Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12322–12326, December 1994
Biochemistry

Modulator factor-binding sequence of the sea urchin early histone H2A promoter acts as an enhancer element

FRANCO PALLA*, CELESTINO BONURA†, LETIZIA ANELLO*, LINDA DI GAETANO†, AND GIOVANNI SPINELLI†‡

†Dipartimento di Biologia Cellulare e dello Sviluppo and *Istituto di Biologia dello Sviluppo del Consiglio Nazionale delle Ricerche, Via Archirafi 20, 90123 Palermo, Italy

Communicated by Max L. Birnstiel, August 22, 1994 (received for review May 25, 1994)

ABSTRACT **The sea urchin early H2A histone gene, like the other four members of the repeating units, is transiently expressed during very early development. To investigate the mechanisms underlying the faithful expression of the early H2A gene, we focused our attention on the modulator element. We showed by DNase I cleavage protection patterns that the modulator includes the upstream sequence element 1 (USE1) and mapped at nucleotides −137 to −108 in the early H2A gene promoter. Functional tests conducted by microinjection into sea urchin embryos then showed that the modulator element binds the transcriptional factor called modulator-binding factor 1 (MBF-1). We found in fact that coinjection of an excess of the MBF-1-binding site, either as the modulator or as the USE1, efficiently impaired the activity of the H2A promoter. An unexpected finding was the expression of the reporter gene from the early H2A promoter at the gastrula stage of embryonic development, when the early histone genes are transcriptionally silent. In addition, we also found that the modulator element was active at the gastrula stage. The potential enhancer activity of the modulator was tested by microinjecting several constructs containing single or multiple copies of the modulator element placed 5′ or 3′ to a thymidine kinase gene (*tk*) promoter in both sea urchin embryos and *Xenopus laevis* oocytes and determining the expression of a reporter chloramphenicol acetyltransferase gene under the control of the linked *tk* promoter. We found that an oligonucleotide bearing the MBF-1-binding site activates the expression of the reporter gene independently of the position and orientation. We conclude that the modulator binds the MBF-1 activator and that it is a transcriptional enhancer of the early H2A histone gene.**

Initiation of transcription by the RNA polymerase II promoters is controlled mainly by the interaction of regulatory factors with components of the preinitiation complex (1) formed from the basal transcription factors and assembled at the basal promoter (2–4). Thus, promoter-bound regulators can control transcription of specific genes (*i*) during cell differentiation and embryonic development and (*ii*) in different tissues. Regulative regions of RNA polymerase II promoters are usually distinct in upstream promoter sequences and enhancers (5). Typical enhancers, such as viral (6, 7) and cellular (8, 9) enhancers, have a modular structure with several binding sites for sequence-specific transcription factors/activators. However, in many cases single activator-binding sites, if multimerized, can act as enhancers when placed at a distance relative to the transcription start site (10–12). In addition, enhancer elements containing a single binding site for activators have been described. This is the case, for instance, for embryonic enhancers determining the stage-specific activation of late β-H1 (13) and late L1 H2B (14, 15) histone genes of sea urchin.

We are interested in the elucidation of the molecular mechanisms that underlie the timing of transcriptional activation of the sea urchin early histone gene subtypes. This gene family is organized in several hundred tandem repeated arrays (16). Each repeating unit contains one copy of each of the five histone genes. Transcription of the early histone gene family occurs transiently in early-cleavage embryos, reaching the maximum at the 32- to 64-cell stage of development and declining to an undetectable level at the gastrula stage (17). The transcriptional elements required for temporal and maximal expression for each of the five genes have been identified by gene transfer methodology (18) and by promoter binding studies. From all of these studies it appears that each gene has evolved its own specific mechanism for proper temporal expression during development (19–24).

