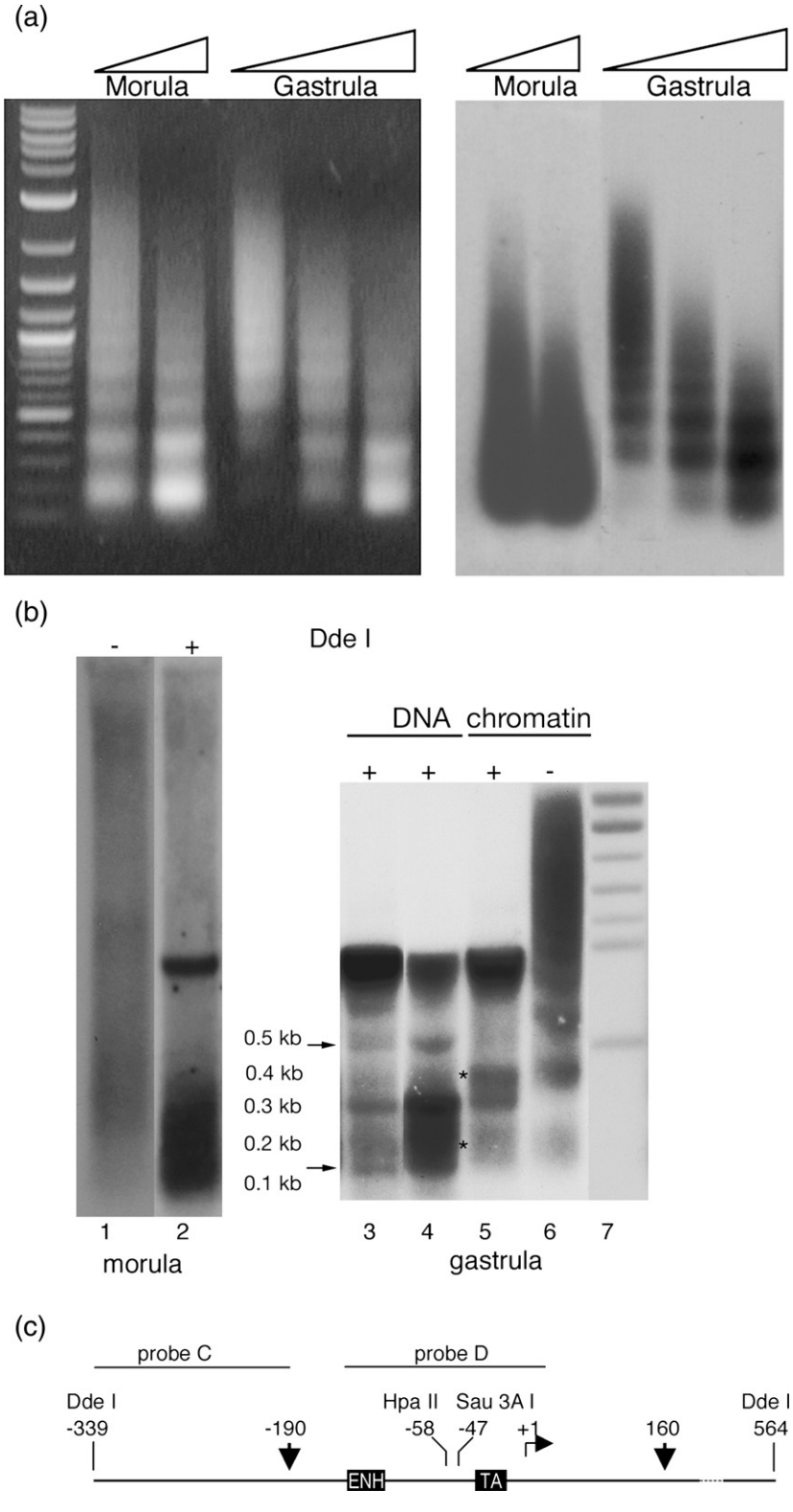

Figure 2. Nucleosome structure analysis of the H2A enhancer promoter region. (a) Nucleosome organization. Nuclei from crosslinked embryos at morula and gastrula stages were digested at 37 °C with MNase (Sigma) for 0, 5 and 1 min (morula), and for 0.5, 1, and 2 min (gastrula). After reversion of crosslinks, nucleosomal DNA was extracted. Digestion products were separated by 1.2% agarose gel, stained with ethidium bromide (left panel), blotted onto nitrocellulose filter and hybridized with probe D. The autoradiograph in the right panel shows a canonical array of nucleosomes only in gastrula nuclei. (b) Chromatin indirect terminal labeling. Nucleosomal DNA from morula (lanes 1 and 2) and gastrula embryos (lanes 5 and 6), obtained as described in (a), were digested to completion with DdeI restriction enzyme. Naked DNA (lanes 3 and 4) was incubated with 0.05 units/ml of MNase for 0.5 and 2 min before digestion with DdeI (lanes 3 and 4). Digestion products were processed as in (a) and hybridized with probe C. The autoradiograph shows cutting sites in naked DNA, indicated by arrows, protected in gastrula chromatin. Asterisks points to bands that probably correspond to mono and di-nucleosomal DNA in the double digested chromatin samples. (c) Schematic presentation of the *H2A* transcription unit with the relevant restriction sites. Arrows points to the MNase cleavage sites protected by nucleosomes in gastrula chromatin.

mediating the repression of the H2A gene expression at gastrula stage. To address this issue, we started from the evidence derived from the nucleosome mapping experiments that placed the TATA box at the border of the nucleosome core. The experimental strategy was based on the assumption that a microinjected histone DNA organizes a chromatin architecture similar to that of the endogenous genes. Thus, interposing a small DNA fragment between the enhancer and promoter of the histone *H2A* gene should induce a translational sliding of the histone octamer relative to the TATA box. The effect of insertion can be determined by expression studies. For our experiment, We used the

