Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 6033–6037, August 1989
Biochemistry

Cis-acting elements of the sea urchin histone H2A modulator bind transcriptional factors

(*Xenopus* oocyte microinjection/chloramphenicol acetyltransferase assays/trans-acting factors/DNase I protection)

FRANCO PALLA*, CATERINA CASANO†, IDA ALBANESE†, LETIZIA ANELLO*, FABRIZIO GIANGUZZA†, MARIA G. DI BERNARDO*, CELESTINO BONURA†, AND GIOVANNI SPINELLI†‡

†Dipartimento di Biologia Cellulare e dello Sviluppo, and *Istituto di Biologia dello Sviluppo del Consiglio Nazionale delle Ricerche, via Archirafi 20-22, 90123 Palermo, Italy

Communicated by Max L. Birnstiel, April 21, 1989

ABSTRACT **Functional tests, performed by microinjection into *Xenopus laevis* oocytes, show that a DNA fragment containing the modulator of the early histone H2A gene of *Paracentrotus lividus* enhances transcription of a reporter gene when located, in the physiological orientation, upstream of the *tk* basal promoter. Gel retardation and DNase I footprinting assays further reveal that the H2A modulator contains at least two binding sites [upstream sequence elements 1 and 2 (USE 1 and USE 2)] for nuclear factors extracted from sea urchin embryos, which actively transcribe the early histone gene set. Interestingly, USE 1 is highly homologous to a cis-acting element previously identified in the H2A modulator of *Psammechinus miliaris* [Grosschedl, R., Mächler, M., Rohrer, U. & Birnstiel, M. L. (1983) *Nucleic Acids Res.* 11, 8123–8136]. Finally, a cloned oligonucleotide containing the USE 1 sequence competes efficiently in *Xenopus* oocytes with the H2A modulator to prevent enhancement of transcription of the reporter gene. From these results, we conclude that USE 1 and perhaps USE 2 in the H2A modulator are upstream transcriptional elements that are recognized by trans-acting factors common to *Xenopus* and sea urchin.**

The sea urchin genome contains several histone gene families that encode the protein subtypes of sperm (S-type) and of cleavage, early, and late stage embryo (1). The synthesis of specific histone protein variants during development is the result of regulatory mechanisms that operate at both the transcriptional and posttranscriptional level (2). The early histone genes in all sea urchin species are tandemly repeated 300–600 times and are organized in quintets (3, 4). Transcription of this gene set occurs upon meiotic maturation and soon after fertilization (5–7). Newly synthesized early histone mRNAs accumulate at the 32- to 128-cell stage. Their transcription is shut off in mesenchyme blastula embryos (8–10). The transition from a transcriptionally active to an inactive state of the early histone genes is accompanied by structural alterations of their chromatin arrangement (11–13).

Molecular genetic analysis of many polymerase II promoters has revealed that optimal and accurate initiation of transcription requires a cooperative interaction of trans-acting factors with multiple cis-acting transcriptional elements (14–17).

Recent studies on the factors involved in the transcriptional control of the sea urchin histone H1 and H2B genes have identified basal and ontogenic transcriptional elements involved in the transition from early to late gene expression during development (18, 19). Furthermore, they have shown that an embryo-specific repressor element, the CCAAT displacement factor, prevents the interaction of a positive trans-acting factor with the CCAAT box of the sperm-specific H2B-1 gene (20) and in so doing may block transcription in the embryo.

