Both basal and ontogenic promoter elements affect the timing and level of expression of a sea urchin H1 gene during early embryogenesis

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Late histone H1- β mRNA accumulates with the correct ontogenic pattern following microinjection of the cloned gene into fertilized sea urchin eggs. Sequences upstream of the gene encoding the sea urchin H1- β protein contain both basal and developmentally regulated elements. One late H1-specific activator sequence (USE IV) is required for the accumulation of mRNA following the blastula stage of development. All late H1 genes also contain a highly conserved GC-rich sequence resembling a low-affinity binding site for the mammalian transcription factor Sp1 that is required for basal expression of the H1- β gene at all stages of embryogenesis. When this GC-rich sequence (GGGCTG) is converted to a perfect core Sp1 sequence (GGGCGG), the H1- β transcripts accumulate to much greater levels and their peak accumulation is shifted to the early blastula stage rather than late blastula and gastrula stages of development. Coincidentally, early H1 genes, whose peak expression is also at the early blastula stage, all contain the same core consensus sequence (GGGCGG). Thus, both gene-specific activator sequences, as well as sequences that resemble sites for general transcription factors, may play a major role in determining the temporal patterns of gene expression during early embryogenesis.

[Key Words: Embryonic activator sequence; ontogenic gene expression]

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The histone multigene family in sea urchins has provided a model system to study the molecular mechanisms that govern temporal gene expression during early embryogenesis. There are several families of histone genes that are each expressed in a unique stage-specific manner in embryos and adult tissues (reviewed in Maxson et al. 1983a). The expression of the early histone genes, which are organized in 300–500 tandem arrays, is limited to the first 10–12 hr of embryogenesis, after which these genes are shut off and never used again in the life cycle of the animal (Maxson et al. 1983a). The late histone proteins are encoded by a smaller multigene family consisting of 2–12 members that are dispersed in irregular arrays (Childs et al. 1982; Maxson et al. 1983b; Knowles and Childs 1986; Knowles et al. 1987). The transcripts encoded by these genes are stored in small quantities in the egg, and the genes are transcribed during the first several cell divisions; however, their transcription rate increases at about the time the early family is inactivated (Knowles and Childs 1984; Knowles et al. 1987; M. Ito et al., in prep.). Late histone transcripts peak in late blastula, and gastrula-stage embryos. Members of the late histone gene family are also transcribed in the adult tissues of the animal (Kemler and Busslinger 1986; Lieber et al. 1986; Halsell et al.

1987). Three major variants of H1 histone proteins are synthesized during early embryogenesis. The α -subtype is encoded by the early genes, and the β - and γ -subtypes are each encoded by a unique sequence late gene (Knowles and Childs 1986; Knowles et al. 1987; Lai and Childs 1988).

We have cloned and sequenced the *Strongylocentrotus purpuratus* late H1- β gene and have determined its temporal pattern of expression during development (Lai and Childs 1988). The gene is coordinately regulated with the H1- γ gene and shares extensive sequence homologies within the 350 bp upstream of the mRNA initiation site. In this paper we have identified some of the elements that are essential for both the basal and maximal expression, as well as the proper temporal expression of the H1- β gene. A series of deletion and point mutants of this gene were microinjected into fertilized sea urchin eggs, and the expression of the cloned DNA was monitored during embryogenesis. At least two distinct components of the promoter appear to be responsible for determining the time of maximal transcript accumulation during embryogenesis. In the wild-type promoter, an upstream sequence element (USE IV) at –288 to –317 further activates basal promoter activity at the blastula stage of development. Basal promoter activity requires

the TATA box, USE 0, and USE I. However, by altering a GC-rich sequence equivalent to a mammalian low-affinity Sp1 site (GGGCTG) (McKnight et al. 1984; Jones et al. 1986) in the USE I of the wild-type H1- β promoter to a sequence equivalent to higher affinity site (GGGCGG) also found in the early H1 promoter, we induce maximal accumulation of H1- β transcripts earlier in embryogenesis. Thus, a single base-pair difference in the sequence of binding sites for general transcription factors can play a major role in determining the temporal pattern of gene expression during embryogenesis.