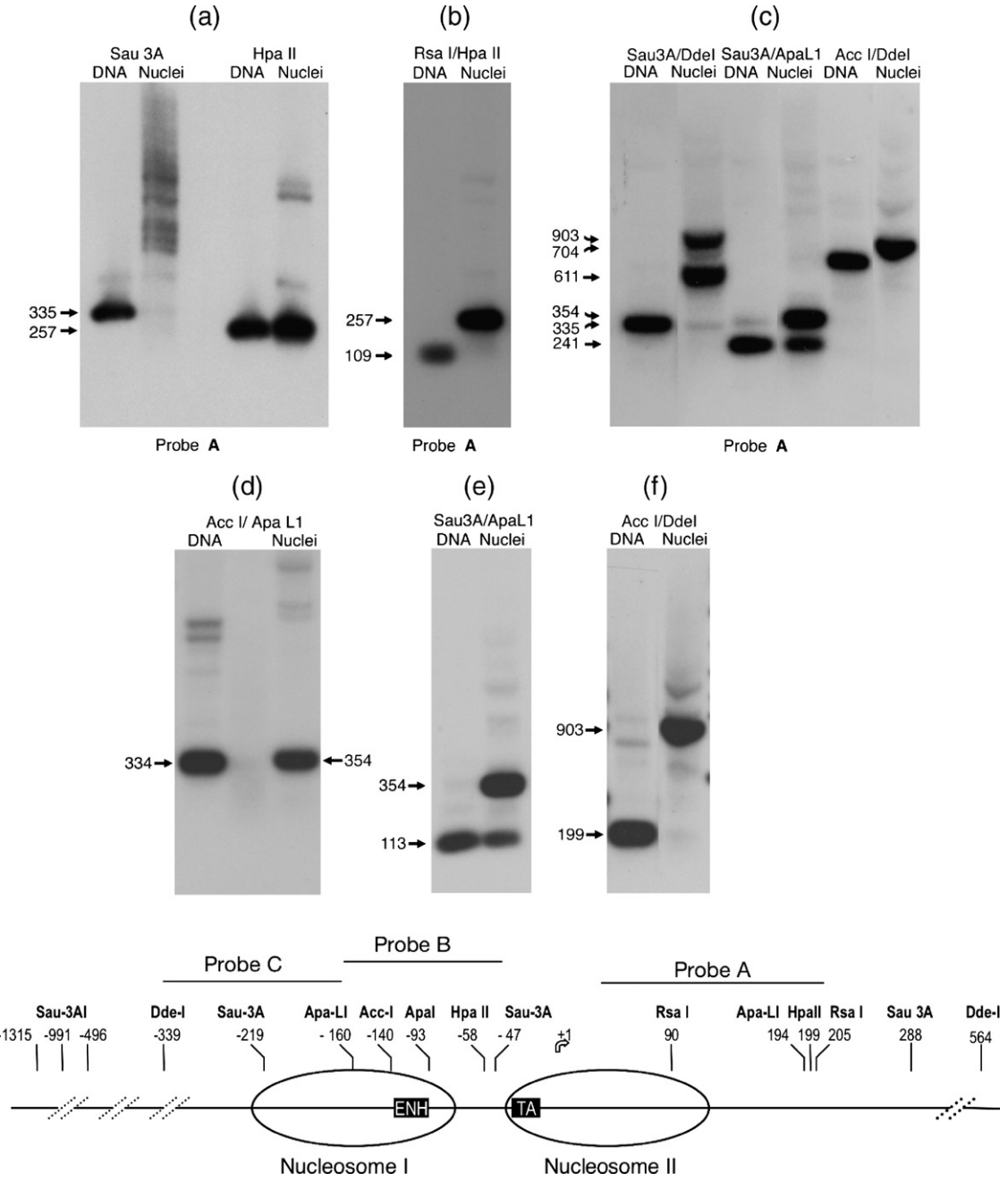

Figure 3. Restriction enzyme accessibility in gastrula chromatin. Nuclei from crosslinked gastrula embryos or naked DNA, were digested with the indicated restriction enzyme. Genomic DNA was extracted after reversion of the crosslinks and either analyzed directly (a) or digested with a second restriction indicated in (b)–(f). Digestion products were processed by Southern blotting hybridization using the probes A, B, and C. The drawing shows the restriction enzymes map of the enhancer promoter region with the two positioned nucleosomes and the location of the probes A, B, and C.

H3-H2A-H1 gene constructs, depicted in Figure 4, one lacking (control) and the other containing a 26 bp polylinker DNA, from bluescript plasmid, inserted between the *H2A* enhancer and promoter. Both *H3* and *H1* genes are driven by minimal promoters, whereas the *H2A* is wild-type. We have already shown that, when all sequence elements responsible for the temporal regulation of this gene are missing, the *H2A* enhancer interacts with the promoter of the α-*H3* gene in the other direction and activates transcription.[33,41] In contrast, the *H1* gene is transcribed at very low level as we have compelling evidence (unpublished observations) indicating that the *H2A* enhancer is blocked by the *sns 5* insulator in the interaction with the downstream *H1* promoter. To distinguish between endogenous and transgene histone transcripts, the two *Paracentrotus lividus* histone gene constructs were microinjected into the closely related sea urchin *Spherechinus granularis.