Several nuclear factor-binding sites are present in the upstream promoters of the early histone genes. Nonetheless, for any of them, transcriptional enhancer activity has not yet been demonstrated. However, the expression of early H2A gene of *Psammechinus miliaris*, tested in *Xenopus laevis* oocytes, depends on the presence and integrity of an upstream element, denoted "modulator" (25, 26), which has been equated to an enhancer (27). In fact, a DNA segment containing this control element can act in frog oocytes in both orientations. Our functional tests performed by microinjection in *X. laevis* oocytes allowed identification in the modulator of the *Paracentrotus lividus* early H2A gene of a cis-acting element, the upstream sequence element 1 (USE1), thus confirming the positive role played by this sequence element in the expression of the H2A gene (28). Promoter binding analysis in nuclear extracts of sea urchin embryos actively transcribing the early histone genes led to the identification of the factor binding to USE1, which has been denoted "modulator-binding factor 1" (MBF-1) (21).

We wished to define the role of the modulator in the activation of early H2A gene expression in the homologous system. We found that the activity of exogenous H2A promoter in microinjected sea urchin embryos relies upon the binding of MBF-1 to the modulator. Furthermore, an oligonucleotide containing the MBF-1-binding site activated transcription of a reporter gene, when placed at a distance and in both orientations relative to the viral tk promoter.§

MATERIAL AND METHODS

DNase I Protection and Electrophoretic Mobility-Shift Assays. Nuclear extracts from *Pa. lividus* at the 32- to 64-cell stage of development were prepared as described (21). DNA fragments (1–2 ng) of the H2A promoter labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase were incu-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: USE, upstream sequence element; MBF, modulator-binding factor; CAT, chloramphenicol acetyltransferase; RT, reverse transcription; tk/TK, thymidine kinase.
‡To whom reprint requests should be addressed.
§The sequence for the H2A promoter reported in this paper has been deposited in the GenBank data base (accession no. M25281).

bated for 20 min at 4°C with 50–150 μg of either bovine serum albumin or nuclear extract in 50 μl of 10 mM Hepes, pH 7.9/60 mM KCl/1 mM dithiothreitol/1 mM EDTA/4% Ficoll. Samples were digested with DNase I under the conditions described in the legend to Fig. 2, extracted with phenol, and loaded on denaturing polyacrylamide gel.

Construction of Plasmids. To construct the H2A-CAT plasmid, the *Acc* I–*Rsa* I DNA fragment from clone pH70 (29), spanning nucleotides −140 to 90 relative to the H2A transcription start site, was subcloned in the chloramphenicol acetyltransferase (CAT) expression vector pBL3-CAT (30). H2A-CAT-INSERT was obtained by cloning a *Sau*3A 258-bp pUC18 fragment in the *Bam*HI site of the polylinker of the H2A-CAT plasmid. MODULATOR-TK-CAT plasmids were constructed by shotgun cloning of ligated double-stranded oligonucleotides bearing the H2A modulator sequence (the sequence is shown in the next section) either upstream or downstream the CAT coding sequences of the tk-70 pBL2 vector under the control of the thymidine kinase gene (*tk*) promoter (30). The copy number and the orientation of the modulator elements were determined by DNA sequence analysis.

Detection of CAT Expression in Injected Sea Urchin Embryos and *Xenopus* Oocytes. Linearized H2A-CAT, MODULATOR-TK-CAT, or TK-CAT plasmids brought to a total concentration of 200 μg/ml were microinjected with sea urchin sperm DNA of roughly 5 kb into sea urchin *Pa. lividus* eggs from a mature female by the procedure of McMahon *et al.* (18). For the competition experiments, seven copies of ligated modulator or USE1 oligonucleotides were coinjected with the H2A-CAT construct. The sequences (5′ to 3′) of the sense strands of the modulator and USE1 oligonucleotides were as follows: modulator, GATCAATCGCCAACAGAGGGAGCTATTCCC; USE1, GTAGACAATCGCCAACAGAGGGA. Expression of the CAT gene in the microinjected embryos at the 32-cell and gastrula stages of development was determined by reverse transcription (RT)–PCR as reported (21). Briefly, nucleic acid samples from a pool of about 30 microinjected embryos either at the 32-cell stage or gastrula stage were incubated with 1 mg of RNase-free DNase per ml for 60 min at 37°C, extracted with phenol/chloroform, and precipitated with ethanol. An amount of RNA samples equivalent to those extracted from 1–5 embryos was reverse-transcribed from a specific downstream CAT primer (see the sequence below). After incubation at 98°C for 5 min to denature the reverse transcriptase, the upstream primer was added, and the resulting cDNAs were amplified by *Taq* DNA polymerase for 30 cycles under the following conditions: denaturation for 1 min at 95°C; annealing for 1 min at 54°C; and extension for 1 min at 72°C. The sequences (5′ to 3′) of the 3′ primers were: ATTCCGGATGAGCATTCATACG, located at nucleotides 196–217 relative to the ATG codon for the H2A-CAT plasmid; and GCACCTTGTCGCCTTGCGTA, located at nucleotides 530–549 relative to the ATG codon for MODULATOR-TK-CAT plasmid. The sequences (5′ to 3′) of the 5′ primers were: TCTAGCCAAGAACCATCGCTTC, located at nucleotides 3–22 relative to the transcription start site of the H2A gene for the H2A-CAT plasmid; and TCACTGGATATACCACCATT, located at nucleotides 14–33 relative to ATG codon of the CAT coding sequence for the MODULATOR-CAT plasmid.