Results

Comparison of H1 upstream sequences

Inspection of the 350 nucleotides immediately upstream of the H1- β mRNA initiation site reveals several sequences in common with H1- γ (Fig. 1). Upstream of the

TATA box is a sequence (USE 0) that is 100% conserved among the three late H1 genes (19/19) and 79% conserved when compared with the early H1 gene (15/19). A GC-rich element (USE I) is present at the same position upstream of H1 genes in many species (Coles and Wells 1985; Perry et al. 1985). The USE I of early genes corresponds to the consensus sequence for mammalian transcription factor Sp1, whereas the USE I of all three late H1 genes differs by a single nucleotide from this consensus. Upstream of USE I is another H1-specific element, AAACAC (Coles and Wells 1985; Perry et al. 1985), which is the core of a highly conserved region of 65 bp in the three late H1 genes. Still farther upstream are two conserved late H1-specific sequences (USE III and USE IV) that extend from -210 to -226 and -288 to -317, respectively (Fig. 1). Each of these sequences is a candidate for an essential element of the H1- β promoter due to the extreme conservation maintained during the greater than 65 million years that *Strongylo-*

Figure 1. Conserved DNA sequences of sea urchin H1 gene promoters. Sequences identical among early or late H1 gene promoters are blocked. Core elements of the TATA box, USE 0, USE I, USE II, among all the H1 genes, and the homologous region of USE III and USE IV for all three late H1 promoters are indicated with darker shading. Numbers on left and right of each sequence indicate the position of the sequence relative to the mRNA initiation site of each gene. (S.p. α), *S. purpuratus* H1- α (Levy et al. 1982); (S.p. β), *S. purpuratus* H1- β (Lai and Childs 1988); (S.p. γ), *S. purpuratus* H1- γ (Knowles et al. 1987); (L.p. γ), *L. pictus* H1- γ (Knowles and Childs 1986); (P.m. α) *Psammechinus miliaris* H1- α (Birnstiel et al. 1978).

centrotus purpuratus and *Lytechinus pictus* have survived as independent species (Smith 1981).

Ontogenic activation of microinjected H1 histone genes

Sea urchin cytoskeletal actin genes (Flytzanis et al. 1987), early histone H2A genes (Davidson et al. 1985), and early and late H2B genes (Colin et al. 1988) are all expressed in the proper temporal fashion following microinjection into eggs or one-cell zygotes. The proper spatial expression of the CyIIIa actin gene only in aboral ectoderm cells has been confirmed by in situ hybridization (Hough-Evans et al. 1987). As an initial approach to the identification of *cis*-acting regulatory sequences in the late H1- β gene, we monitored the expression of a microinjected construct containing 1.3 kb of 5'-flanking sequence and 1.0 kb of 3'-flanking sequence. *S. purpuratus* H1 genes were microinjected into *L. pictus* eggs, and properly initiated mRNAs were monitored with an RNase protection assay. *L. pictus* embryos are known to contain all the *trans*-acting factors required for the proper expression of *S. purpuratus* histone genes because interspecies hybrids between these two sea urchin species regulate both maternal and paternal histone genes properly (Maxson and Egrie 1980). Endogenous H1- γ mRNA was used as a control for recovery of RNA in each sample. The results (Fig. 2) demonstrate that

H1- β mRNA accumulates at about the appropriate embryonic stage following microinjection of the H1- β construct. H1- β transcripts appeared at low levels 8 hr post-fertilization and accumulated for the next 10 hr. RNA from uninjected *L. pictus* embryos did not protect a fragment the size of *S. purpuratus* H1- β mRNA (see Figs. 4 and 5). Quantitative analysis of these data revealed that the transcripts derived from the injected H1- β gene accumulated to levels three- to fivefold higher than the endogenous H1- γ mRNA (Table 1). The H1- β transcripts do, however, decrease in abundance at later stages somewhat more rapidly than the endogenous late H1 mRNAs. This difference may be due either to post-transcriptional regulation, known to affect histone transcripts in a variety of systems (Osley et al. 1981; Maxson and Wilt 1982; Heintz et al. 1983; Weinberg et al. 1983) or to differences in the ability of these genes to compete with other genes for limiting quantities of *trans*-acting factors late in embryogenesis. We also monitored the amount of injected DNA during development (Table 1). Late H1- β DNA is replicated during the first 30 hr of embryogenesis, as are many linear DNA molecules (Flytzanis et al. 1985, 1987). Independent experiments with an early H1 DNA construct demonstrate that this DNA is also replicated during the first 30 hr of embryogenesis, yet transcription from this template occurs only for the first 10–12 hr, as expected (Z. Lai and G. Childs,

Figure 2. Ontogenic expression of microinjected *S. purpuratus* H1- β DNA. (A) About 3500 molecules of linearized H1- β DNA (3.0 kb *SalI* fragment in M13mp19) were microinjected into each *L. pictus* one-cell zygote. Injected embryos were collected for extracting RNA at each indicated time. RNA of 30 embryos from each time point was probed with Sp6 transcripts complementary to *S. purpuratus* H1- β mRNA and *L. pictus* H1- γ mRNA, digested with RNase A and RNase T1; protected fragments were then resolved on 5% acrylamide–7 M urea gels. (Lane S.p. gastr.) A control using 0.5 μ g *S. purpuratus* gastrula RNA (30 hr); (lane L.p. gastr.) the signal from 0.125 μ g *L. pictus* gastrula RNA (30 hr). All samples including controls contained both probes in the hybridization reaction. (B) Diagram of hybridization probes and the sizes of the expected protected fragments. For the *L. pictus* H1- γ probe, the multiple bands are probably due to the template that was not completely digested with *StyI*.