* We used the expression of the α-*H3* transgene as internal control of the timing of α-*H2A* transcription. The results are shown Figure 4. In agreement with an earlier report, the *H2A* gene followed the temporal regulation of the endogenous

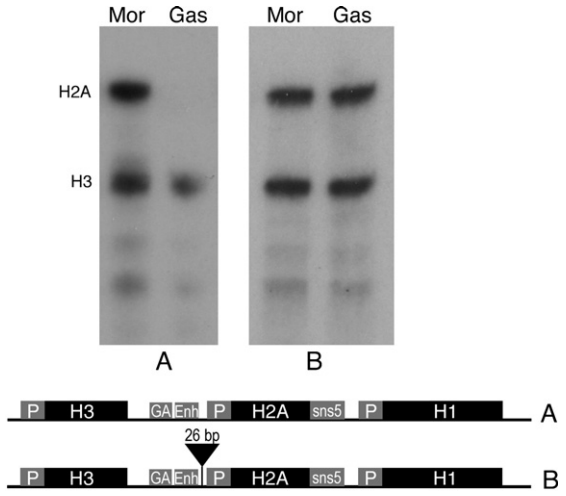

Figure 4. Testing the effect of a 26 bp insertion between enhancer and promoter on the expression of the *H2A* gene in transgenic sea urchin embryos. Two *P. lividus* constructs denoted A and B, schematically drawn below the autoradiographs, consisting of the α-*H3* and α-*H1* genes both driven by the basal promoter, and the wild-type α-*H2A* gene containing all regulative sequences, were microinjeceted in *S. granularis* zygotes. Embryos were raised and RNA extracted at morula (Mor) and gastrula (Gas) stages. RNase protection was carried out by hybridizing antisense labeled RNA transcribed *in vitro* from *P. lividus H3* and *H2A* subclones with total RNA from 30 injected embryos. The two RNA probes were added together to the hybridization mix. Arrows point to the protected 409 nt and 357 nt RNA bands, respectively for the *H2A* and *H3* transcripts. Increasing the distance between enhancer and promoter by 2.5 DNA gyres causes up-regulation of the *H2A trans*-gene at gastrula stage.