We have focused our attention on the early H2A gene of the sea urchin *Paracentrotus lividus* for several reasons. The 5′ flanking region of this gene contains, upstream to the basal transcriptional elements, a sequence§ that possesses sequence similarity to the modulator element present in a similar location in the early H2A gene of the closely related sea urchin species *Psammechinus miliaris*. Functional studies of the region containing this sequence, carried out by microinjection into *Xenopus laevis* oocytes, have shown that it contains positive cis-acting elements for H2A transcription, with enhancer activity (21–23). Further studies of the histone gene chromatin conformation later revealed that active transcription of the early H2A gene of both sea urchin species is related to the appearance of a nuclease hypersensitive site just at the border of the modulator (24, 25). Upon transcriptional inactivation, this site disappears and the histone gene cluster reverts to the native chromatin state. As suggested by a large body of evidence, the presence of these domains in the neighborhood of active genes is often correlated with the binding of regulatory proteins to short stretches of DNA (26).

To gain more information on the cis- and trans-acting factors involved in the transcriptional control of the early H2A gene, we fused a DNA region containing the H2A modulator to the *tk* regulatory region, cloned the chloramphenicol acetyltransferase (CAT) reporter gene upstream, and tested the activity by microinjection into *Xenopus* oocytes. In addition, DNase I footprinting was used to determine the binding sites of sea urchin nuclear factors. Finally, we carried out homologous and heterologous competition in *Xenopus* oocytes with both the histone H2A DNA fragment containing the modulator and with an oligonucleotide corresponding to one of the two identified binding sites for sea urchin nuclear factors.

MATERIALS AND METHODS

Preparation of the Nuclear Extracts. The collection of gametes from *Pa. lividus*, culture of embryos, and isolation of nuclei were carried out as previously described (11). After centrifugation through a 1.0 M sucrose cushion, the purified nuclear pellet from 32- to 64-cell stage embryos was washed once in 10 mM NaCl/10 mM Tris·HCl, pH 7.4/0.5 mM phenylmethylsulfonyl fluoride, pelleted, and resuspended in 10 mM Tris·HCl, pH 7.4/200 mM NaCl/200 mM KCl/0.5 mM phenylmethylsulfonyl fluoride.

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Abbreviations: CAT, chloramphenicol acetyltransferase; USE, upstream sequence element.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25281).

The sample was kept in an ice bath for 1 hr with frequent shaking and then was centrifuged at 3000 × *g* for 5 min. The supernatant was dialyzed overnight in the cold room against 10 mM Tris·HCl, pH 7.5/70 mM NaCl/1 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/20% (vol/vol) glycerol and then was centrifuged at 14,000 × *g* for 10 min. The supernatant was divided into small aliquots and stored at −80°C. The protein concentration in the nuclear extracts was determined by standard procedures (27).

Construction of Plasmids and Preparation of Probes. The Mi H2A tk-70, Mr H2A tk-70, and pUC MH2A plasmids, which contain the −219/−49 base-pair (bp) fragment of the H2A gene (see Fig. 1), were obtained by cloning the corresponding *Sau*3A fragment into the *Bam*HI site of the tk-70 (a kind gift of R. Cortese, European Molecular Biology Laboratory, Heidelberg) and pUC 8 vectors, respectively. To construct the pUC USE 1 plasmid, the following oligonucleotide, which spans nucleotides −134/−116 of the H2A segment, was synthesized: 5′ GATCGCCAACAGAGGGAGCT 3′ as was its complementary sequence: 5′ GATCAGCTCCCTCTGTTGGC 3′. After annealing, the double-stranded oligonucleotide was cloned into the *Bam*HI site of the pUC 19 vector. The orientation of the inserts was determined by DNA sequence analysis.

Gel Retardation Assay. Aliquots of the end-labeled histone DNA fragment were incubated with the nuclear extract in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/70 mM NaCl/0.5 mM dithiothreitol at 37°C for 30 min. In some assays, unlabeled *Escherichia coli* DNA, fragmented to an average size of about 400 bp by sonication, was added to the incubation mixture. At the end of the incubation, the samples were analyzed by electrophoresis as described by Strauss and Varshavsky (28). The gel was dried under reduced pressure and autoradiographed by using x-ray film and an intensifying screen at −80°C.

