MOLECULAR AND CELLULAR BIOLOGY, June 1989, p. 2315–2321
0270-7306/89/062315-07$02.00/0

Vol. 9, No. 6

An Embryonic Enhancer Determines the Temporal Activation of a Sea Urchin Late H1 Gene

ZHI-CHUN LAI, DANIEL J. DEANGELO, MAURIZIO DILIBERTO, AND GEOFFREY CHILDS*

Department of Molecular Genetics, Albert Einstein Medical School, 1300 Morris Park Avenue, Bronx, New York 10461

Received 7 December 1988/Accepted 22 February 1989

Normal development requires that individual genes be expressed in their correct temporal patterns, but the mechanisms regulating this process during early embryogenesis are poorly understood. We have studied the early and late sea urchin histone genes during embryogenesis to address the molecular mechanisms controlling temporal gene expression. By measuring the changes in expression of cloned H1-β DNA constructs after microinjection into fertilized one-cell zygotes, we demonstrated that a highly conserved 30-base-pair segment of DNA between positions −288 and −317 (USE IV) is responsible for the transcriptional activation of this late histone gene at the late blastula stage. In this report, we demonstrate that an oligonucleotide corresponding to USE IV acts as an embryonic enhancer element capable of activating the simian virus 40 early promoter in a stage-specific manner. Using an in vivo competition assay and in vitro DNase I footprinting and mobility shift assays, we also identified a protein(s) that interacts with this enhancer. Results of the competition assay suggested that this factor acts to stimulate transcription of the H1-β gene. The factor was found to be stored in mature eggs as well as in all embryonic stages examined. The mobility of the factor found in eggs, however, differed from that of the embryonic form, which suggested that posttranslational modification occurs after fertilization.

A fundamental problem common to all multicellular organisms is the need to regulate genes in both temporal and spatial manners. The different histone multigene families of sea urchins have provided a convenient system for studying the mechanisms of temporal gene expression during early embryogenesis. Expression of the early or embryonic histone genes, which are encoded by 300 to 500 tandem arrays, is confined to a period up to the blastula stage of development, about 12 h after fertilization (reviewed in 37). The late histone gene family consists of 2 to 12 dispersed irregular arrays (6, 26, 29, 30, 33, 38) whose transcripts are expressed at low basal levels up until the blastula stage, when their transcription rates increase (23, 28). Levels of late histone gene transcripts peak in late-blastula- or gastrula-stage embryos (5, 20, 28, 30, 33). In addition, these late genes are used in the adult tissues of the organism, whereas the high-copy-number early gene family is transcriptionally silent (34).

Our current understanding of the molecular basis of gene activation during embryogenesis depends largely on the development of transient and permanent transformation systems. Cloned genes encoding specific products can be introduced into eggs, and regulation of their expression can be observed during embryogenesis (7, 11, 31, 40, 49). It has been demonstrated that relatively small sequences of DNA promoter regions can correctly direct embryonic gene expression specific to stage and cell lineage. A *Xenopus* actin gene with 2.8 kilobases of upstream sequences, the first two exons and the associated intron, can direct correct expression in myotomes (49). In sea urchins, the mechanisms of temporal and spatial gene expression can also be investigated by injecting cloned DNA constructs into eggs or one-cell zygotes and then monitoring the expression of the injected DNA during early embryogenesis (11, 40). Several different genes have been shown to be properly regulated when reintroduced into eggs by this procedure (8, 11, 14, 21, 25, 33, 48). They are expressed in cells of the proper lineages as well as at the proper stages of early embryonic development. We have previously demonstrated that injection of the late H1-β gene of *Strongylocentrotus purpuratus* into *Lytechinus pictus* zygotes results in low basal transcription up until the blastula stage of development and that transcripts then accumulate during the next 10 h, paralleling the activity in the endogenous gene (33). Using in vitro mutagenesis, we were also able to demonstrate that a highly conserved late H1 gene-specific sequence of 30 base pairs (bp) (USE IV) plays a major role in activation of the gene at the late blastula stage of development (33). The sea urchin gene transfer system therefore appears to be of general use for studying the mechanism of embryonic gene activation.

Although there are many examples of virus- and tissue-specific enhancers (27, 44, 45), few have been discovered to function during early embryogenesis. A 74-bp enhancer sequence located about 700 bp upstream of the *Xenopus* GS17 gene promoter is sufficient to activate either homologous or heterologous promoters at the mid-blastula transition (31). Although there is no direct evidence for *trans*-acting factors that interact with this enhancer, these factors must be of maternal origin and stored as a protein or mRNA, since there is no embryonic transcription before the mid-blastula transition. We were prompted to ask whether the embryonic activator sequence of the late H1-β gene (USE IV) could act as an enhancer element, since it is considerably upstream of the RNA initiation site (−288 to −317) and might therefore be shared with other genes activated at the same time during embryogenesis. In this report, we demonstrate that a 33-bp synthetic oligonucleotide corresponding to USE IV can activate a heterologous promoter, the simian virus (SV40) early promoter, at the correct time during embryogenesis when placed downstream of the reporter chloramphenicol acetyltransferase (CAT) gene. A putative positive-acting enhancer binding protein was detected both in vitro and in vivo. This protein was maternal in origin and found during all embryonic stages tested, but the maternal

* Corresponding author.

form of the protein(s) behaved differently from the embryonic form on nondenaturing low-ionic-strength polyacrylamide gels.

MATERIALS AND METHODS

DNA constructs. A 33-bp oligonucleotide corresponding to USE IV of *S. purpuratus* H1-β was constructed by annealing oligonucleotides (5′-AAGTTTTAAATCTGATTCTGTAAC TGTAAGTTT-3′ and 5′-AAACTTACAGTTACAGAATC AGATTTAAAACTT-3′) synthesized on an Applied Biosystems automated DNA synthesizer. Gel-purified 33-mer was phosphorylated with T4 polynucleotide kinase, catenated by treatment with T4 DNA ligase (36), and cloned into the vector M13mp7. Clones with one and three copies of USE IV were obtained and digested with *Bam*HI to release the insert fragment. This material was ligated into *Bam*HI-treated pSV2CAT or pA10CAT4N to generate constructs 2, 3, 5, and 6 (Fig. 1). Other constructs were described by Lai et al. (33).