gene, as it was expressed at morula and repressed at gastrula stage. Since the *H2A* enhancer is constitutively active, the α-*H3* transgene was transcribed at both developmental stages. Strikingly, insertion of the 26 bp polylinker sequence allowed expression of the α-*H2A* gene also at gastrula stage (Figure 4(b)). An interpretation of this finding is that the 26 bp insertion modified the positioning of the nucleosome from the basal promoter, exposing the TATA box and allowing the assembly of the transcription machinery.

Down-regulation of α-*H2A* gene correlates with the recruitment of histone post-translational modifications in the promoter and insulator region

To better understand the relationship between chromatin structure and down-regulation of the α-*H2A* gene at gastrula stage we carried out ChIP analysis at regions of the promoter and insulator previously shown to be involved in transcriptional repression.[33] Firstly, we determined the association of HDAC to the chromatin of active and silent α-histone genes. For the ChIP experiments we used commercially available mouse anti-HDAC-1 antibodies. Because no records of the use of these antibodies in sea urchin was available, we preliminarily tested the mouse anti-HDAC-1 specificity against the sea urchin protein. The mouse anti-HDAC-1 stained in nuclei extracts from gastrula embryos a protein band of the expected size for the sea urchin HDAC-1,[42] and specifically reacted with the *P. lividus* HDAC-1 expressed in *E.coli* from the cloned gene (Figure 5(a)). The result of the ChIP experiment shown in Figure 5(b), indicates that the levels of HDAC-1 occupying the *H2A* promoter and *sns* 5 insulator increased markedly upon repression at gastrula stage. This evidence suggests that histone de-acetylation is required for the assembly of a repressed chromatin domain in the *H2A* transcription unit.

To further support the involvement of HDAC-1 in the mechanism of down-regulation, we inhibited its function by culturing the embryos in the presence of trichostatin (TSA) and valproate (VPA) and determined the expression of the α-*H2A* gene by Northern blot. Western blot analysis of histones from control, TSA, and VPA embryos carried out with antibodies against hyperacetylated H4 showed an increased level of acetylation upon drug treatment (not shown). We limited our analysis to embryos up to 14 h of development, because at later stages, when the control were at gastrula stage (20–22 h post-fertilization), the drugs caused several abnormalities and embryos failed to gastrulate. At 14 h post-fertilization, control and treated embryos were both at the mesenchyme blastula stage, allowing a comparative analysis of gene expression. The result presented in Figure 5(c) demonstrates that inhibition of histone de-acetylation caused only a slight increase in the abundance of α-*H2A* mRNA at 6 and 9 h of development, implying that at the early blastula stage the majority of the tandemly repeated α-histone genes are in an open chromatin configuration. By contrast, at mesenchyme blastula we found up-regulation of its expression in embryos cultured in the presence of the HDAC inhibitors (Figure 5(c)), while in the normal embryos transcription of the α-*H2A* gene was drastically reduced.

Next we investigated by ChIP experiments, whether down-regulation was linked to a modification of the pattern of acetylation and methylation of K9 of H3 in the *H2A* regulatory regions. The results are shown in Figure 5(c). We found a tight correspondence between histone genes transcription at morula stage and, respectively, the recruitment of H3K9ac and very little associations of H3K9me2 (the modifications are reported according to a recent nomenclature[43]) to the promoter and insulator. Conversely, transcriptional silencing at gastrula stage correlated, as expected, with a reciprocal pattern of modifications in the same regions, i.e. H3K9me2 association and a reduced H3 K9 acetylation (Figure 5(c)).