Microinjection in *X. laevis* oocytes and CAT assays was as described (21).

RESULTS

DNase I Cleavage Protection Patterns (Footprints) of the Early H2A Gene Promoter. The modulator was previously identified in the early H2A regulative region of *Ps. miliaris* by mutational analysis performed in frog oocytes (25–27). Nucleotide sequence comparison of the early H2A upstream promoter regions of *Ps. miliaris* and *Pa. lividus* revealed two extended blocks of conserved sequences respectively centered on the modulator and on the CCAAT box (Fig. 1).

Previous promoter binding studies in nuclear extracts prepared from embryos at the 32- to 64-cell stage have mapped an upstream sequence element denoted USE1 at nucleotides −117 to −129 within the *Pa. lividus* H2A promoter (28). An oligonucleotide containing this sequence element was then shown to bind a transcription factor denoted MBF-1 (21). However, when we recently prepared DNase I cleavage protection patterns of the H2A promoter fragments with different nuclear extract preparations and with higher protein concentrations, we reproducibly observed that the USE1 sequence element was included in a larger protected region. This is clearly evident from the results presented in Fig. 2. DNase I footprinting of sense and antisense strands of a modulator-containing early H2A promoter fragment in nuclear extracts from embryos actively transcribing the early histone genes allowed delineation of the boundaries of the protected sequence of the modulator (Fig. 2). From these results and from those of the functional tests described below, we conclude that the modulator element of the early H2A promoter is centered between nucleotides −137 and −108.

The Modulator Is an Essential Transcriptional Element of the Early H2A Promoter. As mentioned before, the site of binding of the transcription factor MBF-1 was previously shown to correspond to the USE1 sequence element. The DNase I protection patterns shown in Fig. 2 indicate that the interacting sequence of the modulator extends beyond the USE1 sequence element. Therefore, it is expected that MBF-1 binds to the modulator element and activates transcription of the early H2A histone gene. To provide evidence that binding of MBF-1 to the modulator is essential for high-level activity of the early H2A promoter, we carried out competition experiments in microinjected sea urchin embryos. To this end an H2A DNA fragment spanning nucleotides −140 to 90, which includes the modulator element and the CCAAT box, was fused to the CAT reporter gene. The resulting H2A-CAT construct, depicted in Fig. 3*C*, was microinjected into *Pa. lividus* eggs that were fertilized and tested for expression of the CAT gene at the 32-cell and gastrula stages. In a parallel experiment, CAT expression was determined in 32-cell-stage sea urchin embryos coinjected with the same construct and an ≈70-fold excess of either ligated USE1 or modulator oligonucleotides.