Table 1. Quantitative analysis of transcript levels from injected *S. purpuratus* H1- β genes and the endogenous H1- γ gene during early embryogenesis

Postfertilization (hr)	Injected H1-DNA (genes per embryo) ^a	Injected H1-mRNA (molecules per embryo) ^b	Endogenous H1-mRNA (molecules per embryo) ^b
6	2.8×10^4		
8		3.1×10^6	0.1×10^6
10	5.9×10^4		
16	9.8×10^4	8.1×10^6	1.2×10^6
20	11.0×10^4	5.2×10^6	1.6×10^6
30		3.0×10^6	1.1×10^6

^a The number of injected templates remaining in embryos of a given age was determined by hybridization to M13 DNA, as described in Methods.

^b H1- β and H1- γ mRNA was quantitated as described in Methods.

unpubl.). We conclude that the continued expression of injected late H1 genes represents proper transcriptional activity and is not due to the difference in replication or stability of different injected DNA templates.

Deletion analysis of the H1- β promoter

A series of deletions that progressively removed DNA sequences at the 5' end of H1- β was used for the preliminary identification of upstream sequences required for proper regulation in vivo (Fig. 3A). Each of these deletion constructs was microinjected into the same batch of fertilized eggs, and the number of transcripts that accumulated by 20 hr postfertilization was quantitated relative to endogenous late H1- γ mRNA. The degree of amplification of each construct was also determined to ensure that the absence of H1- β transcripts with any given mutant was not due to loss of DNA or lack of amplification. Many experiments of this type indicate that as long as the injected DNA is amplified within a broad concentration range, the same intrinsic transcriptional capacity of the template is maintained (i.e., the same result is obtained if the number of templates remaining is reversed for the -1300 and -469 mutants in Fig. 3A). Measurements made by Flytzanis et al. (1987) also support this argument. After carefully measuring the number of molecules of CyIIIa-CAT actin fusion DNAs compared with the activity of the gene in many batches of eggs and embryos, these workers conclude that DNA is saturating with respect to the ability to activate the CAT genes. Removal of H1- β sequences 5' to -329 has no effect on the level of H1- β transcripts that accumulate by 20 hr. However, removal of the sequences from -329 to -106 or -93 consistently reduced the number of accumulated transcripts to 30% that of wild type. These deletions remove sequence elements II, III, and IV. Deletion mutants from which USE I is removed are not transcribed to detectable levels.

Although the number of H1- β transcripts peaks at about 20 hr, the previous experiment does not address the effect of these deletions on the temporal expression of this gene. Sequences upstream of -329 could be required for proper timing rather than maximal expression. To test this possibility, the temporal pattern of expression of several deletion constructs was tested (Fig. 3B). In deletion mutants up to -329, the expression pat-

tern was indistinguishable quantitatively and qualitatively from that of a construct with 1.3 kb of upstream sequences. However, deletion down to -106 showed a basal level of H1- β transcripts at all developmental times tested. That is, without sequences distal to -106, the gene is expressed at the same low level as the wild-type gene for the first 10 hr of development but subsequently fails to accumulate additional transcripts because it is not transcriptionally activated as is the wild-type gene (Knowles and Childs 1984).