Discussion

Sea urchin α-histone genes were cloned more than three decades ago,[44,45] and since then they have

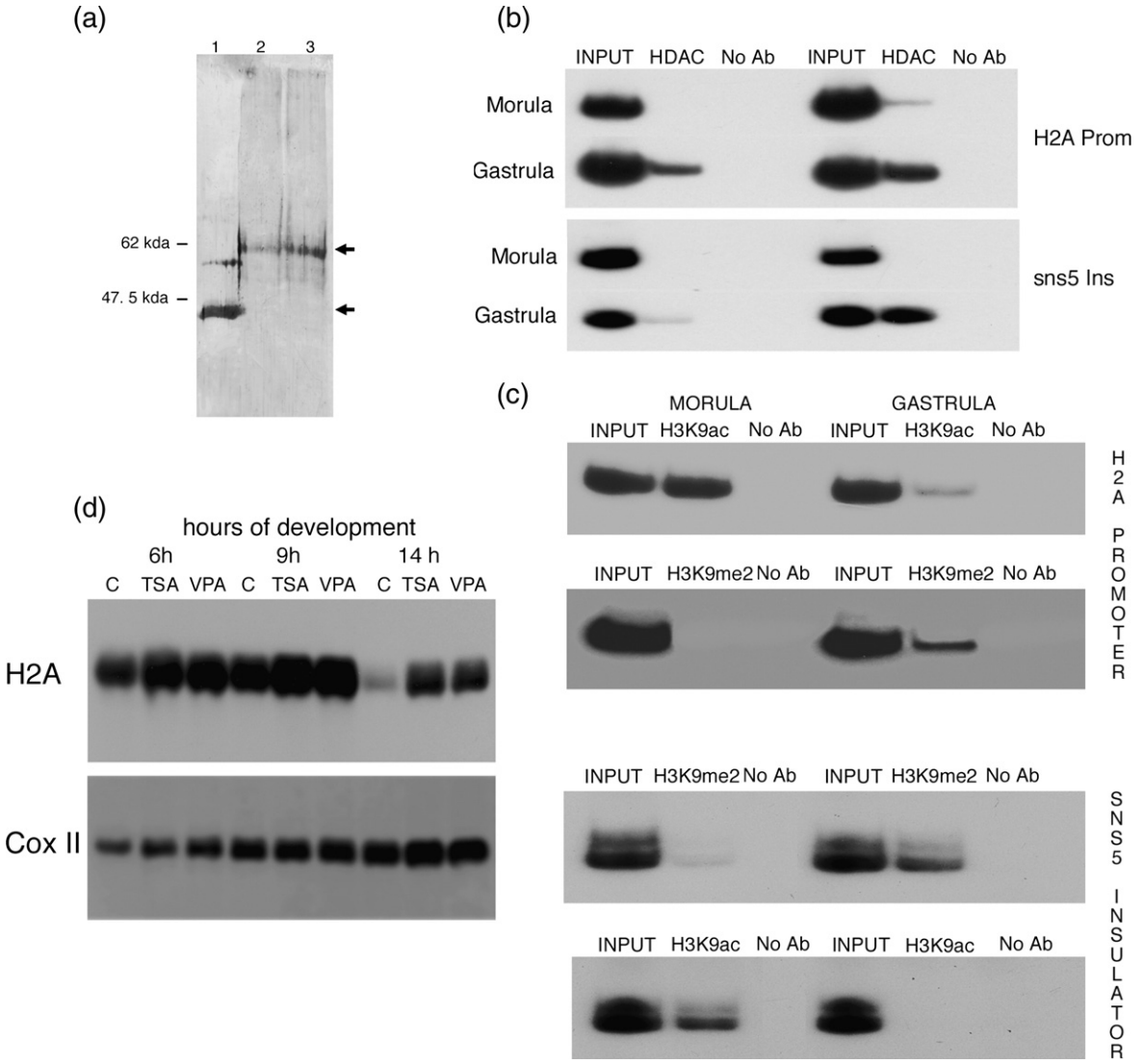

Figure 5. Histone deacetylase recruitment and histone tail modifications to the promoter and *sns* 5 insulator of the α-*H2A* gene at morula and gastrula stages. (a) Western blot analysis showing the reaction of the mouse anti-HDAC-1 antibodies with a sea urchin HDAC-1 fragment expressed in *E.coli* (lane 1) and with the nuclear extracts from morula (lane 2) and gastrula (lane 3) embryos. Arrows point to the detected bands. Chromatin immunoprecipitation experiments were carried out as in Figure 1(b), with antibodies recognizing the HDAC-1 (b); the H3K9ac or H3K9me2 histone tail modifications (c). The positions of PCR primers for promoter and insulator sequences are shown in Figure 1(c). Amplifications were performed, respectively, for 25 and 30 cycles for HDAC-1, and 25 cycles for the histone tail modifications. (d) H2A expression in control and TSA and VPA -treated embryos in developing sea urchin embryos. The RNA blot was hybridized with the labeled H2A and after striping the probe with Cox II. The autoradiographic image shows up-regulation of histone H2A expression upon inhibition of histone de-acetylation.

