Promoter activity of the sea urchin (*Paracentrotus lividus*) nucleosomal *H3* and *H2A* and linker *H1* α-histone genes is modulated by enhancer and chromatin insulator

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ABSTRACT

Core promoters and chromatin insulators are key regulatory elements that may direct a transcriptional enhancer to prefer a specific promoter in complex genetic loci. Enhancer and insulator flank the sea urchin (*Paracentrotus lividus*) α-histone *H2A* transcription unit in a tandem repeated cluster containing the five histone genes. This article deals with the specificity of interaction between the *H2A* enhancer-bound MBF-1 activator and histone gene promoters, and with the mechanism that leads the *H1* transcripts to peak at about one-third of the value for nucleosomal *H3* and *H2A* mRNAs. To this end, *in vivo* competition assays of enhancer and insulator functions were performed. Our evidence suggests that the MBF-1 transcription factor participates also in the expression of the *H3* gene and that the *sns5* insulator buffers the downstream *H1* promoter from the *H2A* enhancer. Altogether, these results provide a clear demonstration of the enhancer-blocking function of a chromatin insulator in a natural gene context. In addition, they suggest that both the *H2A* enhancer and the *sns5* insulator may account for the diverse accumulation of the linker H1 versus the core nucleosomal histones during early development of the sea urchin embryo.

INTRODUCTION

The gene regulatory information encoded in the primary DNA sequence is interpreted and transmitted to the transcription machinery, that it is assembled at the promoter, by the binding of transcription factors to the appropriate sequence elements (1,2). Among these, enhancers are described as DNA elements that increase the level of transcription of the associated gene in a position- and distance-independent manner relative to the transcription start site (3). As a consequence, in complex genetic loci with multiple promoters, mechanisms ought to be put in place to make an enhancer prefer one specific promoter. Indeed, some transcriptional enhancers can discriminate between core promoters that contain either a *TATA* box or a DPE element (4). For instance, in *Drosophila*, the *AE1* enhancer preferentially interacts with the *ftz* promoter rather than with the *Scr* promoter, in spite of its intergenic position and comparable distance from both promoters; in transgenic embryo, promoter competition dictates the *IAB5* enhancer, placed between two divergently transcribed transgenes, to preferentially activate transcription from the *TATA*-containing promoter (5,6). In addition, in the sea urchin *Hemicentrotus pulcherrimus*, a combination of the upstream and either *TATA*-containing or *TATA*-less core promoter sequences seems to contribute to the establishment of the specific spatial and temporal expression profiles of developmentally regulated genes (7,8).

Besides promoter competition, chromatin insulators may also be involved in promoter selectivity by a given enhancer. Most of these regulatory elements may have two activities: (i) a boundary function blocks the spread of the heterochromatin into the euchromatic region and protects the transgenes from the negative influence of chromatin at the site of insertion; and (ii) an enhancer-blocking activity that restricts enhancer function in one direction and only when interposed between the enhancer and promoter (8–13). The DNA replication-dependent sea urchin early or α-histone gene cluster represents an interesting model system to investigate the specificity of function of an enhancer element in close proximity of different gene promoters. These genes are development-regulated and organized in a single large cluster made up of ~2000 tandem repeat units, each containing one copy of each of the five histone genes, in the order

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5′-*H2B-H3-H2A-H1-H4*-3′ (14,15). As embryogenesis occurs, transcription of these genes is limited to the rapid early cleavage and reaches its maximum at the morula/early blastula stage. After hatching, they become repressed and are maintained in such a transcriptional state for the whole life cycle of the animal (16).

We have previously described the *cis*-regulatory sequences and the necessary transcription factor for the timing of transcription of the α-*H2A* gene during the embryogenesis of the sea urchin *P. lividus*. In the 5′ region, a 30-nucleotide-long regulatory sequence termed modulator or simply *M30* (17,18) specifically binds the MBF-1 activator (19). The *H2A* modulator has a bidirectional enhancer activity, both in the homologous and heterologous (*Xenopus laevis* oocytes) system (20,21). Worth mentioning is the capability of tandem copies of the MBF-1-binding sites to activate transcription from a viral promoter independent of distance and orientation (21,22). As shown in this article (Figure 1), this enhancer function is maintained also by a single MBF-1 recognition sequence. Remarkably, the MBF-1 regulator, although essential for *H2A* transcriptional activation, is constitutively bound to the *H2A* enhancer, which in a transgene construct can elicit transcription from a viral promoter also after silencing of the endogenous α-histone genes at the gastrula stage (23,24).

Down-regulation at the gastrula stage relies on the functional interaction between the 5′ dispersed *GA* repeats, located upstream of the enhancer, and the *sns5* insulator placed at the 3′-end of the *H2A* transcription unit (24). The repressed *H2A* gene is characterized by the specific positioning of two nucleosomes in the promoter/enhancer region, histone de-acetylase recruitment, and histone H3K9 dimethylation in the insulator and 5′ regulatory sequences (25). Interestingly, the *sns5* element contains the enhancer blocker *sns* that, in an enhancer-blocking assay, restricts enhancer function in a directional and polar manner in both sea urchin and mammalian cells (22,26,27). In addition, *sns5* exhibits the other property of insulators, the ability to block repressive chromatin effects on the flanking regions of transgenes (28).