Microinjection of sea urchin one-cell zygotes. The procedure used to inject zygotes was essentially that of McMahon et al. (40) exactly as described by Lai et al. (33). Constructs 1, 2, and 3 (Fig. 1) were linearized with *Apa*I, and constructs 4, 5, and 6 (Fig. 1) were linearized with *Bgl*II. The wild-type H1-β DNA construct (33) was linearized with *Pst*I.

CAT assays. Injected embryos were collected along with about 1,500 uninjected embryos of the same stage. Embryos were lysed in 250 mM Tris hydrochloride (pH 7.8) by three freeze-thaw cycles (40). CAT enzyme activity was then assayed as described elsewhere (40). Percent acetylation was calculated by counts of acetylated chloramphenicol divided by total counts, which were measured by scintillation counting of appropriate regions of the thin-layer chromatography plates. CAT activities were relative to the average background level. Authentic bacterial CAT enzyme (Sigma Chemical Co., St. Louis, Mo.) was used as a positive control.

RNase protection assays. Preparation of probes, hybridization to embryonic RNA samples, and subsequent analysis of protected fragments were done essentially as described by Melton et al. (41), with modifications as described by Lai et al. (33).

Preparation of whole-cell extracts. All extracts were made from a single mating, using dejellied eggs from one female and sperm from one male. Embryos grown to the appropriate stage at a 1% dilution at 15°C were harvested by centrifugation and washed once with Ca^{2+}-Mg^{2+}-free seawater and once with 1.5 M dextrose. The embryos (or eggs) were suspended in 10 volumes of nuclear lysis buffer (75 mM NaCl, 0.5 mM EDTA, 12.5 mM Tris hydrochloride [pH 7.5], 0.1 mM phenylmethylsulfonyl fluoride, 0.01 M β-mercaptoethanol). Embryos were then homogenized in a Dounce homogenizer (A pestle), and 1/10 volume of 4 M $(NH_4)_2SO_4$ was added. The resulting homogenate was rotated at 4°C for 30 min and centrifuged (10,000 rpm for 15 min in an SS34 rotor; Ivan Sorvall, Inc., Norwalk, Conn.). To the supernatant was added 4.8 ml of saturated $(NH_4)_2SO_4$ per 4 ml of volume, and the preparation was rotated in the cold for 1 to 2 h. This solution was centrifuged, and the protein pellet was suspended in a minimum amount of dialysis buffer (20% glycerol, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.1 mM EDTA, 100 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and dialyzed until the conductivity reached that of 0.1 M KCl. Protein concentrations were determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.).

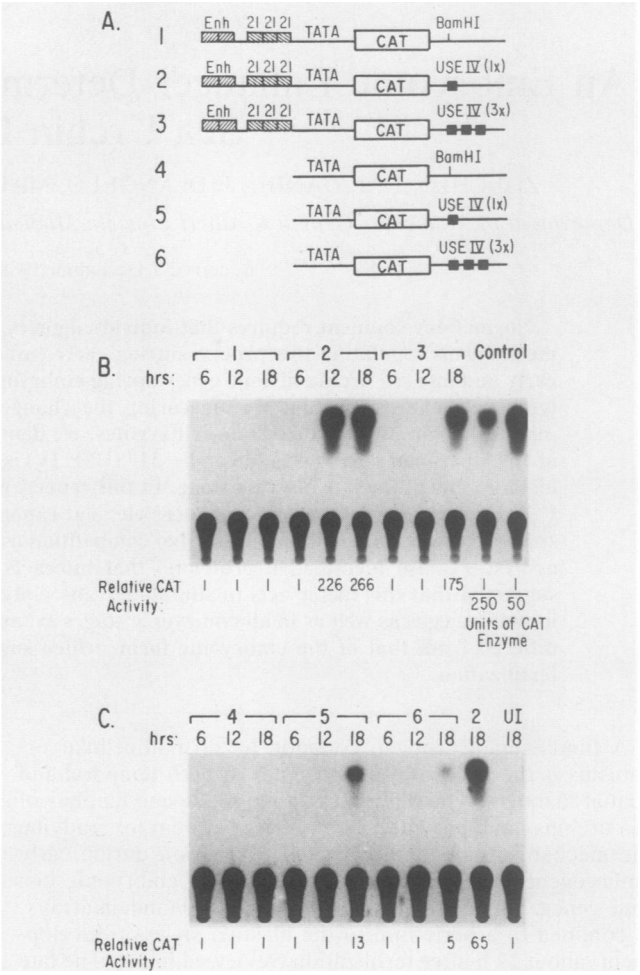

FIG. 1. Enhancer activity of USE IV oligonucleotides. (A) Constructs used to test the ability of USE IV to activate heterologous promoters: 1, pSV2CAT; 2, pSV2CAT with one copy of a 33-bp USE IV oligonucleotide cloned into the *Bam*HI site; 3, same as construct 2 except that three copies of USE IV were cloned in the *Bam*HI site; 4 to 6, same as constructs 1 to 3, respectively, except that the vector pA10CAT4N was used. This vector does not contain the 21-bp repeats and the SV40 enhancer (22). (B and C) Results of CAT assays of microinjected DNA constructs. Linearized DNA (2 pl of a 25-ng/μl solution) was introduced into fertilized one-cell *L. pictus* zygotes, embryos were collected at the indicated times after fertilization, and extract equivalent to 50 injected embryos was assayed for CAT enzymatic activity. Bracketed numbers indicate the DNA constructs injected. Control lanes contained known quantities of authentic CAT enzyme; lane UI represents activity from the same number of uninjected embryos.