represented a model system for the identification of the *cis*- sequence elements involved in the regulation of transcription during development. The modulator of the α-*H2A* was the first regulatory sequence shown to be essential for *H2A* expression and to be capable of enhancing transcription when placed in the inverted orientation with respect to the promoter.[31,46,47] This evidence, first obtained in the *Xenopus laevis* system, was confirmed in the homologous sea urchin embryo by microinjection. By these experiments it was irrefutably demonstrated that the modulator can be equated to an enhancer.[25,27] However, further studies from our laboratory showed that the enhancer activity of the modulator and the expression of its activator MBF-1, though being absolutely necessary for maximum expression of transgenes, was not confined only to the transcription period of the endogenous *H2A* gene.[28] These findings led to the paradoxical conclusion that the modulator is a constitutive enhancer of a developmentally regulated sea urchin histone α-*H2A* gene.[19] The results described here extend these observations. We have in fact presented compelling evidence that the MBF-1 activator is constitutively associated to the chromatin, and yet, the *H2A* gene is down-regulated at gastrula stage.

Down-regulation of transcription in eukaryotes occurs by the binding of repressors that act by either

preventing the binding of activators, or by quenching the activation surface of nearby transcription factors.[48–50] Such negative regulatory mechanism cannot be applied to the α-*H2A* gene for the following reasons. First, repression of *H2A* relies on the functional interaction of the 5′ dispersed multiple sequence elements, the GA repeats situated upstream of the enhancer, and the *sns* 5 insulator placed at the 3′ of the transcription unit.[33] Second, the negative regulatory function of the insulator is position-dependent, in that, if it is moved upstream of the GA repeats, expression of a reporter gene driven from the *H2A* promoter-enhancer occurs also at gastrula stage.[33] Third, as we have shown here, binding of the MBF-1 transcription factor to its site occurs regardless of the transcriptional state of the *H2A* gene. Finally and very important, MBF-1 bound to a repressed promoter maintains the capability to *trans*-activate, in that, it can elicit transcription from the *H3* minimal promoter in the other direction (Figure 4 and Di Caro *et al.*[33]). Altogether, these results strongly indicate that down-regulation of the *H2A* histone gene does not depend on the binding of repressor molecule(s) that either interfere with MBF-1 binding or inhibit its activity. We do not understand the reason for MBF-1 transcriptional activator occupying its binding site in the repressed chromatin template, but several reports show that sequence-specific regulatory proteins are able to bind to their target sequences in silenced chromatin. For instance, the Gal4 transcription factor can reside in its binding site in repressed promoters[51] and the heat shock factor (HSF), can associate to the promoter of a *hsp26* transgene even when the gene is silenced by the Polycomb protein PcG in *Drosophila* cells.[52] In addition, studies on the mechanism of SIR2-dependent silencing in yeast have demonstrated LexA binding to its sites linked to a *URA3* gene integrated at the heterochromatic telomere or *HMR* mating type locus.[53]

The maximum rate of transcription of the sea urchin α-histone genes at early blastula stage is roughly one transcription event per gene per minute, assuming that all histone genes are active.[54] Because this transcription rate is lower than the theoretically possible rate of transcript production in this organism[55] (but similar to the transcription rate of single copy genes, such as *Spec*1[56]), it is possible that histone genes are also regulated by copy selection. According to this mechanism, only a portion of the genes could be expressed during early cleavage. Consequently, only a fraction of the histone gene chromatin should present an open conformation. The multicopy rRNA genes are regulated by copy selection. The active and inactive rRNA genes have been identified on the basis of differential protection of the DNA from psoralen crosslinking and the resulting differential electrophoretic mobility of the DNA. The transcribed copies are more crosslinked than the inactive genes.[57] These observations have led to the suggestion that the rDNA genes are largely devoid of nucleosomes when transcriptionally active, whilst they are organized in nucleosomes when are not expressed. Similar studies carried out in sea urchin, have shown that the early histone genes were more accessible to psoralen crosslinking when active than inactive. However, they failed to reveal a bimodal distribution of psoralen crosslinking at either transcription states, strongly suggesting that these genes have a homogeneous chromatin structure.[58] Based on these observations, we conclude that the constitutive association of the MBF-1 activator to the enhancer and the specific positioning of nucleosome on the promoter, reflect a similar chromatin architecture of all *H2A* genes of the cluster.