DNase I Footprinting Assays. The sense strand of the histone DNA fragment containing the modulator was labeled by linearizing the pUC MH2A construct with *Eco*RI, incubating with terminal transferase and [α-^{32}P]ddATP, and then cleaving with *Hin*dIII. One nanogram of fragment was incubated with 10 μg of nuclear extract in a final volume of 7 μl in the presence of a 500-fold excess of unlabeled sonicated *E. coli* DNA. At the end of the incubation, the samples were treated with DNase I, in the presence of 2.5 mM $MgCl_2$, for 5 min at 37°C. The reaction was terminated by the addition of EDTA to 5 mM, and the samples were loaded onto a 4% polyacrylamide gel and electrophoresed as described.

The gel was autoradiographed without drying. The regions of interest were then excised from the gel and eluted in 6 volumes of electrophoresis running buffer containing 0.5% SDS overnight at 37°C with shaking. The DNA was purified from the eluate by DEAE-cellulose chromatography, lyophilized, and analyzed by electrophoresis on a sequencing 9% polyacrylamide gel. DNase I digestion of naked DNA was performed as follows: 4 ng of the 3′ end-labeled DNA fragment was digested, in the presence of 8 μg of *E. coli* DNA, for 5 min at room temperature. Sequence reactions were performed by standard procedures (29).

Microinjection and CAT Assays. *X. laevis* oocytes were injected with the various plasmids at the same total DNA concentration (indicated in the legend to figures), which was achieved by the addition of pUC 19 carrier DNA. The CAT assays on cytoplasmic extracts were carried out on the same number of injected *Xenopus* oocytes and performed as described (30).

RESULTS

The Sea Urchin H2A Modulator Enhances the Activity of the *tk* Promoter in the *Xenopus* Oocytes. To investigate the functional role of the upstream region of the early H2A gene, we tested the effect of a DNA segment containing the modulator on the activity of a heterologous promoter. A 172-bp *Sau*3A fragment, which extends from position −219 to −49 relative to the 5′ end of H2A and includes the entire modulator (see Fig. 1*A*), was inserted in either orientation in the tk-70 vector, a derivative of the pBLCAT2 construct (31) that contains the *tk* promoter region, spanning nucleotides −70/+53, fused to the reporter CAT gene (see Fig. 1*B*). This *tk* promoter region retains only the proximal SP1 binding site and the TATA box (32). The resulting constructions (Mi H2A tk-70 and Mr H2A tk-70) and the vector alone were microinjected into the nucleus of *X. laevis* oocytes, and the CAT enzymatic activity was determined in the oocyte extracts. The results are shown in Fig. 2. In agreement with previous reports (33), the tk-70 vector is poorly transcribed in *Xenopus* oocytes (Fig. 2*A*, lane a). On the other hand, the presence of the 172-bp histone DNA fragment in the physiological orientation, upstream of the Sp1 binding site in the tk-70 vector (Mr H2A tk-70 construct of Fig. 1*B*) resulted in about 20-fold activation of transcription of the reporter gene (Fig. 2*A*, lane b). The histone DNA fragment in the reverse orientation (Mi H2A tk-70 construct of Fig. 1*B*) had no effect on the activity of the basal *tk* promoter (Fig. 2*A*, lane c).

We next carried out competition experiments in *Xenopus* oocytes coinjected with a constant amount of the test construct (Mr H2A tk-70) and increasing amounts of modulator DNA cloned in plasmid pUC (pUC MH2A constructs). Plasmid pUC19 was added to all samples to equalize the DNA concentration. The result is presented in Fig. 2*B*. At low molar ratios of competitor to test DNA, little effect (Fig. 2*B*, lanes a and b) was observed, whereas at higher molar ratios an almost complete inhibition of CAT activity was obtained, comparable to the basal activity of the tk-70 vector alone. Coinjection of about a 4-fold molar excess of competitor DNA resulted in a drastic reduction of transcription of the test gene (Fig. 2*B*, lane c). This inhibitory effect was not due to plasmid sequences because, as shown in lane d of Fig. 2*B*, the presence of an equal amount of pUC 19 carrier did not affect CAT expression of the microinjected Mr H2A tk-70. In conclusion, the 172-bp histone DNA fragment contains upstream transcriptional elements that are recognized by transacting factors present in the *Xenopus* oocyte.