To identify the CAT transcripts, the RNA samples were reverse-transcribed first, and the resulting cDNA was amplified by PCR. To make sure that the PCR products reflected the relative transcriptional level in the microinjected embryos, an internal control RNA, transcribed *in vitro* from the H2A-CAT-INSERT template (see Fig. 3*C*) was coreverse-transcribed and coamplified with the RNA samples of the

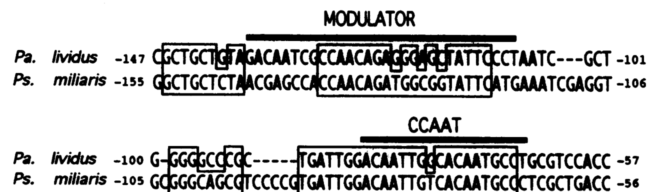

FIG. 1. Nucleotide sequence comparison between the upstream promoters of the early H2A histone gene of *Pa. lividus* and *Ps. miliaris*. To enhance identity, gaps, indicated by dashes, have been introduced in the *Pa. lividus* sequence. Thick overlines indicate the protected regions deducted from DNase I footprinting experiments (Fig. 2 and data not shown).

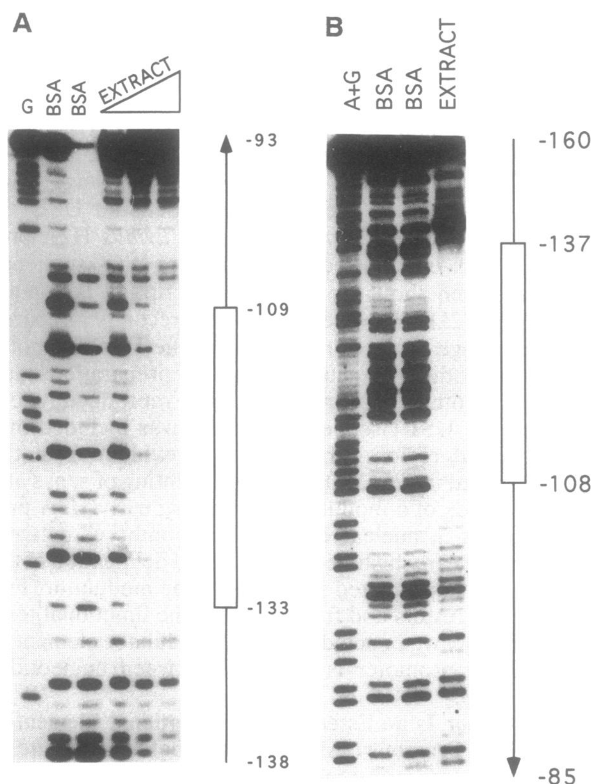

FIG. 2. DNase I cleavage protection patterns of the early H2A promoter fragment by nuclear extracts from 32-cell-stage and 64-cell-stage embryos. Deletion mutant subclones of the H2A promoter were utilized to label coding (*A*) and noncoding (*B*) strands at the 5′ end. The labeled fragments were respectively incubated with 50–150 μg (*A*) and 100 μg (*B*) of nuclear extracts and digested with 5 μg of DNase I for 3 min at room temperature. As a control, the labeled DNA fragments were incubated with 100 μg of bovine serum albumin (BSA) and digested with DNase I at the same conditions. The digestion products were analyzed together with the cleavage products of the G+A sequence reaction on denaturing polyacrylamide gel.