Effects of point mutations and short internal deletions

Deletion analysis showed that elements II, III, or IV, individually or together, are required for the accumulation of H1- β transcripts in late blastula, whereas USE I seems to be required for basal transcription. To test the effect of USE 0 or USE I alone on H1- β transcription, we constructed a set of site-directed mutants in the H1- β gene. As with the deletion mutations, a single batch of eggs was injected with wild-type and mutant constructs, and the abundance of H1- β was monitored relative to endogenous late H1- γ mRNA at 20 hr postfertilization (Fig. 4). Mutations that change three conserved cytidine residues in USE 0 (-52 to -50) to adenosines (Fig. 4, lane 7) or the conserved ACG sequence in USE 0 (-49 to -47) to GAT (Fig. 4, lane 6) both abolished transcription. We conclude that USE 0 is an essential element of the H1- β promoter. This sea-urchin-specific sequence may replace the requirement for the CCAAT sequence found in the same position of H1 genes in other species (Coles and Wells 1985). When three guanines of USE I (-85 to -83) were changed to adenosines, all transcription from H1- β was abolished (Fig. 4, lane 5). Thus, despite the deviation from the Sp1 core consensus sequence, USE I is also an essential element of the H1- β promoter. We also tested the effect of changing the thymidine in the late USE I sequence GGGCTG (-81) to a guanine, creating a consensus Sp1 site. Surprisingly, this mutation resulted in as much as five-fold fewer transcripts accumulating at 20 hr postfertilization than the wild-type construct (Fig. 4, lane 4). We also tested the effects of changing the TAT of the TATA box (positions -33 to -31) to GGG (Fig. 4, lane 11). Although this mutation decreased the levels of transcript accumulation to 30%

Figure 3. Transcriptional activity of the microinjected *S. purpuratus* H1-β gene and 5' deletion constructs. (A) About 3500 linearized molecules of each construct were microinjected into *L. pictus* one-cell zygotes. Embryos were collected for extracting RNA or DNA at 20 hr postfertilization. RNA and DNA were manipulated as described in Methods. The 5' end of each construct is indicated. The number of H1-β transcripts accumulated at 20 hr postfertilization was calculated as described in Methods and normalized to the endogenous H1-γ mRNA in each sample. (B) Indicated constructs (−372; −329; −106) were microinjected, and embryos were collected at each time point postfertilization for preparing RNA, which was manipulated as described in Methods. RNA equivalent to 30 embryos was used in each hybridization reaction. (Lane *S.p. gastr.*) 0.5 μg *S. purpuratus* gastrula (30 hr) RNA; (lane *L.p. gastr.*) 0.125 μg *L. pictus* gastrula (30 hr) RNA.

of wild type, we see no evidence of aberrant initiation sites.

The sequences upstream of USE I required to induce mRNA accumulation above basal levels were further investigated by using short internal deletions of each element. Deletion of the entire USE II element between −121 and −155 had a significant effect (40–60% wild-type expression) (Fig. 4, lane 8). Deletion of a 10-bp segment of USE III had a smaller, but still reproducible, effect on H1-β mRNA accumulation (Fig. 4, lane 12). A 30-bp deletion of USE IV, on the other hand, resulted in only basal level accumulation (i.e., the same as in 8-hr embryos) of H1-β mRNA (Fig. 4, lane 13). Each mutant construct was amplified to the same extent as the wild-type construct. Quantitative analysis of these data are presented in Table 2.

We tested the idea that USE IV is required for transcriptional activation of the H1-β gene at the blastula stage by examining the temporal expression of this deletion construct (Fig. 5). At all time points (10, 16, and 20 hr postfertilization), only low levels of transcripts accu-

mulate. Using the same batch of eggs and the same probes, embryos injected with the wild-type construct accumulate H1-β mRNA between 10 and 20 hr postfertilization, as expected. We conclude that USE IV is required for the activation of the H1-β gene at the late blastula stage of development.

Effect of the *Sp1* mutation on temporal expression

We expected that conversion of the USE I sequence in the H1β gene to an authentic Sp1 high-affinity core-binding site might act to increase transcript abundance. As the early H1 gene contains the identical core Sp1 site, we measured the temporal expression of this construct. The data shown in Figure 6A and quantitated in Figure 6B (right panel) demonstrate that the pattern of transcript accumulation from this construct is now more typical of an early gene than a late gene. Transcripts are most abundant 10 hr postfertilization, decline by 16 hr, and are further reduced by 20 hr. The Sp1 construct is replicated during embryogenesis to the same extent as

Figure 4. Analysis of point mutations and internal deletions within the *S. purpuratus* H1- β gene promoter. About 3500 linearized DNA molecules of each construct were microinjected into *L. pictus* one-cell zygotes. Embryos were collected for making RNA at 20 hr postfertilization. (Lane tRNA) 5 μ g tRNA was used to hybridize to the mixed H1- β H1- γ probes; (lane s.p. gast.) 0.5 μ g *S. purpuratus* gastrula (30 hr) RNA; (lane L.p. gast.) 0.125 μ g *L. pictus* gastrula (30 hr) RNA; (lanes 1,3,9) RNA samples from uninjected embryos. Remaining lanes correspond to the point mutation and internal deletion constructs described in the lower part of the figure. Each experimental lane contains RNA equivalent to 30 embryos.