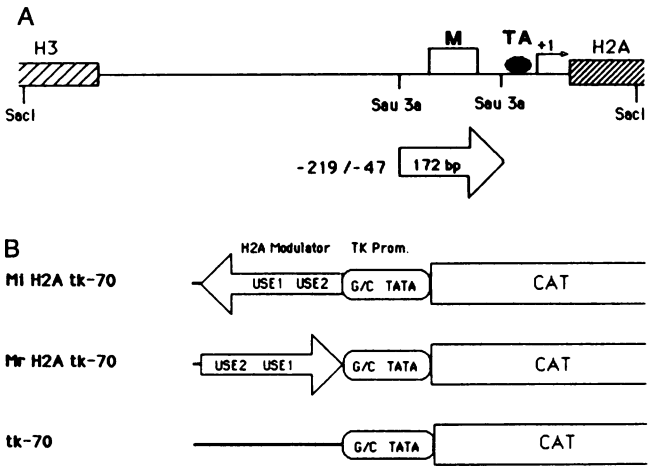


FIG. 1. (*A*) Map of the *Sac* I fragment from the PH70 *Pa. lividus* early histone DNA. The 172-bp *Sau*3A fragment used in the assays described below is shown below the map. M, modulator sequence; TA, TATA box. (*B*) CAT constructs microinjected into *X. laevis* oocytes. The transcriptional elements of the *tk* region are indicated. USE 1 and USE 2 (upstream sequences 1 and 2) refer to the DNase I-protected sequences of the H2A fragment shown in Fig. 4. Arrows indicate the orientation of the inserts.