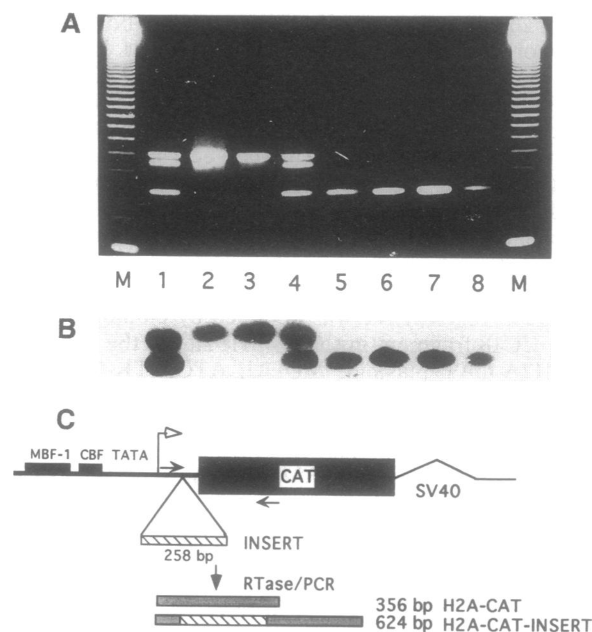

FIG. 3. Competition of the H2A promoter activity by the MBF-1-binding sequence in coinjected sea urchin embryos. (*A* and *B*) Linearized H2A-CAT construct was microinjected into *Pa. lividus* eggs that were fertilized and allowed to develop to the 32-cell stage (lanes 1 and 5) and gastrula stage (lanes 4 and 8). In parallel, sea urchin eggs were coinjected with the same construct and a 70-fold excess of ligated USE1 (lanes 2 and 6) or modulator (lanes 3 and 7) oligonucleotides, and embryos were harvested when they reached the 32-cell stage. Nucleic acids were extracted from a pool of 30 microinjected embryos and processed for RT–PCR (lanes 1–4) and PCR (lanes 5–8). An amount of RNA or total nucleic acids equivalent to those extracted from one or three embryos, respectively, was amplified. The amplification products and a 123-bp ladder DNA marker (lane M) were analyzed by gel electrophoresis. (*A*) Ethidium bromide staining of the agarose gel. (*B*) DNA blot of a similar gel hybridized with labeled antisense CAT RNA. (*C*) Schematic drawing of the H2A-CAT and H2A-CAT-INSERT plasmids and the expected RT–PCR products.

microinjected embryos. The expected PCR products were 356 bp and 624 bp for the H2A-CAT and H2A-CAT-INSERT, respectively. The results presented in Fig. 3 show that an H2A promoter fragment that included the MBF-1 and CCAAT factor binding sites promoted CAT expression in microinjected embryos at the 32-cell stage of development. Indeed, the bands of 356 bp and 624 bp were detected by RT–PCR in the RNA samples from the 32-cell-stage (Fig. 3 *A* and *B*, lane 1) and, surprisingly, from the gastrula-stage (lane 4) injected embryos. By contrast, the activity of the H2A promoter was drastically reduced in embryos coinjected with either USE1 (lane 2) or modulator oligonucleotides (lane 3). Inclusion or omission of a 1000-fold excess of sonicated genomic DNA to the cloned DNA constructs did not affect the transcription levels (our unpublished results), and hence competition of transcription seen after addition of USE1 or modulator oligonucleotides is a sequence-specific effect.

The roughly 600-bp DNA band detected in the samples not subjected to competition (Fig. 3*A*, lanes 1 and 4) was occasionally observed in different experiments and appeared to be a PCR product of the internal control RNA. Strangely enough, this doublet DNA fragment was never seen in the PCR products of the samples blocked by competition.

The expression of the reporter gene occurring at the gastrula stage suggests that this DNA fragment lacks the sequence elements required for temporal regulation (Fig. 3 *A* and *B*, lane 4). However, when we used an early H2A promoter fragment spanning nucleotides −226 to 40 for the microinjection experiments, we obtained identical results (not shown). Therefore, the regulative sequences needed at the gastrula stage to inactivate expression of the early H2A histone gene are not located in the upstream promoter or in the mRNA leader region. To be sure that the H2A-CAT DNA was present in all microinjected embryos, an aliquot of the nucleic acid samples was withdrawn before DNase I digestion and amplified with *taq* DNA polymerase without a reverse transcription step. As shown in lanes 5–8 of Fig. 3 *A* and *B*, the expected PCR fragment of 324 bp was obtained for all samples. From these results we conclude that the activity of the H2A promoter relies upon the binding of transcription factor MBF-1 to the modulator element.