Preparation of nuclear extracts. Nuclear extracts were prepared from hatched mesenchyme blastula-stage embryos by the procedure of Morris et al. (42), which results in transcriptionally competent extracts.

DNase I footprinting. DNA fragments were labeled in the coding and noncoding strands by filling in the appropriate 5′ overhangs with the Klenow fragment of DNA polymerase. Probe (5,000 cpm) was mixed with 100 μg of crude nuclear extract in 25 μl of binding buffer (0.01 M Tris hydrochloride [pH 7.5], 0.08 M NaCl, 4% glycerol, 0.01 M β-mercaptoethanol, 1 mM EDTA) containing 1 μg of poly(dI-dC) · poly(dI-

dC) and incubated on ice for 30 min. The samples were brought to 1.5 mM $MgCl_2$ and digested with 5 μg of DNase I for 90 s on ice. The reaction was terminated by addition of stop buffer (0.6 M NaCl, 0.2% sodium dodecyl sulfate, 10 mM EDTA), phenol-chloroform extracted, and ethanol precipitated. The DNA was then run on an 8% polyacrylamide–7 M Urea sequencing gel and visualized by autoradiography.

Gel retardation analysis. Binding reactions were carried out by incubating radiolabeled double-stranded oligonucleotides with 20 μg of whole-cell extracts, using the conditions described above for DNase I footprinting. After the 30-min incubation on ice, samples were applied to low-ionic-strength 5% polyacrylamide gels (15, 17) in TBE buffer (25 mM Tris [pH 8.0], 25 mM H_3BO_3, 1 mM EDTA). Gels were electrophoresed at 25 mA at 4°C, dried on 3MM paper (Whatman, Inc., Clifton, N.J.), and autoradiographed. For competition studies, the indicated quantities of competitor were added to the reaction mixtures before addition of the extract. Both the specific and nonspecific competitor DNAs were synthesized oligomers of approximately 30 bp in length and were used unligated.

RESULTS AND DISCUSSION

The promoter of the gene encoding the histone H1-β protein contains several sequences that have been conserved among sea urchin species that diverged between 30 and 40 million years ago (46). One of these conserved sequences, a 30-bp region referred to as USE IV (upstream sequence element), is responsible for the transcriptional activation of the gene at the blastula stage of development (33). Other conserved sequences of its promoter are responsible for low basal levels of transcription observed during the period of development up to the blastula stage (33). When USE IV is deleted, the H1-β gene is expressed at low basal levels throughout early embryogenesis. To investigate whether USE IV acts independently as an embryonic stage-specific enhancer, a 33-bp double-stranded oligonucleotide corresponding to the USE IV element of the *S. purpuratus* H1-β gene was cloned in either one or three tandem copies into the *Bam*HI site at the 3′ side of the bacterial CAT gene in the vector pSV2CAT (18) or a derivative vector, pA10CAT4N (22). The resulting plasmids were microinjected into fertilized one-cell *L. pictus* zygotes, and CAT enzymatic activity was assayed during subsequent early embryonic development. The half-life of CAT enzyme in sea urchin embryos has been estimated to be about 40 min (13). This short half-life ensures that detection of CAT enzymatic activity during a given stage of embryogenesis is indicative of relative transcription during that interval. The vectors pSV2CAT and pA10CAT4N were both inactive during the first 18 h of embryogenesis (Fig. 1). Addition of one or three copies of the USE IV oligonucleotide downstream of the CAT gene, however, resulted in activation of the SV40 early promoter by 18 h after fertilization (Fig. 1), coincident with the time of maximal accumulation of H1-β transcripts (32). Occasionally, we observed that activation occurred as early as 12 h postfertilization, as seen in the experiment with the construct containing one copy of USE IV (Fig. 1). Thus, the 33-bp USE IV element acts as a stage-specific enhancer element to determine proper regulation of expression during early embryogenesis.

Like other enhancers (27, 44, 45), USE IV appears to require a combination of *cis*-acting elements for maximal activity. In the presence of only a TATA box (pA10CAT4N), the activity of USE IV is 5- to 10-fold lower than that of a similar construct (pSV2CAT) containing the six Sp1 binding sites and the SV40 enhancer. Sea urchin embryos contain an octamer binding protein (1) that could interact with the SV40 enhancer to increase the activity of USE IV. We suspect that the inactive SV40 enhancer just upstream of the TATA box or the multiple Sp1 binding sites in the 21-bp repeats act as proximal promoter elements in sea urchins that alone are inactive or exhibit extremely low activity unless they are near an authentic sea urchin enhancer. Similarly, the maximal activity of the USE IV enhancer in the context of its homologous promoter requires the presence of other basal promoter elements (33). The USE IV enhancer is unusual in that a single copy appears to be at least as effective as three copies (44). The inability of three copies of USE IV to stimulate transcriptional activity further could be due to the interaction of more than one nuclear factor with each USE IV monomer, to the presence of as yet undetected negative control sites, or to the fact that the spacing of tandem USE IV elements is inappropriate. Slot blot quantitation (see reference 33 for methods) confirmed that all of the six constructs shown in Fig. 1 replicated to similar degrees during embryogenesis (data not shown). Thus, the inactivity of the pSV2CAT construct was due neither to its inability to replicate nor to preferential loss of injected DNA.

Another unusual property of USE IV is its ability to activate transcription at very low levels in the presence of only the TATA box. This property raises the possibility that the factors binding to this element may interact with TATA box-binding proteins to activate transcription. Similar activation is achieved when an octamer element is placed within 70 bp of the start site of transcription in absence of other regulatory elements except a TATA box (50). Maximal activity of the H1-β gene promoter, however, requires the presence of multiple basal DNA elements, including USE 0, USE I, USE II, and USE III, as well as the USE IV enhancer (33). Moreover, the wild-type H1-β–CAT fusion construct is able to stimulate transcription severalfold better than are any of the heterologous SV40 promoter-sea urchin enhancer constructs used in this study (data not shown).