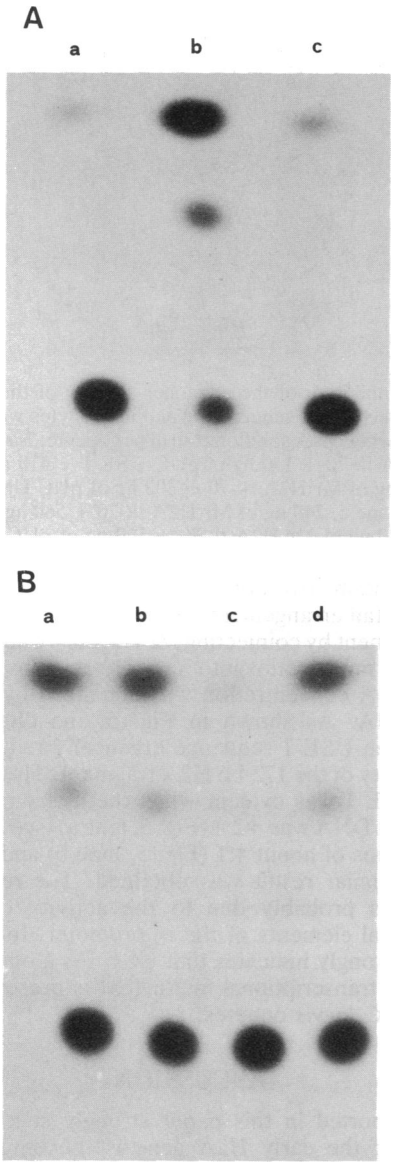


FIG. 2. Effect of the H2A fragment containing the modulator on the activity of the *tk* promoter. Autoradiographs of thin-layer chromatographs are shown. (*A*) *Xenopus* oocytes were microinjected with 30 nl of the following constructs per oocyte (see Fig. 1*B*): tk-70 (lane a); Mr H2A tk-70 (lane b); Mi H2A tk-70 (lane c). The DNA concentration was 240 ng/μl. (*B*) Homologous competition carried out by coinjecting a DNA solution (30 nl per oocyte, 1 μl) containing the test construct and the pUC MH2A construct. Lane a, 240 ng of Mr H2A tk-70 + 75 ng of pUC MH2A + 525 ng of pUC 19. Lane b, 240 ng of Mr H2A tk-70 + 150 ng of pUC MH2A + 450 ng of pUC 19. Lane c, 240 ng of Mr H2A tk-70 + 600 ng of pUC MH2A. Lane d, 240 ng of Mr H2A tk-70 + 600 ng of pUC 19. The CAT enzymatic activity was determined as described (30).

DNA-Binding Proteins in the Sea Urchin Nuclear Extract Interact with H2A Modulator Sequences. To investigate whether the 172-bp DNA region is also the site of binding for some sea urchin nuclear proteins, we prepared nuclear extracts from 32- to 64-cell stage embryos, which actively transcribe the early histone gene set. Gel retardation assays (34, 35) were carried out by incubation of the terminally labeled 172-bp fragment with the extract followed by electrophoretic fractionation of the samples. These assays provided preliminary evidence that some extract components interact with our probe to form discrete DNA–protein complexes (Fig. 3*A*, lane b), perhaps surprisingly, even in the absence of competitor DNA. The upper, slowly migrating

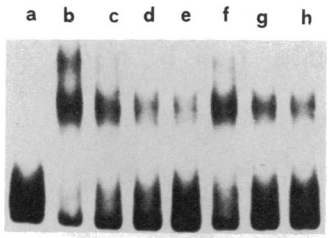
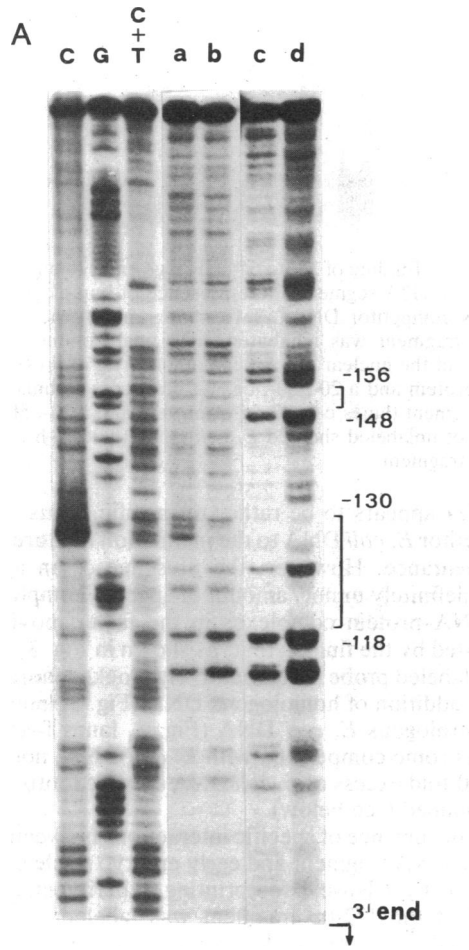


FIG. 3. Binding of 32- to 64-cell stage embryo nuclear extract to the 172-bp H2A segment in the presence of homologous and heterologous competitor DNAs. Two nanograms of the 3′ end-labeled 172-bp fragment was incubated, in a final volume of 8 μl, with aliquots of the nuclear extract containing 2 μg of protein (lane b), 2 μg of protein and a 20-, 50-, or 80-fold excess of unlabeled 172-bp H2A segment (lanes c–e), 2 μg of protein and a 20-, 50-, or 80-fold excess of unlabeled sheared *E. coli* DNA (lanes f–h). Lane a, free 172-bp fragment.

complex appears to be rather unspecific. Thus, addition of competitor *E. coli* DNA to the incubation mixture leads to its disappearance. However, the presence of an appreciable, albeit definitely minor, amount of specific complexes among the DNA–protein complexes in the faster moving band is suggested by the finding that, as shown in Fig. 3, the binding of the labeled probe is competitively blocked more efficiently by the addition of homologous DNA (Fig. 3, lanes c–e) than of heterologous *E. coli* DNA (Fig. 3, lanes f–h). Although there is some competition with *E. coli* DNA, note that even at a 500-fold excess of *E. coli* DNA specific footprint patterns are obtained (see below).