The five histone genes are coordinately expressed during early development. Despite this need for nucleosome assembly in the newly replicated chromatin, the number of *H1* linker mRNA molecules is less than the value for histone nucleosomal mRNAs, being about half, as determined by kinetic and hybridization studies (29,30) or even less (this article). In this article, we have investigated the specificity of the *H2A* enhancer and the molecular mechanism that allows differential transcription of linker versus core histones genes during sea urchin development. In principle, the *H2A* enhancer-bound MBF-1 activator could interact, at least, with the promoters of the neighboring *H3* and *H1* genes, unless promoter competition and the 3′ located *sns5* insulator restrain the activity of the *H2A* enhancer specifically to the associated *H2A* gene. Our results exclude competition between core promoter elements and indicate that the MBF-1 transcription factor participates also in the expression of the *H3* gene in the resident chromatin. In addition, we show that the enhancer-blocking activity of the *sns5* insulator buffers the downstream *H1* promoter from the *H2A* enhancer. These results provide evidence for insulator action in a normal genomic context and suggest that both the *H2A* enhancer and the *sns5* insulator are involved in the different regulation of nucleosomal core and linker histone transcription during early development of the sea urchin embryo.

MATERIALS AND METHODS

DNA constructs

The Δp*H3-H2A*-Δp*H1* DNA plasmid, containing the core promoters of *H3* and *H1*, and a wild-type *H2A* transcription unit, was constructed by polymerase chain reaction (PCR) amplification of histone DNA cluster Ph70 and cloning in the pBS vector. The *M30-CAT* plasmids were constructed by shotgun cloning of ligated double-stranded oligonucleotides bearing the *H2A* modulator sequence either upstream or downstream the chloramphenicol acetyltransferase (CAT) coding sequences of the *tk*-70 pBL2 vector under the control of the *thymidine kinase* gene (*tk*) promoter (31).

A dominant negative construct was obtained by fusing the MBF-1 DNA-binding domain encoding sequences (19) to those of the Engrailed repressor domain cloned in the CS2 + nls expression vector. All DNA clones were checked by sequencing.

Microinjection of DNA constructs, double-strand oligonucleotides and synthetic RNA

Microinjection in *P. lividus* and *Sphaerechinus granularis* was conducted as previously described (32). Approximately 5000 molecules of the desired plasmid DNA were injected into the zygote, together with Texas Red-conjugated dextran added at a concentration of 5% in a 2 pl volume of 30% glycerol.

In the *in vivo* competition experiments, double-stranded *M30*, *M30mut*, or *BoxA* 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 a molar ratio of the specific genetic element to construct of 50:1. In the competition experiments for the endogenous histone genes, the purified double-strand oligonucleotides were mixed with glycerol and Texas Red-conjugated dextran, and injected at a final concentration ranging from 1 to 15 ng/μl.

The oligonucleotide sequences used in the competition assays are listed in Supplementary Table S1A.

For mRNA injection, dn-MBF-1 and control CS2 + nlsEn (33) constructs were linearized and transcribed *in vitro* using the Sp6 mMessage mMachine kit (Ambion). Capped mRNAs were resuspended in ultrapure RNase-free water (Gibco) and 2 pl, corresponding to the amounts of 0.1, 0.5 and 1.0 pg, respectively, were injected. Injected embryos at the desired stage were harvested and total RNA extracted.

RNase protection assay

Total RNA samples, extracted from microinjected embryos, were hybridized with antisense ^{32}P-labelled RNA probes. The *H3*, *H2A* and *H1* antisense RNA probes did not protect the endogenous *S. granularis* RNA bands. Hybridization conditions, RNase digestion and gel fractionation of the RNase resistant hybrids were as described (22).

Real-time quantitative PCR

The amounts of histone gene transcription in control and injected embryos at morula stage were evaluated as described (34). Briefly, total RNA from batches of 150 microinjected embryos was extracted by using the High Pure RNA Isolation kit (Roche). RNA samples were treated with reagents provided by the Turbo DNA-free kit (Ambion) and resuspended in a final volume of 30 μl. Reverse transcription into cDNA was performed in an 80 μl reaction using random hexamers and the TaqMan Reverse Transcription Reagents kit (Applied Biosystems). The resulting cDNA sample was further diluted and the equivalent amount corresponding to one embryo was used as template for Q-PCR analysis. Q-PCR experiments were performed from two different batches and all reactions were run in triplicate on the 7300 Real-Time PCR system (Applied Biosystems) using SYBR Green detection chemistry (Applied Biosystems). ROX was used as a measure of background fluorescence and MBF-1 mRNA (19) was used to normalize all data, in order to account for fluctuations among different preparations. At the end of the amplification reactions, a 'melting-curve analysis' was run to confirm the homogeneity of all Q-PCR products. Calculations from QPCR raw data were performed by the RQ Study software version 1.2.3 (Applied Biosystems), using the comparative C_t method ($\Delta\Delta C_t$).

The oligonucleotide sequences, length and predicted amplicon size are described in Supplementary Table S1B.