Enhancer Activity of the Modulator. We first tested whether multiple copies of the modulator placed 5′ or 3′ to a heterologous promoter could activate transcription of the reporter gene. Unfertilized *Pa. lividus* eggs were microinjected with the constructs (depicted in Fig. 4*A*) respectively bearing three copies of the modulator oligonucleotide 5′ to a *tk* promoter (construct a) and two copies in head-to-head arrangement 3′ to the CAT coding sequence (construct b). Sea urchin eggs were also injected with the TK-CAT vector as a negative control. CAT expression was monitored by RT–PCR assays in embryos at 32-cell and gastrula stages. The *tk* promoter was not active in sea urchin embryos as shown (Fig. 4*B*) by the lack of PCR products in the RNA sample from embryos microinjected with the TK-CAT plasmid (construct c in Fig. 4*B*, lane 4). By contrast, a major amplified DNA band of the

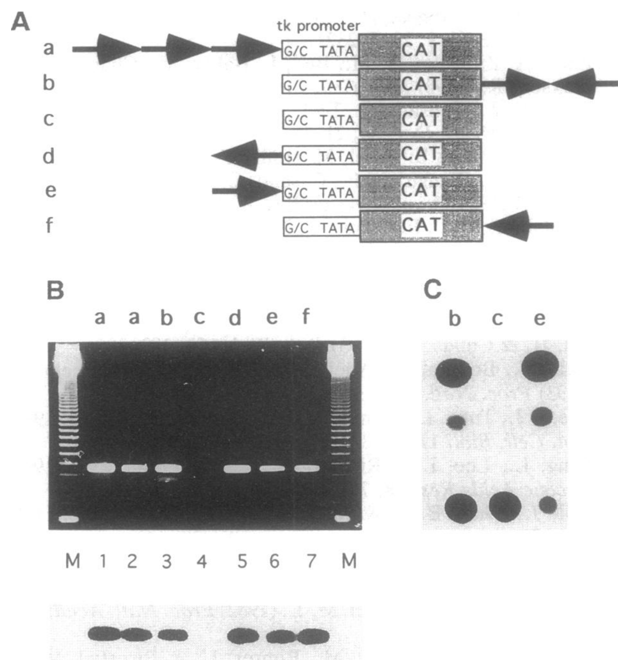

FIG. 4. Enhancer activity of the H2A modulator. (*A*) Drawings of TK-CAT constructs containing either multiple or single copies of the modulator oligonucleotide. The location, orientation, and arrangement of the modulator elements are indicated by arrows. (*B*) Constructs a–f were microinjected into sea urchin eggs. CAT transcripts were detected by RT–PCR in embryos at the 32-cell stage (lanes 1 and 3–7) and, for construct a, also at the gastrula stage (lane 2). The oligonucleotide primers were located in the CAT coding sequence. (*B Upper*) Ethidium bromide staining of the agarose gel. (*B Lower*) Autoradiogram of DNA blot of the same gel hybridized with labeled antisense CAT RNA. (*C*) Autoradiogram of the CAT assays performed on extracts of *X. laevis* oocytes injected with constructs b, c, and e.

expected size of 536 bp is generated by RT–PCR of the RNA samples from embryos at the 32-cell-stage (lane 1) and gastrula-stage (lane 2) embryos injected with construct a, in which three copies of the modulator were placed 5′ to the *tk* promoter. An identical result was obtained with two copies of the modulator oligonucleotide cloned downstream to the CAT coding sequence (construct b, lane 3).

We next asked whether a single MBF-1-binding site could activate transcription from a remote location and in both orientations. As shown in Fig. 4*B*, the expected DNA band of 536 bp was generated by RT–PCR of the RNA samples from 32-cell-stage embryos injected with the constructs in both orientations containing a single modulator element located upstream (constructs d and e, lanes 5 and 6) to the *tk* promoter and downstream to the CAT coding region (construct f, lane 7). To make sure that the PCR products were generated from reverse transcription of RNA molecules and not from residual contamination of the injected DNA, PCR amplifications of the RNA samples were also carried out without performing the reverse transcription step. No PCR products were obtained in such cases (not shown).

In summary, these results indicate that the modulator element of the H2A promoter activates transcription from a heterologous promoter independently of position and orientation of a linked gene and that MBF-1 is active also at the gastrula stage.

The modulator element was first identified in *X. laevis* oocytes (25–27), where an activity related to the MBF-1 activator was also demonstrated (21, 28). It was of some interest, therefore, to determine whether the enhancer activity of the modulator element is also conserved between sea urchin and *Xenopus*. To this end, constructs b, c, and e of Fig. 4, containing one or two copies of the modulator located 5′ to the *tk* promoter and 3′ to the CAT coding sequence, were microinjected in frog oocytes that were tested for CAT enzymatic activity. The results shown in Fig. 4*C* indicate that in *Xenopus*, like in sea urchin, the early H2A modulator activated expression of the reporter gene even when located at a remote position relative to the *tk* promoter.