The occurrence of specific interactions between the 172-bp histone DNA fragment and early embryo nuclear proteins is provided by DNase I footprinting experiments. The sense strand of the 172-bp fragment was labeled at the 3′ end, incubated with the nuclear extract in the presence of a 500-fold excess of *E. coli* DNA to minimize unspecific interactions, then digested with increasing amounts of DNase I, and fractionated on a low ionic strength polyacrylamide gel. The DNA was recovered from the retarded DNase I-treated DNA–protein complexes and analyzed on polyacrylamide gels as described in *Materials and Methods*, in parallel with aliquots of naked 172-bp DNA treated with increasing amounts of DNase I (Fig. 4*A*, lanes a and b) and with a Maxam and Gilbert sequence ladder of the same region. The results shown in lanes c and d of Fig. 4*A* clearly demonstrate that two regions, referred to as USE 1 and USE 2 in Fig. 4*B*, appear to be specifically bound by nuclear factors, with some nucleotides immediately adjacent to, or within, the binding regions becoming more sensitive to the enzyme. A complex cutting pattern is also seen upstream at −156, suggesting that either the two footprinting activities modify the DNA structure upstream or that additional binding proteins are involved. As shown in lane d of Fig. 4*A*, this pattern of DNase I cleavage is not modified by doubling the amount of enzyme. The complete nucleotide sequence of the 172-bp fragment is presented in Fig. 4*B*, where the regions whose pattern of DNase I cleavage is altered upon interaction with nuclear extract components are in boldface and are underlined. As shown in Fig. 4*C*, the USE 1 sequence corresponds to a highly conserved portion of the H2A flanking sequence, originally identified by Birnstiel and coworkers (23) in *Ps. miliaris* and for which a strong homology was found with several viral cis-acting elements.

The H2A Modulator USE 1 Sequence Is a Binding Site for a Transcriptional Factor in *Xenopus* Oocytes. To investigate whether USE 1 corresponds to a binding site for a trans-acting factor, a double-stranded oligonucleotide corresponding to residues −134/−116 of Fig. 4*B* was cloned into the pUC 19 vector (pUC-USE 1). Sequence analysis showed that



C

USE 1

P. lividus	E-H2A (-130/-118)	CCAACAGAGGGAG
P. lividus	E-H2A (-216/-205)	TCAACAGAGCGAG
P. miliaris	E-H2A (-137/-125)	CCAACAGATGGCG
P. lividus	E-H3 (-204/-192)	TAGACAGAAGGCT
L. pictus	L-H3 (-165/-155)	CGAACAGATGGAC

FIG. 4. (*A*) DNase I mapping of the protected sites in the DNA–protein particles. The autoradiographic pattern of DNase I-treated free probe and of the 172-bp fragment labeled at the 3′ end of the sense strand incubated with the nuclear extract, digested with DNase I, and further processed as described in *Materials and Methods* is shown. Lanes: a and b, free probe digested with DNase I at 1.5 and 3.5 ng/μl; c and d, DNA recovered from DNA–protein particles treated with DNase I at 3.5 and 7 ng/μl. The indicated sequence reactions are shown in the lanes at the left. (*B*) Nucleotide sequence of the sense strand of the −219/−49 region of H2A. The protected regions, USE 1 and USE 2, are in boldface and are underlined. The nucleotides that become hypersensitive to DNase I are indicated by an asterisk. (*C*) Sequence homologies between USE 1 and 5′ flanking regions of sea urchin histone genes. Numbers refer to the putative histone mRNA start site. E, early; L, late.