RESULTS

A single copy of the MBF-1 activator-binding site enhances transgene expression

As reported previously, the MBF-1 activator has been identified as being capable of specifically binding a 30-bp sequence of the modulator, and tandem copies of this sequence, denoted as *M30*, enhanced transcription from a viral promoter in a position- and orientation-independent manner in transgenic sea urchin embryos (22). To gain more details on the promoter specificity of the MBF-1 activator, we assessed, in the first place, whether a single copy of the *M30* sequence was capable of activating the basal *tk* promoter. The constructs schematically drawn in Figure 1 were microinjected into sea urchin zygotes, embryos were raised, and expression of the reporter *CAT* gene was determined at the gastrula stage by RNase protection assays with RNA samples from the same number of injected embryos. The results shown in Figure 1 demonstrate that the MBF-1-binding sequence enhanced transcription from the viral promoter to a similar extent as did multiple copies. Furthermore, this enhancer activity was displayed independently of location and orientation and occurred also at 2.7-kb distance from the *tk* promoter.

Promoter specificity of the MBF-1 activator

To elucidate the role of the MBF-1 activator in the expression of the early histone genes, we inhibited its binding to the *H2A* enhancer by performing an *in vivo* competition assay. This assay involves the titration of a given DNA-binding factor by molar excess of tandem copies of a *cis*-regulatory sequence. We have routinely used this
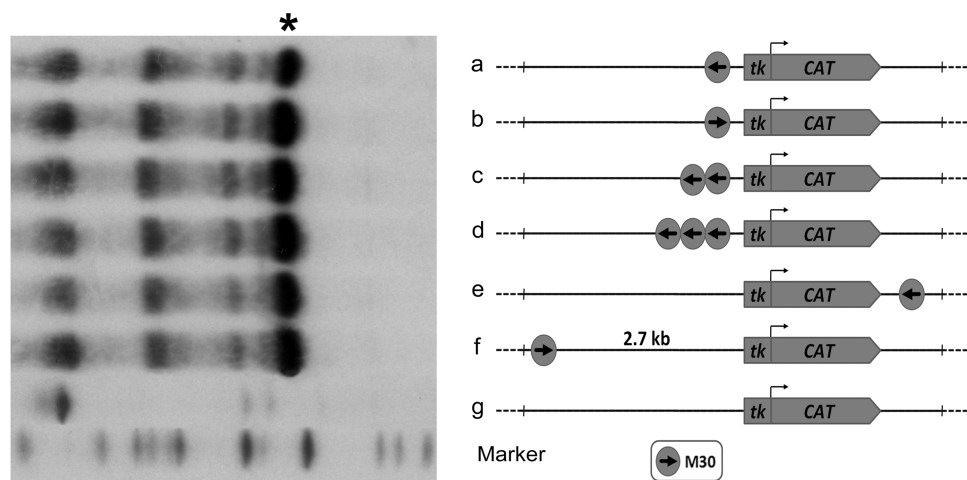
Figure 1. A single copy of the MBF-1 activator binding site enhances transgene expression. The *M30-tk*-CAT transgenes, bearing one, two or three copies of the 30 bp *H2A* modulator sequence in different location and orientation, were microinjected into sea urchin zygotes. Total RNA from 30 to 50 gastrula stage embryos, 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' section. Asterisk indicates the protected RNA band for the CAT transcript.
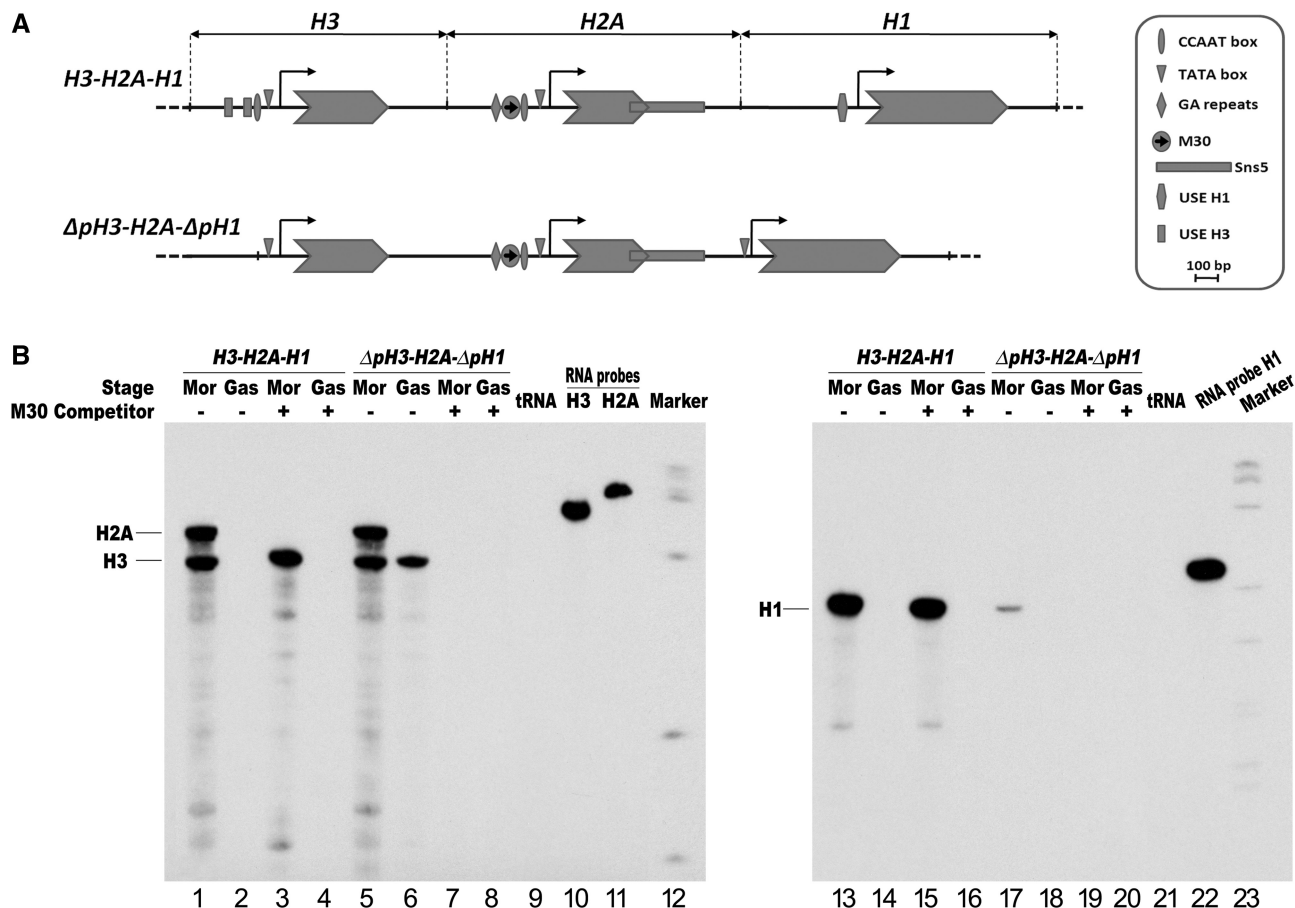
Figure 2. *In vivo* competition assay to knock-down the *H2A* enhancer function in transgenic embryos. (**A**) Annotated map of the *P. lividus* wild type and deletion mutants early *H3*, *H2A* and *H1* histone genes, highlighting the *cis*-regulatory sequence elements. The horizontal black line and arrow-shaped boxes represent, respectively, the genomic DNA and coding sequences, while the bent arrows denote the putative transcription start site. (**B**) The *P. lividus* histone gene constructs, orientated as in the endogenous histone gene repeat, were co-injected with excess of the modulator binding site (*M30*) into *S. granularis* zygotes. RNase protection was carried out by hybridizing antisense ^{32}P-labelled RNA, transcribed *in vitro* from *H3*, *H2A* and *H1* subclones, with total RNA exctracted from 25 injected embryos at morula (Mor) and gastrula (Gas) stages. The two *H2A* and *H3* probes were hybridized together. The protected 409, 357 and 209 nt RNA bands, respectively, for the *H2A*, *H3* and *H1* transcripts are indicated.