To test for the presence of transcription factors that interact with the USE IV enhancer, we devised a series of in vivo competition assays. Authentic wild-type H1-β DNA was injected either alone or in the presence of excess quantities of DNA fragments that contained the USE IV enhancer, the basal promoter (TATA box, USE 0, USE I, USE II, and USE III), or part of the H1-coding sequence. The DNA was injected into fertilized one-cell *L. pictus* zygotes. When the embryos reached the late blastula stage, 20 h after fertilization, H1-β transcripts were quantitated relative to endogenous late H3 transcripts by an RNA protection assay (Fig. 2). Both the fragment containing USE IV (fragment A) and that containing the basal promoter (fragment B) were capable of inhibiting the accumulation of H1-β transcripts, whereas the fragment spanning the coding region (fragment C) had no effect on H1-β gene transcription. The most likely explanation for this inhibition is that the 5′ sequences of the H1-β gene contain *cis*-acting elements that interact with positively acting transcription factors required for the expression of the H1-β gene. Thus, USE IV appears to be a positively acting element, which indicates that it is recognized by one or more positively acting transcription factors.

We did not observe stoichiometric depression of the H1-β gene transcription with coinjected competitor DNA molecules. Inhibition of H1-β gene transcription by 50% required about a 15-fold molar excess for fragment A and a 35-fold

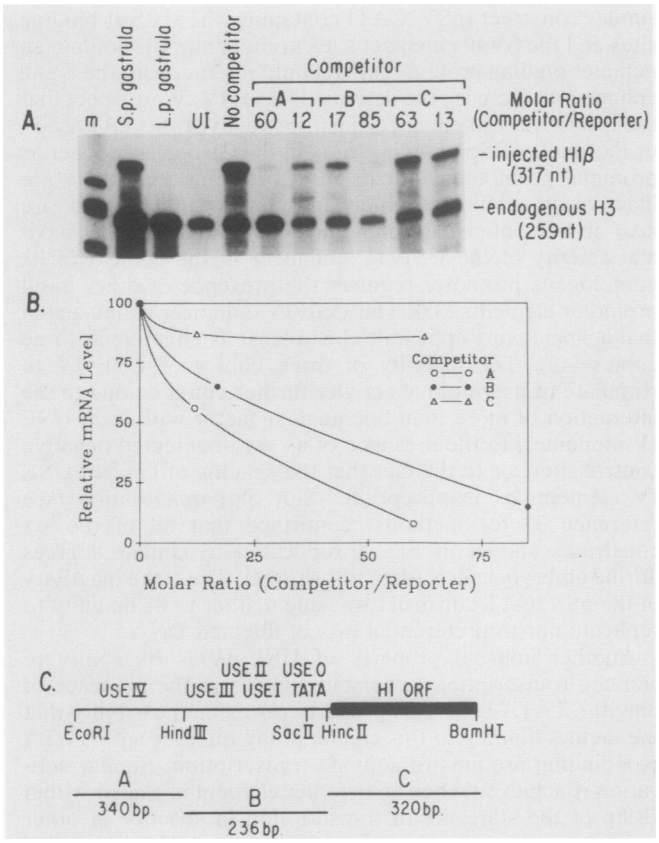

FIG. 2. Inhibition of H1-β gene expression with 5'-flanking DNA fragments as competitors in vivo. (A) H1-β DNA (3.0-kbp *Sal*I fragment in M13mp19) (9) at a concentration of 10 μg/ml was microinjected into fertilized one-cell *L. pictus* zygotes alone or in the presence of the indicated molar excess of purified fragment A, B, or C (diagrammed in panel C). Embryos were grown until they reached the late blastula stage of development (18 h), RNA was prepared, and the equivalent of 30 embryos was probed with Sp6 transcripts complementary to *S. purpuratus* H1-β (33) and *L. pictus* late H3 (34) to quantitate correctly initiated transcripts (41). Lanes: M, ^{32}P-labeled ϕX174 *Hae*III digest as marker; S.p. gastrula, 0.5 μg of authentic *S. purpuratus* 30-h gastrula-stage RNA; L.p. gastrula, 0.125 μg of authentic *L. pictus* 30-h gastrula-stage RNA; UI, signal from uninjected embryos. (B) Quantitation of the data shown in panel A. Different exposures of autoradiograms of the data were scanned with an LKB Ultroscan laser densitometer and plotted. (C) Map of the H1-β gene showing the three competitor fragments, A, B, and C, used in panel A. ORF, Open reading frame.