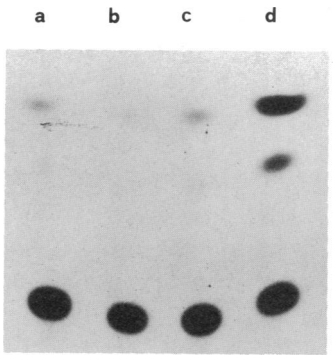


FIG. 5. Inhibition of the enhancer activity of the 172-bp H2A fragment by the USE 1 sequence. *Xenopus* oocytes were coinjected with the following DNA solutions (30 nl per oocyte, 1 μl). Lane a, 240 ng of Mr H2A tk-70 + 150 ng of pUC USE 1 + 410 ng of pUC 19. Lane b, 240 ng of Mr H2A tk-70 + 300 ng of pUC USE 1 + 260 ng of pUC 19. Lane c, 240 ng of Mr H2A tk-70 + 560 ng of pUC USE 1. Lane d, 240 ng of Mr H2A tk-70 + 560 ng of pUC 19.

the clone contains two tandem copies of the oligonucleotide in a head-to-tail arrangement. We next carried out a competition experiment by coinjecting Mr H2A tk-70 and pUC-USE 1 at different molar ratios into *Xenopus* oocytes. Once again the total DNA concentration was equalized by addition of pUC 19 DNA. As shown in Fig. 5, the oligonucleotide containing the USE 1 sequence drastically reduced the enhancer activity of the 172-bp H2A fragment. The competitive effect of USE 1 was evident when the molar ratio of competitor to test DNA was ≈2:1 (Fig. 5, lane a) (see *Discussion*). At molar ratios of about 4:1 (Fig. 5, lane b) and 8:1 (Fig. 5, lane c), a similar result was obtained. The residual CAT expression is probably due to the activity of the basal transcriptional elements of the *tk* promoter. In conclusion, this result strongly indicates that USE 1 is a binding site for a conserved transcriptional factor that is present in limited amounts in *X. laevis* oocytes.

DISCUSSION

The data reported in this paper strongly suggest that the modulator of the early H2A gene is the binding site for transcriptional factors. Evidence for this comes from the highly reproducible finding that the 172-bp H2A upstream segment, fused to the basal *tk* promoter, enhances CAT expression. Furthermore, coinjection of the 172-bp fragment cloned in pUC 8 (pUC MH2A) with Mr H2A tk-70 drastically reduced expression of the CAT reporter gene (Fig. 2). As reported by Grosschedl and Birnstiel (21), the region of the *Ps. miliaris* early H2A gene containing the modulator was the first polymerase II far upstream element identified to have enhancer activity in both orientations. The interpretation of our results is complicated by two facts. (*i*) The modulator segment used in our studies is not identical to the previously studied modulator of *Ps. miliaris* as it includes the A segment with two inverted CAAT motifs (Fig. 4*B*, near position −80; see refs. 21 and 22). (*ii*) The wild-type *tk* gene contains one inverted CAAT sequence, which is lacking in the basal *tk* promoter used in our experiments. As a consequence, recombination of the *Pa. lividus* upstream sequences in the physiological orientation (but not in the reverse orientation) may simply activate the transcription of the reporter gene by reconstituting the *tk* regulatory regions. However, the observation that the cloned USE 1 sequence can compete

efficiently with the modulator–tk construct suggests that the sequences upstream of the inverted CAAT motifs rather than the CAAT motif have a major enhancing effect on the transcription from the basal *tk* promoter.