approach to knockdown the function of regulatory sequences of the *sns5* insulator and of histone and *Hbox12* gene promoters. As demonstrated in several instances, the effect on transgene expression is identical to that obtained by the mutation of the same sites (24,27,34).

Concatameric ligation products containing on average six tandem copies of the *M30* oligonucleotide were co-injected with the *P. lividus H3-H2A-H1* histone gene constructs, depicted in Figure 2A, into the sea urchin *S. granularis* zygotes, to distinguish between endogenous and transgene histone transcripts. Embryos were raised and the expression of the injected genes was detected by RNAse protection assays. All three injected genes followed the embryonic temporal expression profile of the endogenous histone genes, i.e. they are highly transcribed at morula stage and silenced at gastrula stage (lanes 1, 2, 13 and 14). As a control, we used the mutant *M30* sequence (*M30* mut), which as reported cannot bind the MBF-1 protein translated *in vitro* (19,21). Injection of *M30* mut at the same doses as *M30*

had no effect on histone gene transcription (Supplementary Figure S1). Conversely, the injected *M30* sequence selectively inhibited transcription of the *H2A* gene, while it did not affect the expression of both *H3* and *H1* genes at the morula stage (lanes 3 and 15). These results would suggest that the *H2A* enhancer prefers to interact with the cognate promoter.

To assess whether competition between core promoter elements is one of the molecular mechanisms that lead the MBF-1 activator to selectively interact with the *H2A* promoter, we performed the *in vivo* competition assay for the *H2A* enhancer function on the deletion mutant Δp*H3-H2A*-Δp*H1* (Figure 2A). In such a construct, the *H2A* transcription unit is a wild type. All the regulatory sequence elements upstream the *TATA* box were deleted from the *H3* promoter (31,35), while the 5′ deletion of the linker *H1* promoter occurred up to half of the essential regulatory sequence *USE* (36).