Table 2. Quantitation of H1- β mRNAs derived from injected wild-type and mutant constructs

	Experiment 1 ^a			Experiment 2 ^a	
	H1- β DNA genes per embryo ^b	H1- β mRNAs per embryo	H1- β gene activity (%) ^c	H1- β mRNAs per embryo	H1- β gene activity (%) ^c
Wild type	2.1×10^4	5.0×10^6	100	1.2×10^6	100
TATA ^d	1.9×10^4	1.5×10^6	29	—	—
USE 0 ^e	2.6×10^4	$<5 \times 10^4$	<1.0	$<10^4$	<1.0
USE 0 ^f	1.1×10^4	$<5 \times 10^4$	<1.0	$<10^4$	<1.0
Sp1 ^g	3.6×10^4	1.1×10^6	22	3.0×10^5	25.2
USE I ^h	1.6×10^4	$<5 \times 10^4$	<1.0	$<10^4$	<1.0
Δ USE II	1.2×10^4	1.9×10^6	39	6.9×10^5	58.0
Δ USE III	1.9×10^4	3.1×10^6	62.0	—	—
Δ USE IV	1.5×10^4	9.8×10^5	16.8	—	—

Primary data for each construct are shown in Fig. 4. Mutant constructs are described more completely in Methods and the text.

^a Experiments 1 and 2 represent data from two separate groups of injections using different batches of eggs and sperm.

^b Refers to the number of template DNA molecules remaining 20 hr postfertilization.

^c Fraction of H1- β transcripts relative to the wild-type construct.

^d Changes TAT at position -33 to -31 to GGG.

^e Changes ACG at position -49 to -47 to GAT.

^f Changes CCC at position -52 to -50 to AAA.

^g Changes T at position -81 to G.

^h Changes GGG at position -85 to -83 to AAA.

Figure 5. Effect of USE IV on the temporal expression of the H1- β gene. (A) Approximately 600 linearized DNA molecules of the wild-type (–1300) and Δ USE IV deletion constructs were microinjected into each one-celled zygote, and embryos were collected at 10, 16, and 20 hr postfertilization. RNA was isolated as described and hybridized with *S. purpuratus* H1- β and *L. pictus* late H3 riboprobes. The late H3 transcripts accumulate with the same ontogenic pattern as the late H1 transcripts. (Lane M) Radiolabeled *Hae*III-digested X174 DNA markers; (lane *S.p. gast.*) 0.5 μ g *S. purpuratus* gastrula (30 hr) RNA; (lane *L.p. gast.*) *L. pictus* gastrula (30 hr) RNA. Each experiment contained the RNA equivalent to 30 embryos. (B) Diagram of hybridization probes and the sizes of the expected protected fragments.

the wild-type DNA (Fig. 6B, right panel). This confirms that the decrease in transcript abundance between 10 and 20 hr is not due to the loss of template DNA. In Figure 6B (left panel) is an independent experiment also demonstrating the early pattern of transcription for the Sp1 construct. We also include data showing the expression from microinjected wild-type DNA, using the same batch of eggs for the injections. Not only is the pattern of transcription different between these two DNAs that differ by a single base pair, but the Sp1 construct reproducibly accumulates four- to eightfold more H1- β mRNA. Although different batches of eggs exhibit variable levels in the absolute number of transcripts that accumulate, within a single batch of eggs the relative levels and ontogenic accumulation from different templates does not vary. Thus, the single-base change affects the temporal pattern of expression of this gene, and differences in expression cannot be attributed to differential stability or replication of the injected DNA.

There are two possible explanations that could explain the precipitous decline in mRNA abundance using the Sp1 construct. One reason could be due to post-transcriptional control, as mentioned previously. As many as 17×10^6 H1- β transcripts accumulate at the peak of expression—far more than the embryo normally contains. Alternatively, the decrease in H1- β Sp1 mutant transcription could also be explained by the stage-specific inactivation of a factor specific for the sequence GGGCGG or to the appearance of a negative regulatory

protein that recognizes this sequence but not GGGCTG. This may also explain the shutoff of early, but not late, H1 genes in blastula at 10 hr. These arguments can be addressed by using fusion constructs whose transcripts are not subject to post-transcriptional regulation.