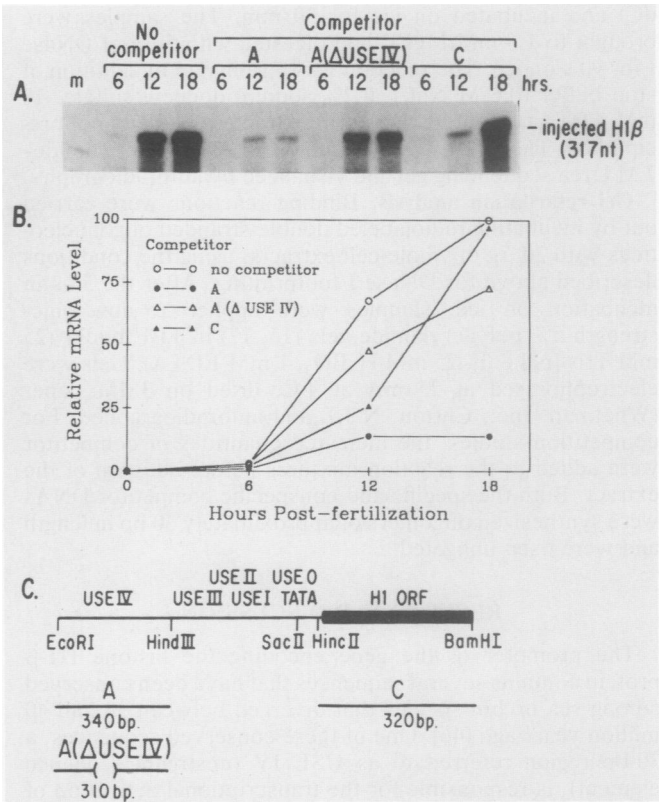

FIG. 3. Inhibition of the temporal accumulation of H1-β transcripts with 5'-flanking DNA containing USE IV in vivo. (A) H1-β DNA (10 μg/ml) was microinjected alone (no competitor) or in the presence of a 60-fold (fragment A), 65-fold [fragment A(ΔUSEIV)], or 63-fold (fragment C) molar excess of the indicated DNA fragment, and embryos were collected for preparation of RNA at the indicated times after fertilization. Correctly initiated transcripts were detected from the RNA equivalent of 20 embryos as described in the legend to Fig. 2. (B) Quantitation of the results shown in panel A. (C) Diagram of the H1-β gene indicating the fragments used as competitors. Fragments A and C were isolated from a wild-type construct; A(ΔUSEIV) is the same *Eco*RI-*Hin*dIII fragment as in panel A except that it was prepared from a construct with a 30-bp deletion of USE IV (33).

molar excess for fragment B (Fig. 2B), possibly because of subsaturating amounts of the injected reporter gene. Alternatively, competitor DNA fragments can form mixed concatemers with reporter gene fragments (14, 39), some of which themselves can act as enhancers on adjacent H1-β promoters. Similar experiments with the actin CYIIIA gene, however, resulted in closer to idealized stoichiometric decreases (35). These investigators included carrier sea urchin DNA in their injections, which could provide spacers between competitor DNA and reporter DNA, thereby eliminating the possible enhancer activity of the competitor DNA. Twenty embryos were collected at each time point and used to quantitate the total amount of microinjected DNA (reporter and competitor DNAs cannot be distinguished by hybridization analysis). Slot blot hybridization confirmed that the injected DNA was replicated during embryogenesis and that the total amounts present with all competitors were similar (data not shown).

We also examined the temporal regulation of expression of the H1-β gene microinjected in the presence of excess quantities of competitor DNA fragments; one containing an intact enhancer sequence, the same fragment with a 30-bp deletion of USE IV, and a coding-region fragment (Fig. 3). Coinjection of the DNA fragment containing the intact USE IV element resulted in basal expression of the gene during the first 18 h of embryogenesis, whereas the other two fragments had no effect on the accumulation of H1-β transcripts during this same developmental interval. The altered expression pattern of this wild-type gene in the presence of coinjected USE IV mimicked the phenotype of USE IV deletions (33) and provides further evidence that the regulatory protein(s) interacting with this element play a major role in controlling the temporal expression of this gene. Transcripts accumulated during the interval between 6 and 12 h postfertilization even in the presence of excess quantities of fragment A (Fig. 3) but not during the ensuing 6 h of development. We are not sure whether other parts of the H1-β promoter also confer some temporal expression or

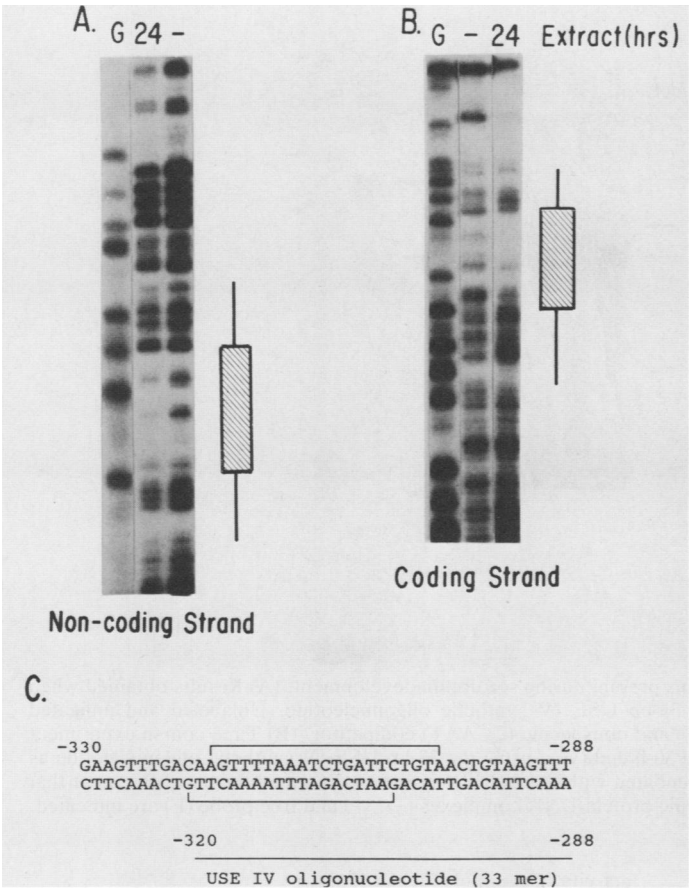

FIG. 4. DNase I footprint analysis of the USE IV region. Nuclear extracts prepared from 24-h postfertilization mesenchyme blastula-stage embryos (100 μg) were used to protect a DNA fragment extending from −227 to −372 from DNase I digestion (lane 24). Other lanes: G, Maxam-Gilbert sequencing reaction on the fragment used as a marker; −, DNase reaction without added nuclear extract. Shaded boxes represent regions protected from DNase I digestion. (C) Diagram of the nucleotide sequence of the USE IV element indicating the protected sequences and the oligonucleotide that contains enhancer activity.

whether this activity represents a general increase in nuclear transcription in embryos of this age.