We have already described that the expression of *H3* driven by only the *TATA* box and other core promoter elements is up-regulated in a *H3-H2A* transgene (24).
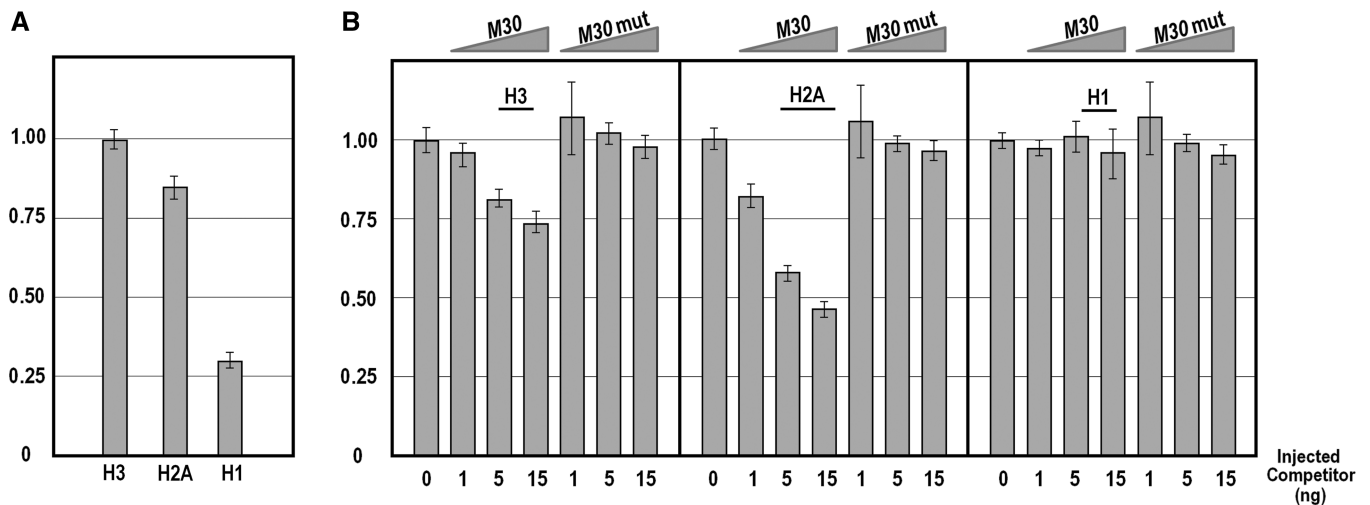
Figure 3. *In vivo* competition assay for endogenous *H3*, *H2A* and *H1* histone gene expression. (**A**) Relative abundance of histone mRNAs in the *P. lividus* embryo at morula stage. A similar prevalence is detected for the two nucleosomal *H3* and *H2A* mRNAs, while the *H1* linker histone mRNA peaks at about one-third of the value for the formers. (**B**) Endogenous histone gene expression analysis carried out in embryos at morula stage microinjected with excess of the *M30* sequence element or the mutated *M30* mut oligonucleotide as a control. Graphs show *n*-fold changes in mRNA expression level of histone genes based on the threshold cycle number (C_t) of injected embryos compared to that of the uncompeted control embryos. C_t numbers were normalized for the endogenous MBF-1 in the same sample. Data were derived from two independent microinjection experiments and each bar represents the average of triplicate samples from the two batches of embryos.

The result displayed in Figure 2B extends this observation, by showing that the *H2A* enhancer-bound MBF-1 transcription factor is most probably responsible for the up-regulation. In fact, while the *H2A* gene followed the time of expression of the endogenous gene, the core promoter of the *H3* gene, which by itself displays a barely detectable transcriptional activity (not shown), gave rise to comparable levels of transcripts. Significantly, co-injection of an excess of the *M30* enhancer abolishes transcription of both genes (Figure 2B, lanes 5–8), suggesting that the *TATA* boxes and other core promoter elements of *H3* and *H2A* genes do not compete for the interaction with the MBF-1 activator.

A different result was obtained with the *H1* gene driven by the mutated *H1* promoter. The core promoter elements displayed a very low transcriptional activity and did not respond to the *trans*-activating signal emanated by the MBF-1 transcription factor (Figure 2B, lanes 17–20), suggesting that *H1* gene expression is autonomously regulated. This independence from the *H2A* enhancer can be explained either with a core promoter competition mechanism or by the action of the *sns5* insulator located at 3′ of the *H2A* gene, between the *H2A* enhancer and *H1* promoter.

The MBF-1 activator is involved also in the transcription of the early H3 gene

The results displayed in Figure 2 seem to be in apparent contrast. In one case, we have observed a lack of influence of the *M30* competitor on the function of the wild type *H3* promoter. In the other case, we showed the interaction of the MBF-1 activator with the *H3* core promoter. To gain insights on this issue, we have determined by quantitative PCR (Q-PCR) whether competition of the *H2A* enhancer

activity affected, and to what magnitude, the expression of the endogenous *H2A*, *H3* and *H1* genes. The histone mRNAs prevalence in the un-competed embryos, shown in Figure 3A, indicates that, in the sea urchin *P. lividus* at morula stage, the *H1* linker histone peaks at about one-third of the value for the nucleosomal *H3* and *H2A*. As expected, the mRNA levels for *H2A* decreased with the microinjection of increasing amounts of the wild type MBF-1-binding site. Once again, the mutant *M30* sequence had no effect. Conversely, the number of mRNA molecules for the linker histone did not change with the rise of either *M30* or *M30* mut competitors, confirming the independence of *H1* transcription from the *H2A* enhancer. Very interestingly, we found that inhibition of MBF-1 binding reduced also the *H3* gene transcripts, although to a lesser extent than the *H2A* mRNA.

In order to obtain clear-cut evidence on the involvement of the MBF-1 activator in the expression of the *H3* gene, we performed a more direct experiment to knock down its function. To this end, we made a dominant negative construct, termed dn-MBF-1, in which the DNA-binding domain of MBF-1 (19) was joined to the repression domain of *Drosophila* engrailed (33). An *in vitro* transcribed mRNA was injected into the sea urchin zygote and the expression of *H3*, *H2A* and *H1* genes analyzed by Q-PCR at morula stage. The results obtained (Figure 4) are in accordance with the *in vivo* competition analysis described above. In fact, we found a dose-dependent negative effect of the MBF-1 forced repressor on the expression of the nucleosomal *H2A* and *H3*, but not *H1* genes. Once again, transcription of *H3* was less affected compared with *H2A*.