Discussion

Our goal is to understand how related genes are transcribed at different times during early embryogenesis. Our approach to this question is to study the regulated expression of the sea urchin late H1- β gene following introduction of the cloned DNA into developing embryos. In this paper we have shown that single-base differences in the binding site for general transcription factors not only can influence the levels of expression but the temporal accumulation as well. Late histone H1 genes are properly activated using this gene transfer system. The effects of mutant constructs on the accumulation of H1 transcripts argue that we are monitoring transcriptional events and that sequences within our constructs are sufficient for regulated expression of this gene. Deletion analysis of the H1- β promoter region demonstrated that 5' sequences up to –106 are sufficient for activation of transcription and basal expression of this gene. These sequences include a TATA box and two upstream sequence elements, USE 0 and USE I. Point mutations in the TATA box, USE 0, or USE I demonstrate that these sequences are each elements of the promoter. Similarly, the USE I element of a chicken H1 gene is essential for transcription in *Xenopus* oocytes or HeLa cells (Younghusband et al. 1986). Proteins bound at these sites would be required as part of a transcription complex that acted as a basal level promoter.

We utilized short internal deletion mutations as a preliminary means of assessing the role of other conserved elements of the late H1 upstream region. We observed reproducible decreases in H1- β mRNA accumulation with a USE II deletion and small but reproducible decreases with a USE III deletion. Deletion of the chicken USE II element has a negligible effect on transcript accumulation when assayed in frog oocytes or HeLa cells (Younghusband et al. 1986). The extreme conservation of the core USE II sequence from sea urchins to humans (Perry et al. 1985) argues that this sequence serves some function. Perhaps its role is to serve as an element involved in cell-cycle regulation by analogy with a different sequence that serves this role in yeast histone genes (Osley et al. 1986). USE III, a late gene-specific sequence could be required for activation in adult tissues rather than embryos. USE IV, on the other hand, seems to be required for the induction or accumulation of H1- β transcripts between 10 and 20 hr postfertilization. Late histone genes are transcriptionally activated at this time (Knowles and Childs 1984). Without this sequence element, only basal levels of mRNA accumulate throughout embryogenesis (Fig. 5). We minimized the potential effects of proteins binding on different sides of the DNA helix by constructing the USE IV deletion so that precisely 30 bp of conserved sequences was re-

Figure 6. Temporal expression of the H1β Sp1 construct during early embryogenesis. (A) About 3500 linearized DNA molecules of the Sp1 the H1β construct were microinjected into each one-cell zygote, and embryos were collected at each time point for making RNA as described in Methods. RNA equivalent to 30 embryos was hybridized to both *S. purpuratus* H1-β and *L. pictus* H1-γ riboprobes. Protected products were resolved on 5% acrylamide-7 M urea gels. (Lane *S.p. gast.*) 0.5 μg *S. purpuratus* gastrula (30 hr) RNA; (lane *L.p. gast.*) 0.125 μg *L. pictus* gastrula (30 hr) RNA; (lane M) 5' end-labeled *HaeIII*-digested X174 DNA that served as a size marker on this gel. (B) (Left panel) wild-type H1-β and Sp1 mutant DNAs were separately microinjected into the same batch of eggs, and H1-β mRNA and endogenous H1-γ mRNA were processed and quantitated as described before. (Right panel) Quantitative analysis of the data shown in A. DNA equivalent to 20 embryos was fixed to nitrocellulose using a slot blot apparatus and hybridized to nick-translated M13mp19 DNA probe, as described in Methods. Quantitation was relative to known quantities of M13 DNA applied to the same filter.

moved. The size of the conserved region is large enough to accommodate more than one sequence-specific DNA-binding protein. Point mutations and binding studies will be required to see whether this is the case. Additional studies will also be required to determine whether this element can induce expression on heterologous promoters in an orientation- and position-independent manner.

Perhaps our most provocative finding was the observation that a single-base mutation creating an authentic high-affinity core Sp1-binding site not only affects the number of transcripts that accumulate but the temporal accumulation as well. One model that can explain this data requires that late H1 genes normally require Sp1 (or the sea urchin equivalent of this mammalian transcription factor) for transcription during the first 10 hr of development, but they compete very poorly with many other genes (including early H1) for this transcription factor due to the low-affinity binding site. Transcripts accumulate in late blastula-stage embryos at least in part because of the activity of USE IV. Creating an authentic high-affinity binding site for the factor in the late H1-β gene allows the injected DNA to compete effectively for this factor, with the result being high levels