Of some interest is the finding that MBF-1 binding occurs despite the positioning of a nucleosome on the *H2A* enhancer at gastrula stage. As factor binding would be more difficult when the DNA elements of the target site are oriented facing the histone octamer,[59] the rotational positioning of the nucleosome should be such as to expose these sequences on the surface of the particle. A second nucleosome is positioned on the basal promoter. The accessibility to restriction enzyme digestion with the production of a single digestion band (Figure 3), strongly suggests a highly specific phased nucleosome on the TATA box containing promoter in all repressed *H2A* genes of the histone gene cluster. Incorporation of the TATA sequence into a nucleosome dramatically reduces transcription initiation, presumably because the orientation of the TATA sequence relative to the surface of the histone core affects the access of TBP.[60,61] We suggest that stereochemical constraints on binding of the general transcription factor TFIID might be the mechanism that impairs the bound MBF-1 activator to elicit transcription from the *H2A* promoter at gastrula stage. This hypothesis is supported by the evidence shown here (Figure 4), that the insertion of a 26 bp sequence between the modulator and the TATA box led to up-regulation of the *H2A* gene a gastrula stage. Although we have no proof for nucleosome positioning on the TATA box of the *trans*-gene, the most obvious interpretation of our finding is that the insertion caused changing of the translation or rotational position of the nucleosome.

Correlation between chromatin structure and transcriptional competence is now well established. Histone modifications and combinations thereof are believed to mark local chromatin for activation or silencing of gene activity. Thus, acetylation of certain lysine residues and methylation of K4 of histone H3 are required for gene activation, whereas deacetylation and methylation of K9 of H3 are usually associated with repression.[62] The results of the ChIP experiments of the regulatory regions, promoter and insulator, are in line with these findings. Our results represent to our knowledge the first demonstration in sea urchin of the associations between specific histone modifications and defined functional outcomes. They also highlight a dynamic role of the genomic insulator *sns 5* in the regulation of the α-*H2A* gene expression.

Insulators are specialized DNA sequences that upon binding of specific chromosomal proteins define the boundary between differentially regulated loci and shield promoters from the influence of neighbouring regulatory elements.[63,64] Insulator elements, such as the HS4 of the chicken β-*globin* locus, protect transcriptional active regions from the silencing effects of surrounding heterochromatin by recruiting transcriptional activators that associate with histone-modifying complexes.[65] The barrier activity of HS4 is associated with a peak of histone acetylation over the insulator element independently of the expression status of the β-*globin* gene.[66] In contrast, the sea urchin *sns* 5 insulator switches types of histone modifications, acetylation or methylation of K9 of H3, depending on the transcriptional state of the *H2A* gene. These observations suggest the interesting possibility that K9-H3 acetylation of *sns* 5 insulator participates in maintaining open the chromatin of the *H2A* transcription unit till the early blastula stage, whereas de-acetylation and methylation of K9-H3 induces chromatin condensation after hatching. In addition, the type of histone modification recruitment according to the transcriptional competence, probably implies the involvement of developmental stage-specific insulator proteins. Work aimed at the identification of these insulator proteins is in progress.

Material and Methods

Embryo culture and nuclei purification

Adults of *P lividus* and *S. granularis* were collected along the Sicilian coast and either maintained in a tank at 16 °C or utilized immediately. Embryos were cultured at 18 °C and when they reached the desired stage of development, they were incubated with 1% formaldehyde for 10 min. Crosslinking was stopped by the addiction of glycine to a final concentration of 0.125 M. Nuclei for nucleosome mapping were purified according to a published protocol with slight modifications.[67] Briefly, collected embryos were resuspended in 20 volumes of 0.5 M sucrose, 10 mM Tris-HCl (pH 7.5), and dissociated by homogenizing with ten strokes of the loosely fitting pestle of a Dounce homogenizer. Cells were lysed by adding an equal volume of buffer A (20 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.5), 0.2 mM PMSF, 1% Triton X-100) and nuclei harvested by centrifugation at 2000*g* for 10 min.