In summary, these results confirmed that the MBF-1 transcription factor is absolutely necessary for the
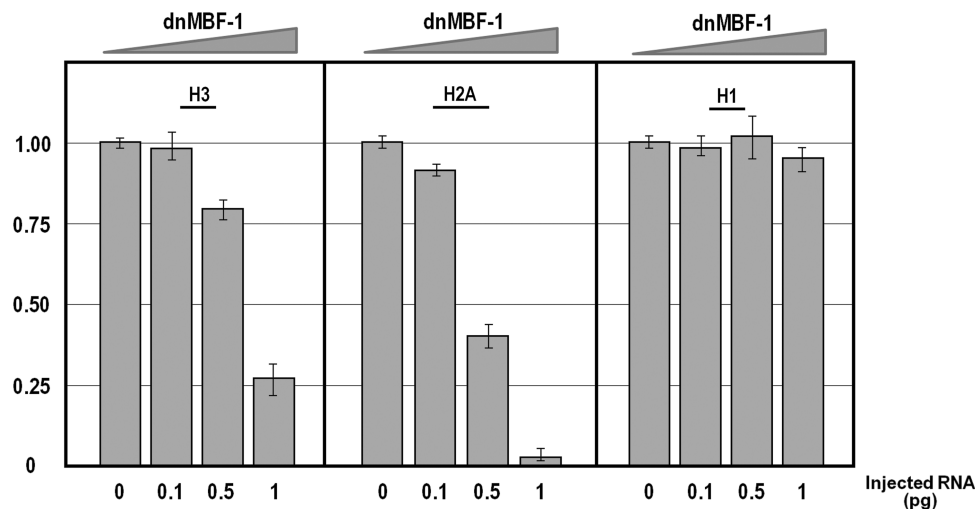
Figure 4. Knock-down MBF-1 function by microinjection of a synthetic mRNA encoding for as dominant repressor (dnMBF-1). Increasing amounts (0.1–1 pg) of the chimeric RNA were injected in *P. lividus* zygotes and RNA extracted from embryos at morula stage. Graphs show *n*-fold changes in mRNA expression level of histone genes based on the threshold cycle number (C_t) of dnMBF-1 injected embryos compared to that of the uninjected control embryos. C_t numbers were normalized for the endogenous MBF-1 in the same sample, by amplifying a fragment of the coding region external to the DNA binding domain. Data were derived from two independent microinjection experiments and each bar represents the average of triplicate samples from the two batches of embryos.

activation of the *H2A* gene. In addition, they suggest that the MBF-1 activator is involved also in the transcription of the upstream *H3* gene and has no role on the expression of the linker *H1* gene.

The sns5 insulator confers transcriptional independence to the linker H1 histone gene promoter

The location of the *sns5* insulator between the *H2A* enhancer and *H1* promoter (Figure 1) prompted us to examine its possible involvement in the mechanism of enhancer specificity. Wild type and mutated *H3-H2A-H1* three gene constructs were microinjected, respectively, in the absence and in the presence of excess *BoxA*-ligated oligonucleotides. As reported, *BoxA* is one of the *cis*-acting sequences absolutely required for the enhancer blocking and silencing function of the *sns5* insulator at gastrula stage (24,27). This is further shown in Figure 5. The competitor *BoxA* up-regulated the *H2A*, while the wild type *H3* gene followed the temporal regulatory program of the endogenous gene (lanes 1–4). In addition, the inhibition of the insulator activity did not influence the constitutive *trans*-activation of the *H3* core promoter (lanes 5–8). What is most important, however, is the effect of the competition of the insulator activity on the mutant *H1* promoter. In this case, although it appeared that the expression of the wild type *H1* was not affected, we did find a strong *trans*-activation of the mutated *H1* transgene, at both early and late developmental stages, caused most probably by the inter-action of MBF-1 with the *H1* core promoter (lanes 13–20).

It should be noted that, as for the wild-type *H3* transgene, the RNase protection assay did not reveal possible subtle differences in the transcription of the wild type *H1* transgene between the un-competed and competed samples. For this reason, to eventually validate the regulatory role of the *sns5* insulator, we looked at the expression by Q-PCR of the endogenous histone genes upon microinjection of the competitor *BoxA* sequence. As can be seen in Figure 6, both nucleosomal histone *H2A* and *H3* mRNAs did not vary their prevalence at the different doses of competitor. By contrast, the number of molecules of the linker *H1* mRNA increased monotonically with the augmentation of the *BoxA* oligonucleotide. Altogether, these results represent a strong indication that the *sns5* insulator blocks the *H2A* enhancer in the interaction with the downstream *H1* promoter.