rather than basal transcription during the first 10 hr of development. This model is the opposite of one where somatic (late) *Xenopus* 5S genes bind more tightly to and compete better for limiting quantities of TFIIIA as embryogenesis proceeds (Brown 1984). We are proposing that the somatic (late) family is unable to compete very well early in embryogenesis and the competition is with a general (Sp1 or its equivalent) rather than gene-specific (TFIIIA) transcription factor. In our case, augmented transcription after the early blastula stage seems to result from a late gene-specific element (USE IV). We have previously pointed out the inconsistencies of having the sea urchin histone gene system utilize the simple *Xenopus* 5S gene competition model (Knowles and Childs 1987; M. Ito and R. Maxson, in prep.). In particular, we pointed out that this model cannot explain data where the low copy number gene family (late genes) augments their transcription rates at the blastula stage of development. The use of a late gene-specific sequence, USE IV, to augment and induce transcription beyond the early blastula stage better explains the available data and could, in fact, be shared among many genes that are activated at this stage of embryogenesis (Davidson 1986). If this model is correct, the decline in tran-

script abundance seen with Sp1 mutation at later stages of embryogenesis would result from post-transcriptional events and could be tested using fusion constructs.

Another model consistent with our data is one where distinct factors recognize the core early and late USE I sequence. In this case, the protein binding to the sequence GGGCGG activates transcription only until the early blastula stage but at higher rates than a different protein that binds the sequence GGGCTG. Based on our data, this protein or another that also recognizes the sequence GGGCGG could act as a negative regulator in later stage embryos when the early H1 gene and the Sp1 H1- β construct are not transcribed.

Methods

DNA sequence analysis

DNA was sequenced on M13 vectors [Messing and Vieira 1982], using the method of Sanger et al. (1977). Deletions in M13 constructs used for sequencing and for microinjection were constructed using the procedure described by Dale et al. (1985).

Site-directed mutagenesis

Synthetic oligonucleotides that differ from the wild-type H1- β promoter by a single nucleotide, three nucleotides, or span a region to be deleted were constructed on an Applied Biosystems automated DNA synthesizer. The 3.0-kb *S. purpuratus* H1- β gene in the M13 vector was used as the template for in vitro mutagenesis. Mutants were prepared as described [Zoller and Smith 1983]. All mutations were confirmed by DNA sequence analysis. To sequence site-directed mutations, oligonucleotides shown in Table 3 were used as sequencing primers. (Lowercase letters or vertical lines refer to mutant bases or the site of deletions, respectively. Numbers refer to positions relative to the start of transcription.)

Microinjection of sea urchin one-cell zygotes

The procedure was essentially that of McMahon et al. (1985) and Colin (1986), using modifications worked out by A. Colin, unpubl. Unfertilized eggs were collected after injection of 0.5 M KCl into adult *L. pictus* and kept in Millipore-filtered seawater at 4°C. Shed sperm was removed from male animals with a pasteur pipette and stored on ice. Filtered seawater (FSW) acidified with 1 M citric acid to pH 4.8 was used to remove the surrounding jelly coat of unfertilized eggs. The acidic FSW was

then removed, and dejellied eggs were washed twice with cold FSW. Dejellied eggs were taken up in a pulled siliconized mouth pipette and fixed by electrostatic attraction to 60-mm Falcon dish lids treated for 1 min with 1% protamine sulfate. Micromanipulation was performed using a Nikon-Narishige NT-8 micromanipulator on a Nikon inverted microscope. The pulled needle was broken at the tip, and approximately 2 μ l of 50 ng/ μ l DNA solution was rapidly injected into the cytoplasm of each egg. Eggs were fertilized by the addition of a few drops of 20 $\times$ diluted sperm just prior to injection. Embryos were grown at 15°C. All DNA used for microinjection was linearized by digestion with a restriction enzyme, treated with RNase A and then proteinase K, followed by phenol-chloroform extraction. Following precipitation with ethanol, the DNA was resuspended in 0.2 μ m filtered distilled water. The number of injected molecules was about 3500.

$$\frac{(50 \text{ ng}/\mu\text{l} \times 2 \times 10^{-6} \mu\text{l}) \times (2.3 \times 10^{23} \times 10^{-9} \text{ gm})}{10 \text{ kb} \times 10^3 \times 660}$$

RNase protection assays

The *S. purpuratus* H1- β gene fragment from position - 556 to + 317 was subcloned into pSp64, and an *EcoRI-HindIII* fragment (387 bp) spanning the 3' end of the *L. pictus* H1- γ gene [Knowles and Childs 1986] was subcloned into pSp65. The *L. pictus* late H3 construct was described in Lieber et al. (1986). H1- β , H1- γ and late H3 antisense RNA probes were generated as described by Melton et al. (1984). About 10⁶ cpm of each probe was mixed with the RNA to be analyzed in 1 $\times$ RNase mapping buffer (40 mM PIPES at pH 6.7, 0.4 M NaCl, 1 mM EDTA) and 80% deionized formamide in a 20- μ l reaction volume at 50°C for 18 hr. The reaction was then terminated by the addition of 180 μ l RNase digestion buffer (10 mM Tris at pH 7.5, 5 mM EDTA, 300 mM NaCl), with 20 μ g/ml RNase A and 2 μ g/ml RNase T1. After 60 min at 30°C, 20 μ l of 10% SDS and 50 μ g of proteinase K were added, and the reaction incubated an additional 15 min. After phenol-chloroform extraction and ethanol precipitation, the protected RNA was analyzed on 5% acrylamide-7M urea gels. Samples from microinjected or control embryos were routinely analyzed with both probes in a single reaction mix.