Additional experiments described in this paper suggest that the 172-bp H2A fragment contains binding sites for nuclear factors extracted from embryos in which this gene is actively transcribed. An appreciable, even though small, fraction of the DNA–protein complexes formed upon incubation of the 172-bp DNA with a total nuclear extract is indeed assembled through specific interactions. This was suggested by the different competition efficiency of homologous as compared to heterologous DNA and was clearly revealed by the results of DNase I footprinting assays, which allow us to identify at least two protection sites, USE 1 and USE 2. Protection of these sites is also correlated with an enhanced sensitivity of some neighboring nucleotides.

The finding that at least two sites are involved in stable interactions with nuclear factors and are protected from DNase I attack, even though we detect only one protein–DNA complex in gel retardation assays, might imply that two different proteins bind to the two sites and that distinct complexes with the same electrophoretic mobility are formed, each corresponding to a 172-bp DNA molecule bound by only one factor. Alternatively, both sites may be protected on each 172-bp DNA molecule, by a single factor, or by two distinct proteins whose binding is strongly cooperative. Further investigations will be required to resolve the question.

The most striking observation is that the USE 1 sequence is almost identical to the cis-acting element identified in the modulator of *Ps. miliaris* H2A gene by site-directed mutagenesis by Birnstiel and coworkers (23). Furthermore, the USE 2 sequence identified in this work overlaps with a conserved region that corresponds to the second functional domain, identified in the functional analysis by the same authors, in the *Ps. miliaris* modulator. Therefore, the results of the footprinting experiments confirm the bipartite nature of this cis-acting transcriptional region (23).

The demonstration that USE 1 can very efficiently compete with the enhancing activity of the 172-bp fragment indicates that it represents an upstream regulatory element recognized by a *Xenopus* oocyte transcriptional factor. The apparent more than proportional response of the transcriptional activity of the CAT reporter gene (see Figs. 2*B* and 5) after addition of competing "enhancer" motifs may be a peculiarity of the oocyte injection system. It should be pointed out that *Xenopus* oocyte nuclei are injected with amounts of DNA that are in excess of the endogenous DNA by orders of magnitude. It seems likely therefore that transcription factors become limiting in this system. If the stimulatory activity of the modulator binding and the basal promoter binding proteins is cooperative, as seems likely to be the case, the blocking by competition of even single, important modulator proteins present in limiting concentration may well have rather drastic effects on the transcription of the reporter gene that we observe.

Although we have not directly proved it, our results strongly suggest that the same or a very similar trans-acting factor is present in both the nuclear extract of embryos actively transcribing the histone genes and in *Xenopus* oocytes. This is perhaps not surprising in view of the known high conservation of transcription factors from yeast to man.

Interestingly, the USE 1 sequence is almost exactly repeated farther upstream in the H2A flanking region (Fig. 4*C*). Protection in this area is not observed in our assay, presumably due to its location at the very end of the probe. A very similar sequence is also present in the upstream region of a *Lythechinus* H3 gene, albeit at a greater distance from the 5′ end of the gene (23). By sequence analysis, we find that a very similar sequence is also present in the upstream region of early H3 from *Pa. lividus*, about 165 bp from the TATA box (Fig. 4*C*). The high conservation of the USE 1 sequence among the H2A and the H3 early subtype histone genes of *Pa. lividus* suggests the very interesting possibility that this transcriptional element may be involved in the coordinate expression of this set of genes during sea urchin development.

Experiments are in progress to see whether USE 2 is also a binding site for a second trans-acting factor. Should this be the case, then the cooperative function between the two upstream elements (USE 1 and USE 2) and the basal transcriptional elements, mediated most probably by protein–protein interaction of the factors bound to them, could be responsible for the transcriptional activation of the H2A gene.

We are grateful to Mr. Cascino for his skillful technical assistance and to Prof. Fasano for oligonucleotide synthesis. This work was supported in part by the "Progetto Finalizzato del Consiglio Nazionalle delle Ricerche Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie" and by funds of the Ministero della Pubblica Istruzione.

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