DISCUSSION

The experiments described in this article highlight the regulatory function of the modulator/enhancer and insu-lator flanking the *H2A* transcription unit in the expression of S-phase-dependent histone genes during sea urchin development. These genes are organized in a large cluster and are coordinately regulated during embryo-genesis. Notwithstanding this need, the transcripts of linker and core histones accumulate at the morula stage at different levels, with the *H1* mRNA being, in *P. lividus*, ~30% of the abundance of the two nucleosomal *H3* and *H2A* mRNAs. This differential regulation occurs despite the presence of a strong enhancer in the 5′-flanking region of the *H2A* gene. Although some evidence suggests that the enhancer has a bipartite organization (16), our results indicate that a single binding site for the MBF-1 activator suffices the *trans*-activation of a distant promoter and in both orientations (Figure 1). Because of this property, we raised the issue of whether competition between promoter *cis*-regulatory sequences specifically directs the MBF-1 transcription factor toward the cognate *H2A* promoter.
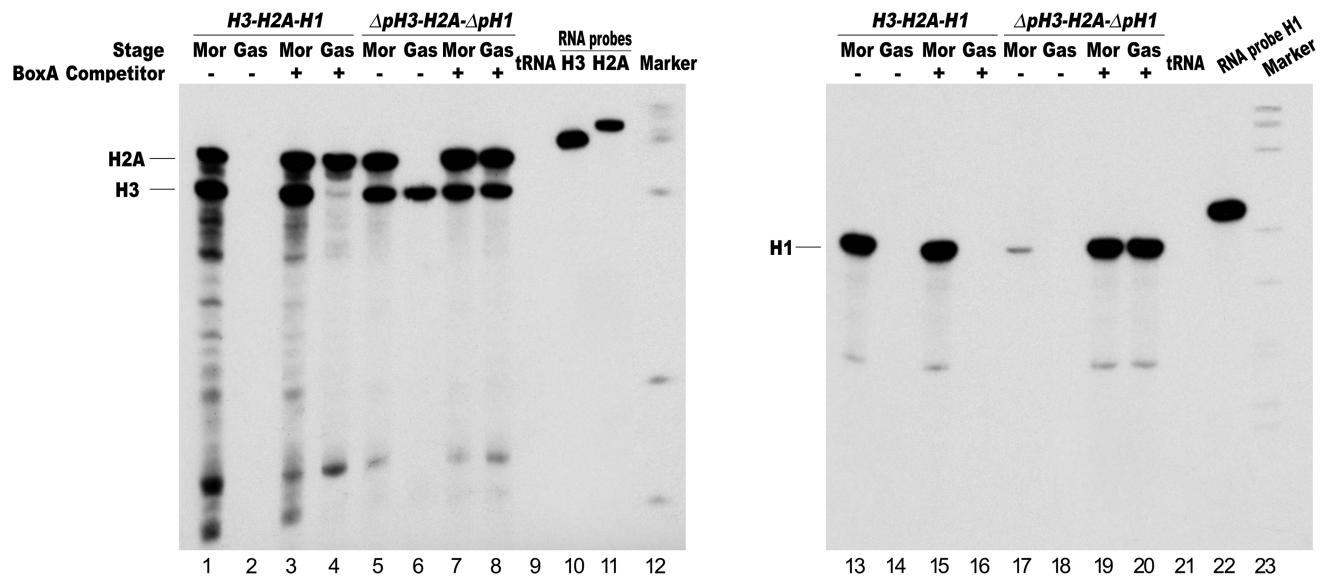
Figure 5. *In vivo* competition assay to inhibit the *sns5* insulator function in transgenic embryos. Wild-type histone gene construct *H3-H2A-H1* and the deletion mutant *ΔpH3-H2A-ΔpH1* (showed in Figure 2A), were microinjected with excess of the *BoxA* sequence element into *S. granularis* zygotes. RNase protection was carried out as for Figure 2. The protected 409, 357 and 209 nt RNA bands, respectively, for the *H2A*, *H3* and *H1* transcripts are indicated.
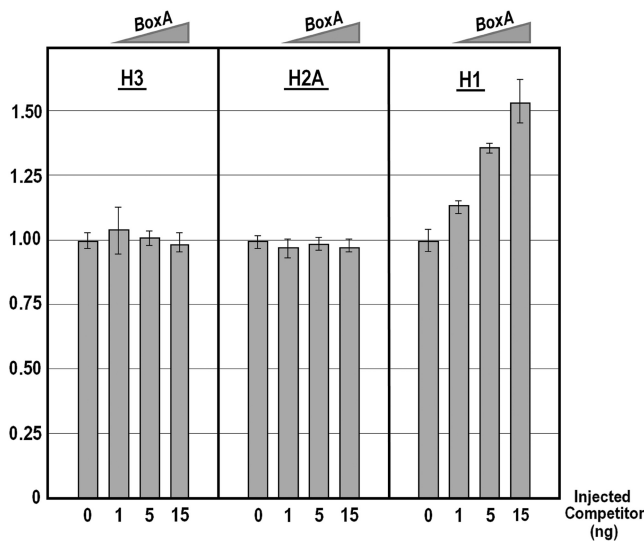
Figure 6. Endogenous gene expression analysis upon impairment of the *sns5* insulator function by *in vivo* competition assay with excess of the *BoxA* sequence element. Graphs show *n*-fold changes in mRNA expression level of histone genes based on the threshold cycle number (C_t) of injected embryos compared to that of the uncompeted control embryos. C_t numbers were normalized for the endogenous MBF-1 in the same sample. Data were derived from two independent microinjection experiments and each bar represents the average of triplicate samples from the two batches of embryos.