DISCUSSION

We have demonstrated that the activity of the early H2A promoter is strictly dependent on the interaction of the transcription factor MBF-1 with the H2A modulator element. In fact, transcription of the CAT gene from a linked H2A promoter that included the modulator and CCAAT elements was drastically reduced in sea urchin embryos coinjected with the same construct and a 70-fold excess of the MBF-1-binding site. These results indicate, therefore, that the modulator is a positive transcriptional element of the H2A gene. The interacting sequence of MBF-1 was previously shown to correspond to the USE1 sequence element (21, 28), which is 12 nucleotides shorter relative to the 3′ end than the modulator element, as it is defined in this paper. However, the similar competitive effect on the activity of the H2A promoter that was observed in sea urchin embryos microinjected with excess of both USE1 and modulator oligonucleotides led us to conclude that MBF-1 interacts with USE1 and the modulator sequence elements with similar affinity.

We were surprised to find that the H2A promoter fragment activated CAT expression at the gastrula stage, when the transcription of the early histone genes has been shut down. Consistent with this observation, promoter binding analysis in nuclear extracts from gastrula-stage embryos showed an identical DNase I protection pattern, with the MBF-1 and CCAAT factor binding sites fully protected (unpublished observation). Furthermore, our results indicate that the MBF-1 activator binds to the modulator and activates transcription of the CAT gene also at the gastrula stage. As far as the temporal expression of the early histone genes is concerned, it appears that each early histone gene bears its own regulative program of expression during embryogenesis. For instance, as for the early H2A gene, the inactivation of the early H2B gene at the gastrula stage seems to depend on regulatory sequences that are not located in the upstream promoter (24). The octamer binding factor involved in the faithful expression of the early H2B gene activates, in fact, the early H2B promoter in early and late cleavage embryos (24). By contrast, the sequence elements for temporal regulation of the early H3, H1, and H4 histone genes are placed in the 5′ flanking regions (19–22). However, only in the case of the early histone H1 gene has a cis-acting element that regulates the temporal expression been identified (20). In conclusion, our results indicate that the sequence elements required for temporal regulation of the early H2A gene are localized either far upstream of the MBF-1 binding site or in the region 3′ to the CAT coding sequence. The latter possibility seems particularly interesting, since we have previously shown that a nuclease hypersensitive site appears in that region at the gastrula stage (31) in proximity to the palindromic sequence known to be essential for 3′ processing of the H2A RNA precursor (32). Alternatively, the regulatory sequences responsible for the inactivation of H2A expression could be located, as suggested for the early H2B gene (24), within the coding sequence. Finally, the possibility still remains that the regulatory sequences lacking in the injected H2A-CAT DNA are involved in posttranscriptional regulation.

The results reported in this paper confirm and extend previous observations made by Birnstiel and coauthors (25–

27). These authors obtained the first evidence for a cis-acting transcriptional element, the modulator, that could activate transcription of the downstream gene in both orientations. We have demonstrated here in both sea urchin and in frog oocyte that an oligonucleotide containing a MBF-1-binding site activates transcription from the *tk* promoter of a linked reporter gene. This activation occurs independently of the position and the orientation of the modulator relative to the promoter. We conclude therefore that the modulator element of the early H2A gene, which is essential for high-level expression of the early H2A histone gene in cleavage stage sea urchin embryos, and enhancers share similar properties and are different names for the same genetic control element.

We are grateful to Mr. D. Cascino and Mr. A. Oliva for technical assistance and to Dr. R. DiMarzo for the synthesis of the oligonucleotides. This work was supported in part by the "Progetto Finalizzato Ingegneria Genetica" and by funds of the "Ministero dell' Università e della Ricerca Scientifica e Tecnologica."