We used DNase I footprinting (16) to detect USE IV sequence-specific DNA-binding activities in embryonic protein extracts. Nuclear extracts derived from 20-h mesenchyme blastula-stage embryos contained an activity that protected a region from −299 to −321 within the USE IV sequence from DNase I digestion (Fig. 4). The protected region was within the 33-bp region demonstrated to have enhancer activity. This enhancer binding protein represents the best candidate for a positive transcription factor that determines the temporal expression of this gene. It is possible that many sea urchin genes that are activated during the blastula stage of development (10, 12) share this enhancer element.

There are several different mechanisms that the embryo could use to activate a stage-specific enhancer. The *trans*-acting factor(s) could either be produced or bind DNA only at the time when the enhancer is active. This mechanism resembles one used by the *Drosophila adh* and *ubx* promoters (2, 19) as well as the sea urchin early H3 gene (M. DiLiberto et al., submitted for publication). Alternatively, the *trans*-acting factor(s) could be made during oogenesis and stored in the egg in a form capable of binding DNA but must be modified at a later time to activate transcription. We addressed this question by using the mobility shift assay (15, 17) with whole-cell extracts prepared from eggs and embryos in different stages (Fig. 5). Using the 33-bp USE IV oligonucleotide as a probe, we looked for sequence-specific DNA-binding proteins in these staged extracts. We found that 17-h late-blastula-stage extracts contained factors that formed specific complexes with this probe (Fig. 5A). These complexes could be effectively competed against by using the USE IV oligonucleotide but not a 28-bp oligonucleotide containing the CCAAT sequence of the early H3 gene. Using equal quantities (20 μg of protein) of extract derived from eggs and from 8-h early-blastula, 17-h late-blastula, and 30-h gastrula embryos, we could estimate how the binding activities of these factors varied during early embryogenesis (Fig. 5B). Binding activity was present in mature eggs, increased in the 8-h and 17-h extracts, and then declined in the 30-h extract. Careful examination of the mobilities of these complexes in eggs and embryos revealed that the egg complexes always migrated faster than did the complexes from embryonic extracts. When gels were run for longer periods of time, the differences could be seen more clearly (Fig. 5C), consistent with the possibility that the binding factor undergoes posttranslational modifications during early embryogenesis. Similar differences were detected in egg and embryonic proteins that bind to the sea urchin actin CYIIIA gene (3). At some time after fertilization, enhancer binding proteins are either replaced or, more likely, posttranslationally modified.

It is tempting to speculate that the activity of the factor is controlled by its posttranslational modification, be it phosphorylation, as in the case of the yeast heat shock factor (47), or glycosylation, as in the case of a variety of RNA polymerase II transcription factors (24). However, modification of the USE IV binding protein(s) preceded the maximal rate of gene transcription by several hours, which raises the possibility that the modification is due to embryonic rather than maternal factors. In vivo, it may be that during the first few cleavage stages, binding to the enhancer does not occur because of the relatively high dissociation constant (3). Activation of the enhancer occurs only when an adequate concentration of binding proteins is reached in the nucleus, which shifts the equilibrium toward binding. Early posttranslational modification of the USE IV binding protein(s) may be required to allow the accumulation of sufficient quantities to enhance gene transcription. Thus, the embryonic genome may be responsible for the control of temporal gene expression.

Another possibility is that other proteins that interact with the USE IV binding protein(s) are involved in activation of the enhancer. Consequently, the USE IV binding protein(s) may be only a part of a larger complex that is needed to activate gene transcription at the proper stage of development (4).

Our method for identifying potential regulatory regions was based on the premise that regulatory sequences would be conserved between distantly related sea urchin species (29). Adjacent to USE IV is another highly conserved late histone gene-specific element, USE III. Deletions of this element resulted in a minor but reproducible decrease in transcription (33). It is possible that the region of DNA encompassing USE III and USE IV is a more potent enhancer than is USE IV alone. This view is supported by the fact that most enhancers are larger than 30 bp and generally interact with multiple factors (43). Moreover, in