Minipreparation of sea urchin embryo RNA and DNA

For each RNase protection assay, 20–30 normal embryos of a given age were collected by mouth micropipette. Embryos were washed in 200 μ l of embryo wash solution (0.5 M NaCl, 10 mM

Table 3. Oligonucleotides used

Sequencing primers			
GC 26:	-183	5'-CATATCTTAATTGAAAAG-3'	-166
GC 33:	-353	5'-GCATTTTATATGAAATT-3'	-337
Preparing mutants			
GC 29:	-42	5'-CACGCATTC g g ATAGCTCTG-3'	-22 TATA box
GC 23:	-58	5'-TTTTCCCCC g a t TCCGCACGC-3'	-38 USE 0
GC 24:	-61	5'-CTGTTTTTCC a a ACGTCCGCA-3'	-41 USE 0
GC 21:	-89	5'-CAGTGGGC g GTGTAGGG-3'	-73 USE I
GC 22:	-94	5'-AGTGTCAGT a a CTGTGTAGG-3'	-74 USE I
GC 25:	-165	5'-AAATCGAAGAG TATTGCCATAA-3'	-111 USE II deletion
GC 31:	-236	5'-AAATTGAAGC CTCTGTCTAA-3'	-207 USE III deletion
GC 32:	-328	5'-AGTTTGACAAG GACCATTTCAGT-3'	-277 USE IV deletion

Tris at pH 7.4, 50 mM EDTA, 10 mM EGTA). Pelleted embryos were then resuspended in 200 μ l 0.3 M NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 0.1% SDS and extracted once with phenol, once with phenol-chloroform, and once with chloroform. RNA was precipitated using 10 μ g carrier tRNA.

For DNA preparation, 20–30 normal embryos were collected and washed as above. Embryos were then resuspended in 50 μ l of 250 mM Tris (pH 7.8), and 50 μ l of 0.1 M EDTA (pH 8), 1% SDS, and 20 μ g proteinase K were added. After incubation at 55°C for 2 hr, nucleic acids were recovered by phenol extraction. RNA was degraded by treatment in 0.3 M NaOH for 1 hr at 65°C, neutralized with 2 M $(\text{NH}_4)_2\text{SO}_4$, and applied to nitrocellulose filters using an S & S Minifold II apparatus (Flytzanis et al. 1987). M13mp19 DNA was labeled by nick translation and used to detect and quantitate injected late H1- β DNA. Slot blots were hybridized using standard procedures (Maniatis et al. 1982).

Quantitation of RNA and DNA

Autoradiograms were scanned using an LKB Ultrosan Laser Densitometer. The absolute numbers of H1- β transcripts were calculated by comparison with the signal generated using the identical probe and 30-hr *S. purpuratus* gastrula RNA. The number of transcripts per embryo equivalent of this RNA preparation was determined by solution RNA probe excess hybridization to be 10^6 . Recovery of RNA was monitored by measuring endogenous *L. pictus* H1- γ mRNA. There is 2.8 ng total RNA in each egg or embryo (Davidson 1986). When 500 ng of total *S. purpuratus* RNA at 30 hr is used as a control, the number of transcripts per embryo would be

$$\frac{\text{AU} \cdot \text{mm}(\text{tested})}{\text{AU} \cdot \text{mm}(\text{control})} \times \frac{500 \text{ ng}}{2.8 \text{ ng}} \times 10^6$$

Number of embryos

The quantitation of endogenous H1- γ transcripts was done using identical procedures.

To measure the amount of injected DNA through early embryogenesis, injected and uninjected embryos were collected to make DNA which, in turn, was immobilized on nitrocellulose along with known quantities of M13 DNA. Nick-translated wild-type M13mp19 double-stranded DNA was used as a hybridization probe. By comparison of hybridization signals, the absolute number of exogenous DNA molecules can be calculated.

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Both basal and ontogenic promoter elements affect the timing and level of expression of a sea urchin H1 gene during early embryogenesis.

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