1. Roeder, R. G. (1991) *Trends Biochem. Sci.* **16,** 402–408.
2. Zawel, L. & Reinberg, D. (1993) *Prog. Nucleic Acids Res. Mol. Biol.* **44,** 67–108.
3. Hori, R. & Carey, M. (1994) *Curr. Opin. Genet. Dev.* **4,** 236–244.
4. Buratowski, S. (1994) *Cell* **77,** 1–3.
5. Müller, M. M., Gerster, T. & Schaffner, W. (1988) *Eur. J. Biochem.* **176,** 485–495.
6. Dynan, W. S. (1989) *Cell* **58,** 1–4.
7. Thompson, C. C. & McKnight, S. (1992) *Trends Genet.* **8,** 232–236.
8. Thanoa, D. & Maniatis, T. (1992) *Cell* **71,** 777–789.
9. Giese, K., Cox, J. & Grosschedl, R. (1992) *Cell* **69,** 185–196.
10. Westing, G. & Schaffner, W. (1988) *EMBO J.* **12,** 3763–3770.
11. Pierce, J. W., Lenardo, M. & Baltimore, D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1482–1487.
12. Shatt, M. D., Rusconi, S. & Schaffner, W. (1990) *EMBO J.* **9,** 481–487.
13. Lai, Z. C., De Angelo, D. J., Di Liberto, M. & Childs, G. (1989) *Mol. Cell. Biol.* **9,** 2315–2321.
14. Zhao, A. Z., Colin, A. M., Bell, J., Baker, M., Char, B. R. & Maxson, R. (1990) *Mol. Cell. Biol.* **10,** 6730–6741.
15. Zhao, A. Z., Vansant, G., Bell, J., Humphreys, T. & Maxson, R. (1991) *Mech. Dev.* **34,** 21–28.
16. Hentschel, C. & Birnstiel, M. (1981) *Cell* **25,** 301–313.
17. Spinelli, G., Gianguzza, F., Casano, C., Acierno, P. & Burckhardt, J. (1979) *Nucleic Acids Res.* **6,** 545–560.
18. McMahon, P. A., Flytzanis, C. N., Hough-Evans, B. R., Katula, K. S., Britten, R. J. & Davidson, E. H. (1985) *Dev. Biol.* **108,** 420–430.
19. Di Liberto, M., Lai, Z. C., Fei, H. & Childs, G. (1989) *Genes Dev.* **3,** 973–985.
20. Fei, H. & Childs, G. (1993) *Dev. Biol.* **155,** 383–395.
21. Palla, F., Bonura, C., Anello, L., Casano, C. & Spinelli, G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6854–6858.
22. Lee, I. J., Tung, L., Bumcrot, D. A. & Weinberg, E. S. (1991) *Mol. Cell. Biol.* **11,** 1048–1061.
23. Tung, L., Lee, I. J., Rice, H. L. & Weinberg, E. S. (1990) *Nucleic Acids Res.* **18,** 7339–7348.
24. Bell, J., Char, B. R. & Maxson, R. (1992) *Dev. Biol.* **150,** 363–371.
25. Grosschedl, R. & Birnstiel, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7102–7106.
26. Grosschedl, R. & Birnstiel, M. L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 297–301.
27. Grosschedl, R., Mächler, M., Rohrer, U. & Birnstiel, M. L. (1983) *Nucleic Acids Res.* **11,** 8123–8136.
28. Palla, F., Casano, C., Albanese, I., Anello, L., Gianguzza, F., Di Bernardo, M. G., Bonura, C. & Spinelli, G. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6033–6037.
29. Spinelli, G., Albanese, I., Anello, L., Ciaccio, M. & Di Liegro, I. (1982) *Nucleic Acids Res.* **10,** 7977–7992.
30. Luckow, B. & Schütz, G. (1987) *Nucleic Acids Res.* **15,** 5490.
31. Anello, L., Albanese, I., Casano, C., Palla, F., Gianguzza, F., Di Bernardo, M. G., Di Marzo, R. & Spinelli, G. (1986) *Eur. J. Biochem.* **156,** 367–374.
32. Birnstiel, M. L., Busslinger, M. & Strub, K. (1985) *Cell* **36,** 349–359.