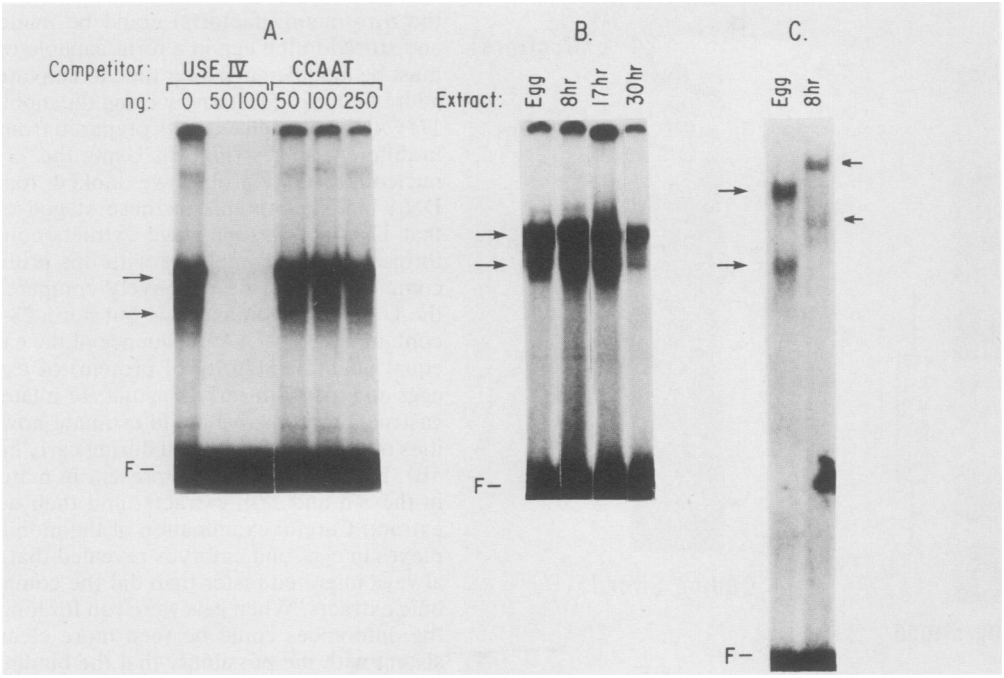

FIG. 5. Gel retardation analysis of the USE IV DNA-binding proteins present during sea urchin development. (A) Results obtained when 20 μg of a 17-h crude whole-cell extract was added to the labeled 33-bp USE IV synthetic oligonucleotide. Unlabeled and unligated oligonucleotide was added in various amounts as the specific (USE IV) and nonspecific (CCAAT) competitor. (B) Time course experiment with 20 μg of crude whole-cell extracts from egg and from 8-, 17-, and 30-h embryos, using the 33-bp USE IV synthetic oligonucleotide as a probe. (C) Results obtained when 20 μg of egg or 8-h extract was incubated with the USE IV probe as described for panel B except that the polyacrylamide gel was run for 5 instead of 1.5 h. Positions of specific protein-DNA complexes (→, ←) and free probe (F) are indicated.

addition to USE III, at least two other regions surrounding USE IV exhibit distinct DNase footprints (data not shown) and might be involved with enhancer activity. The purification and characterization of the USE IV binding factor(s) should shed light on these problems and assist in elucidating the molecular basis of gene activation during embryogenesis.

ACKNOWLEDGMENTS

We thank Jack Lenz for helpful discussions and for suggestions that improved the manuscript.

This work was supported by Public Health Service grants from the National Institutes of Health and by grants from the American Cancer Society. G.C. is the recipient of an Irma T. Hirschl career scientist award, and D.D. is supported by a medical scientist predoctoral training grant.

LITERATURE CITED

1. **Barberis, A., G. Superti-Furga, and M. Busslinger.** 1987. Mutually exclusive interaction of the CCAAT-binding factor and of a displacement protein with overlapping sequences of a histone gene promoter. Cell **50**:347–359.
2. **Biggin, M. D., and R. Tjian.** 1988. Transcription factors that activate the ultrabithorax promoter in developmentally staged extracts. Cell **53**:699–711.
3. **Calzone, F. J., N. Theze, P. Thiebaud, R. J. Hill, R. J. Britten, and E. H. Davidson.** 1988. Developmental appearance of factors that bind specifically to cis-regulatory sequences of a gene expressed in the sea urchin embryo. Genes Dev. **2**:1074–1088.
4. **Carey, M. F., S. P. Gerrard, and N. R. Cozzarelli.** 1986. Analysis of RNA polymerase III transcription complexes by gel filtration. J. Biol. Chem. **261**:4309–4317.
5. **Childs, G., R. Maxson, and L. H. Kedes.** 1979. Histone gene expression during sea urchin embryogenesis: isolation and characterization of early and late messenger RNAs of *Strongylocentrotus purpuratus* by gene-specific hybridization and template activity. Dev. Biol. **73**:153–173.
6. **Childs, G., C. Nocente-McGrath, T. Lieber, C. Holt, and J. Knowles.** 1982. Sea urchin (*L. pictus*) late stage H3 and H4 genes: characterization and mapping of a clustered but non-tandemly linked multigene family. Cell **31**:383–393.
7. **Colin, A. M.** 1986. Rapid repetitive microinjection. Methods Cell Biol. **27**:395–406.
8. **Colin, A. M., T. L. Catlin, S. H. Kidson, and R. Maxson.** 1988. Closely linked early and late H2b histone genes are differentially expressed after microinjection into sea urchin zygotes. Proc. Natl. Acad. Sci. USA **85**:507–510.
9. **Dale, R. M. K., B. A. McClure, and J. P. Houchins.** 1985. A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: application to sequencing the corn mitochondrial 18 S rDNA. Plasmid **13**:31–40.
10. **Davidson, E. H.** 1986. Gene activity in early development, 3rd ed. Academic Press, Inc., Orlando, Fla.
11. **Davidson, E. H., C. N. Flytzanis, J. J. Lee, S. J. Rose, and H. M. Sucov.** 1985. Lineage-specific gene expression in the sea urchin embryo. Cold Spring Harbor Symp. Quant. Biol. **50**:321–328.
12. **Flytzanis, C. N., B. R. Brandhorst, R. J. Britten, and E. H. Davidson.** 1982. Developmental patterns of cytoplasmic transcript prevalence in sea urchin embryos. Dev. Biol. **91**:27–35.
13. **Flytzanis, C. N., R. J. Britten, and E. H. Davidson.** 1987. Ontogenic activation of a fusion gene introduced into sea urchin eggs. Proc. Natl. Acad. Sci. USA **84**:151–155.
14. **Flytzanis, C. N., A. P. McMahon, B. R. Hough-Evans, K. S. Katula, R. J. Britten, and E. H. Davidson.** 1985. Persistence and integration of cloned DNA in postembryonic sea urchins. Dev. Biol. **108**:431–442.
15. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
16. **Galas, D., and A. Schmitz.** 1978. DNAse I footprinting: a simple method for the detection of protein-DNA specificity. Nucleic Acids Res. **5**:3157–3170.