Nucleosome mapping

Nuclei from morula amd gastrula embryos were resuspended in 15 mM NaCl, 65 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris-HCl (pH 7.4) at the concentration of 1 A_{260nm}/ml and incubated at 37 °C with three to five units of micrococcal nuclease (Sigma) in the presence of 1 mM $CaCl_2$. Digestion was stopped by the addition of EGTA to 10 mM final concentration. For restriction enzyme digestion nuclei were resuspended in appropriate buffer and incubated overnight with 50–100 units of the specific enzyme in the presence of 1 mM EGTA. The formaldehyde crosslinking was reverted by 30 min incubation at 37 °C with 50 μg/ml of RNase and 2 h incubation at 56 °C with 50 μg/ml proteinase K in 0.4 M NaCl, 1% (w/v) SDS, before extraction with phenol/chloroform. MNase or restriction enzyme-digested DNA was incubated with a second restriction endonuclease. Digestion products were fractionated onto a 1.5% (w/v) agarose gel, transferred onto a nitrocellulose membrane by Southern blotting and hybridized with a ^{32}P-labelled probe abutting the restriction enzyme site used in the second digestion.

Chromatin immunoprecipitation

Formaldehyde crosslinked sea urchin embryos at morula and gastrula stages were washed three times with cold PBS, collected by centrifugation and incubated, for 10 min on ice, in cell lysis buffer. The nuclei were pelleted by centrifugation at 2000*g* for 5 min, resuspended in nuclear lysis buffer (50 mM Tris (pH 8.1), 10 mM EDTA, 1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF) and incubated on ice for 10 min. Chromatin was sonicated by 15 20-s pulses with the Branson Sonifer 250 at the 2-3 output level. Uniformity of the sonication treatment, quality and quantity of chromatin were confirmed by reversion of crosslinking and running recovered DNA on an agarose gel. The length of the sonicated chromatin ranged from 0.2 to 1 kb. To reduce non-specific background, the samples were incubated with 100 μl of a salmon sperm DNA/protein A agarose slurry for 1 h at 4 °C with agitation. 20% of chromatin, cleared by centrifugation, was withdrawn (input) and processed as the immunoprecipitated chromatin.

For each ChIP experiment 25 μg of DNA containing chromatin was incubated with the pre-immune or the anti-MBF-1 sera and the indicated antibodies against the HDAC-1 and modified H3 histone tail (Upstate Biotechnology) overnight at 4 °C. The same chromatin aliquot was incubated with buffer and used as negative control. The immune complexes were adsorbed to protein A-Sepharose. The beads were washed for 5 min, on a rotating platform, with a low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), 150 mM NaCl), a high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), 500 mM NaCl), a LiCl buffer (0.25 M LiCl,1% NP40,1% deoxycholate, 1 mM EDTA,10 mM Tris, pH 8.0) and twice in 1× TE (10 mM Tris, 0.1 mM EDTA, pH 8.0). The immunocomplexes were eluted with the elution buffer (1% SDS, 0.1 M NaHCO3), digested with RNase at 37 °C and proteinase K in 0.3 M NaCl at 56 °C for 4 h to reverse the crosslinks. DNA from chromatin samples was extracted with phenol/chloroform-and precipitated with ethanol. 3 μl of the immunoprecipitated chromatin and a serial dilution of input DNA were used in PCR reactions with the following sets of primers. *H2A* promoter: (forward) GATGTGCACACCGTGTCGCTGCTGTA; (reverse) ACCGCCGCCGACCCTCTTTG. *Sns*5: insulator (forward) GCTTCTTGGAGGTGTGACCA; (reverse) ACTGTGCGACACAGAGTA Amplification were performed for 25–30 cycles in the presence of 5 μCi of [α-^{32}P]dCTP and the products were analyzed in 6% (w/v) polyacrylamide gels.

Microinjection and expression analysis

The histone DNA constructs described in Figure 4 were generated by inserting the *H1* gene lacking most of the

upstream promoter sequences, downstream of the *H3-H2A* histone DNA.[33] The 26 bp oligonucleotide corresponding to the bluescript plasmid polylinker was inserted in the ApaI restriction site between the H2A enhancer and promoter. DNA microinjection in *S. granularis* zygotes and RNase protection assays were as described.[33] The *P. lividus H2A* and *H3* antisense RNA probes were hybridized together and they did not protect endogenous *S. granularis* RNA bands.[33]

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