Although the activity of the wild type promoter of the *H3* transgene seemed refractory to the inhibition of MBF-1 binding, several lines of evidence substantiate the involvement of the MBF-1 transcription factor in the expression also of the α-*H3* gene, as previously suggested (31). First, we found a robust activation of the *H3*

minimal core promoter that is inhibited by the titration of the MBF-1 factor. Second, we found a reduction of the number of endogenous *H3* and *H2A* mRNA molecules with microinjection of increasing amounts of *M30*, but not the *M30* mutant, competitor (Figure 3). The third evidence is even stronger, in that, the expression of a forced MBF-1 repressor had a severe impact on the transcription level of the two nucleosomal core histone genes (Figure 4). Clearly, the accumulation of the *H2A* transcripts is more affected than that of *H3*, indicating a different responsiveness of their gene promoters to MBF-1 knockdown. A straightforward interpretation of this observation is that the MBF-1 activator is the essential transcription factor for *H2A* gene, as suggested by previous experiments (19), but it only participates, together with other factor(s), in the transcription of the nucleosomal *H3* histone gene. We speculate that one of the factors with which the enhancer-bound MBF-1 activator interacts is the protein complex containing the homeodomain CDP/cut that, as described (37), binds to the *CCAAT* sequence element for the maximum expression of the *H3* gene.

The results described in this article demonstrate that the linker *H1* histone gene is differentially regulated relative to the patterns of core histone gene transcription. In *Drosophila*, the cell cycle-dependent histone genes are tandemly arrayed and coordinately regulated, and the ratio of linker and core histones varies during embryonic development (38). However, the regulatory mechanism involved in the lower accumulation of *H1* gene transcripts, with respect to the nucleosomal histones, are profoundly different between fly and sea urchin. In *Drosophila*, two distinct sets of core promoter recognition factors, the TBP and TBP-related factors TRF2 (39), are responsible for directing transcription, respectively, of the

TATA-containing nucleosome core histone genes and the *TATA*-less linker histone *H1* (40). Although the molecular mechanism is not clear, upstream promoter-bound regulatory factors are probably involved in the interaction with the proper core promoter transcription complex. In the sea urchin *P. lividus*, the *H1* core promoter lacks a canonical *TATA* box and yet, as shown here, there is no competition between core promoter elements for the *H2A* enhancer. Instead, we obtained compelling evidence for the involvement of a chromatin insulator in making the linker *H1* promoter independent from the action of the *H2A* enhancer.

Chromatin insulators are genetic regulatory elements that may possess both a boundary function and a directional enhancer-blocking activity (41). Thus, insulators, by restricting enhancer function, may impart promoter selectivity to a given enhancer in the eukaryotic genome. This role has been demonstrated in several cases. The *Drosophila SF1* insulator, for instance, of the Antennapedia complex, placed in the *scr-ftz* intergenic region, restricts promoter selection by the *ftz*-distal enhancer in transgenic embryos (42) and separates the fushi tarazu from the neighboring *Hox* gene (43). As an additional example, the human and mouse imprinting controlling region (ICR) contain an insulator activity that depends on the binding of the CTCF regulator. In the maternal allele, the CTCF factor binds to the unmethylated ICR and prevents the downstream enhancer from interacting with the upstream *IGF2* promoter. The blocked enhancer can activate transcription of the *H19* gene (44).

Intriguingly, the *P. lividus sns5* DNA fragment, located at the 3′ of the *H2A* transcription unit, has been identified as an essential element for the silencing of the α-*H2A* gene at the gastrula stage (27). In addition, as for the most known vertebrate insulator, the *HS4* insulator of the chicken β-globin locus (13), the *sns5* sequence element displays, on transgene constructs, both enhancer-blocking and boundary activities (22,28). The directional enhancer blocking activity is achieved by the cooperative interactions between all three different protein factors bound to their specific *cis*-regulatory sequences, in that, titration of any of them abolishes the function (24,27). Here, we have shown that microinjection of molar excess of the *cis*-acting *BoxA* element allowed *trans*-activation of the *H1* transgene, driven by the core promoter elements. Most importantly, the *BoxA* competitor injected at the maximum dose (higher concentration is toxic for the embryo) specifically increased the expression of only the endogenous *H1* gene. The most obvious interpretation of this result is that the chromatin insulator *sns5* buffers the downstream *H1* promoter from the activity of *H2A* enhancer-bound MBF-1 transcription factor. The *sns5* insulator should leave also the expression of *H4* and *H2B* genes of the repeating unit not linked to the action of MBF-1 input from the *H2A* promoter. In fact, the high level of expression of these two genes depends on strong promoter upstream elements (45,46).

Altogether, the results presented in this article suggest that the *sns5* insulator is most probably responsible for the different level of accumulation of nucleosomal and linker transcripts during sea urchin embryogenesis. Finally, our findings provide a clear demonstration of the enhancer-blocking function of a chromatin insulator in a natural gene context.

An additional important issue to be clarified concerns the mechanism that prevents the activation of the *H1* gene by the *H2A* enhancer in the adjoining histone gene repeat. As a possibility, the enhancer might not elicit a perceptible effect at the resulting genomic distance of more than 5 kb. Alternatively, it could be speculated that the *H1* gene is flanked, at the 3′, by a supplementary insulator element. Experiments have been planned to distinguish between these two possibilities.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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