17. **Garner, M. M., and A. Rezvin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. Nucleic Acids Res. **9**:3047–3060.
18. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
19. **Heberlein, U., and R. Tjian.** 1988. Temporal pattern of alcohol dehydrogenase gene transcription reproduced by Drosophila stage-specific embryonic extracts. Nature (London) **331**:410–415.
20. **Hieter, P. A., M. Hendricks, K. Hemminki, and E. S. Weinberg.** 1979. Histone gene switch in the sea urchin embryo: identification of late embryonic histone mRNAs and the controls of their synthesis. Biochemistry **18**:2707–2715.
21. **Hough-Evans, B. R., R. R. Franks, R. A. Cameron, R. J. Britten, and E. H. Davidson.** 1987. Correct cell-type-specific expression of a fusion gene injected into sea urchin eggs. Dev. Biol. **121**:576–579.
22. **Imperiale, M. J., R. P. Hart, and J. R. Nevins.** 1985. An enhancer-like element in the adenovirus E2 promoter contains sequences essential for uninduced and E1A-induced transcription. Proc. Natl. Acad. Sci. USA **82**:381–385.
23. **Ito, M., J. Bell, G. Lyon, and R. Maxson.** 1988. Synthesis and turnover of late H2B histone mRNA in developing embryos of the sea urchin *Strongylocentrotus purpuratus*. Dev. Biol. **129**: 147–158.
24. **Jackson, S. P., and R. Tjian.** 1988. O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. Cell **55**:125–133.
25. **Katula, K. S., B. R. Hough-Evans, R. J. Britten, and E. Davidson.** 1987. Ontogenic expression of a *Cyl* actin fusion gene injected into sea urchin eggs. Development **101**:437–447.
26. **Kemler, I., and M. Busslinger.** 1986. Characterization of two nonallelic pairs of late histone H2A and H2B genes of the sea urchin: differential regulation in the embryo and tissue-specific expression in the adult. Mol. Cell. Biol. **6**:3746–3756.
27. **Khoury, G., and P. Gruss.** 1983. Enhancer elements. Cell **33**:313–314.
28. **Knowles, J. A., and G. Childs.** 1984. Temporal expression of late histone messenger RNA in the sea urchin *Lytechinus pictus*. Proc. Natl. Acad. Sci. USA **81**:2411–2415.
29. **Knowles, J. A., and G. Childs.** 1986. Comparison of the late H1 histone genes of the sea urchins Lytechinus pictus and Strongylocentrotus purpuratus. Nucleic Acids Res. **14**:8121–8133.
30. **Knowles, J. A., Z.-C. Lai, and G. J. Childs.** 1987. Isolation, characterization, and expression of the gene encoding late histone subtype H1-gamma of the sea urchin *Strongylocentrotus purpuratus*. Mol. Cell. Biol. **7**:478–485.
31. **Krieg, P. A., and D. A. Melton.** 1987. An enhancer responsible for activating transcription at the midblastula transition in *Xenopus* development. Proc. Natl. Acad. Sci. USA **84**:2331–2335.
32. **Lai, Z. C., and G. Childs.** 1988. Characterization of the structure and transcriptional patterns of the gene encoding the late histone subtype H1-β of the sea urchin *Strongylocentrotus purpuratus*. Mol. Cell. Biol. **8**:1842–1844.
33. **Lai, Z. C., R. Maxson, and G. Childs.** 1988. Both basal and ontogenic promoter elements affect the timing and level of expression of a sea urchin H1 gene during early embryogenesis. Genes Dev. **2**:173–183.
34. **Lieber, T., K. Weisser, and G. Childs.** 1986. Analysis of histone gene expression in adult tissues of the sea urchin *Strongylocentrotus purpuratus* and *Lytechinus pictus*: tissue-specific expression of sperm histone genes. Mol. Cell. Biol. **6**:2602–2612.
35. **Livant, D. L., A. E. Cutting, R. J. Britten, and E. H. Davidson.** 1988. An *in vivo* titration of regulatory factors required for expression of a fusion gene in transgenic sea urchin embryos. Proc. Natl. Acad. Sci. USA **85**:7607–7611.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Maxson, R. E., T. J. Mohon, R. Cohn, and L. Kedes.** 1983. Expression and organization of histone genes. Annu. Rev. Genet. **17**:239–277.
38. **Maxson, R. E., T. J. Mohon, G. Gormezano, G. Childs, and L. H. Kedes.** 1983. Distinct organizations and patterns of expression of early and late gene sets in the sea urchin. Nature (London) **301**:120–125.
39. **McMahon, A. P., C. N. Flytzanis, B. R. Hough-Evans, K. S. Katula, R. J. Britten, and E. H. Davidson.** 1985. Introduction of cloned DNA into sea urchin egg cytoplasm: replication and persistence during embryogenesis. Dev. Biol. **108**:420–430.
40. **McMahon, A. P., T. J. Novak, R. J. Britten, and E. H. Davidson.** 1984. Inducible expression of a cloned heat shock fusion gene in sea urchin embryos. Proc. Natl. Acad. Sci. USA **81**:7490–7494.
41. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA probes from plasmids containing a bacteriophage Sp6 promoter. Nucleic Acids Res. **12**:7034–7056.
42. **Morris, G. F., D. Price, and W. F. Marzluff.** 1986. Synthesis of U1 RNA in a DNA-dependent system from sea urchin embryos. Proc. Natl. Acad. Sci. USA **83**:1–5.
43. **Schaffner, G., S. Schirm, B. Muller-Baden, F. Weber, and W. Schaffner.** 1988. Redundancy of information in enhancers as a principle of mammalian transcription control. J. Mol. Biol. **201**:81–90.
44. **Schirm, S., J. Jiricny, and W. Schaffner.** 1987. The SV40 enhancer can be dissected into multiple segments, each with a different cell-type specificity. Genes Dev. **1**:65–74.
45. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eukaryotic gene transcription. Trends Genet. **1**:224–230.
46. **Smith, A. B.** 1988. Phylogenetic relationship, divergence times, and rates of molecular evolution for camarodont sea urchins. Mol. Biol. Evol. **5**:345–365.
47. **Sorger, P. K., M. J. Lewis, and H. R. B. Pelham.** 1987. Heat shock factor is regulated differentially in yeast and mammalian cells. Nature (London) **329**:81–84.
48. **Vitelli, L., I. Kemler, B. Lauber, M. Birnstiel, and M. Busslinger.** 1988. Developmental regulation of microinjected histone genes in sea urchin embryos. Dev. Biol. **127**:54–63.
49. **Wilson, C., G. S. Cross, and H. R. Woodland.** 1986. Tissue-specific expression of actin genes injected into *Xenopus* embryos. Cell **47**:589–599.
50. **Wirth, T., L. Staudt, and D. Baltimore.** 1987. An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. Nature **329**:174–178.