Activation of a Late H2B Histone Gene in Blastula-Stage Sea Urchin Embryos by an Unusual Enhancer Element Located 3' of the Gene

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In the sea urchin embryo, late histone genes are transcribed at low levels during cleavage and blastula formation and at substantially higher levels in later stages of embryogenesis. To investigate the molecular basis of the stage-specific expression of a late H2B histone gene, we injected mutant genes lacking portions of 5'- and 3'-flanking regions into *Lytechinus pictus* embryos and monitored their expression by RNase protection. A 200-bp region located 489 bp downstream of the mRNA 3' terminus was necessary for the increase in transcription of the late H2B gene at the mid-blastula stage of development. DNase I and methylation interference footprint analyses located only one factor-binding site in this region, and gel mobility shift experiments showed that the DNA-binding activity of this factor (designated H2B abp 1) paralleled the transcriptional activity of the L1 H2B gene. Additional mutagenesis and microinjection experiments located the activator element to a 32-bp DNA segment that includes the H2B abp 1-binding site. These experiments also showed that the 32-bp fragment functions independently of position and orientation and therefore has the hallmarks of an enhancer. That this fragment contains most or all of the L1 H2B gene transcription-stimulatory activity makes it unusual among enhancerlike elements, which generally consist of several clustered factor-binding sites that act additively or cooperatively to affect transcription. The nucleotide sequence of the L1 H2B enhancer element suggests that the *trans*-acting factor that interacts with it is a member of the antennapedia or engrailed class of homeodomain proteins.

We wish to understand the molecular mechanisms that underlie the timing of gene expression early in embryogenesis. The sea urchin histone gene family, comprising two subfamilies whose expression is stage specific, provides a useful model for this purpose. Early (or alpha) histone genes exhibit a sharp peak of transcription during cleavage and blastula formation (31, 45, 46, 59); late histone genes are transcribed at low rates at these early developmental stages and at rates severalfold higher during subsequent embryogenesis, resulting in a 4- to 10-fold accumulation of late histone transcripts between the early and late blastula stages (2, 7, 8, 28, 32, 33, 41, 43). Late histone genes are also expressed in the adult, some in all tissues (somatic variants) and some in specific tissues (tissue-specific variants) (22a, 32, 38).

Gene transfer into sea urchin zygotes has been used widely to identify *cis*-acting elements that participate in the regulation of gene expression during development (10, 18, 26, 27, 36, 37, 39, 55, 57). In this approach, the gene of interest is microinjected into eggs or one-cell zygotes and its expression is monitored during embryogenesis. Such gene transfer studies have shown that injected early H2A, H2B, and H3 histone genes are expressed with temporal patterns similar to those of the endogenous early histone genes (11, 13, 15, 57). In addition, transcripts of injected somatic late H1 and late H2B histone genes accumulate to substantially higher levels in late embryos than in early embryos (11, 36, 37), suggesting that the injected genes are regulated faithfully.

Recently, Lai et al. (36) have shown that an enhancer element located in the 5'-flanking region of a late H1 gene plays a part in the stage-specific expression of the gene, and Barheris et al. have identified a factor, known as tissue-specific activator protein (TSAP), that binds in the promoter region of tissue-specific late H2A and H2B genes and is required for their expression in embryos (4). Here we demonstrate that the ontogenic activation of the L1 somatic late H2B gene (43) depends on an enhancer element located 3' of the gene. An embryonic sea urchin factor, designated H2B activator-binding protein 1 (H2B abp 1), binds specifically and with high affinity to the sequence of GCTGC CAATCATATT within the enhancer element. This factor-binding site contains most of the enhancer activity. The DNA-binding activity of H2B abp 1 changes during development in parallel with changes in the expression of the injected late H2B gene, further supporting the view that this factor plays a key part in the ontogenic activation of the L1 late H2B gene.

MATERIALS AND METHODS

Construction of L1 H2B mutants. The L1 late H2B gene used in these studies is one of several variant late H2B genes characterized in *Strongylocentrotus purpuratus* (30, 43, 44, 49, 56). Plasmid pSpL1 containing the L1 H2B and H4 genes was truncated at -491 relative to the transcription start site of the H2B gene by *Bam*HI digestion to give the subclone designated pSpL1H2BΔ5'-491 (42). The subclone pSpL1Δ3'+535 was constructed by removing a 5-kb *Bgl*II fragment 3' of the L1 late H2B gene (+535 to +5500). This same deletion was made in plasmid pSpL1H2BΔ5'-491 to

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create pSpL1H2BΔ5'-491Δ3'+535, bearing 491 bp of 5'-flanking sequence and 91 bp of 3'-flanking sequence. A series of deletions in the 3'-flanking region (between +535 and +5500) was generated by BAL 31 nuclease digestion (21) of plasmid pSpL1H2BΔ5'-491. Nucleotide sequencing of the breakpoints of these mutants was carried out by the chain termination method (51). All plasmids were linearized 5' of the L1 late H2B gene with *EcoRI* prior to microinjection into *Lytechinus pictus* zygotes.

A 358-bp *HindIII* fragment containing the 3' activator sequence was generated by means of the polymerase chain reaction (PCR), using amplimer oligonucleotides that hybridize at +888+903 (5') and +1228+1245 (3'). The amplified fragment was inserted in the forward orientation into the *HindIII* site in the polylinker 5' of the L1 H2B gene in the construct pSpL1H2BΔ5'-491Δ3'+535 to make the construct designated L1 H2B-5' ACT. The deletion mutants Δ+921 and Δ+972 within the 358-bp fragment were generated by PCR, using 5' amplimers that terminate at +921 and +972, respectively, and a 3' amplimer that spans nucleotides +1228-1245. The mutant-act+pBR (see Fig. 6) was created by inserting a 0.5-kb *HinfI* fragment of pBR322 491 bp 5' of the L1 late H2B mRNA start site. A DNA fragment bearing the H2B abp 1-binding site (see Fig. 6A, boxed region) was then cloned immediately 5' of the pBR fragment in either the forward [act (+936+967)] or reverse [act (+936+967) reverse] orientation.

Microinjection of histone genes into sea urchin eggs. DNA was injected into *L. pictus* one-cell zygotes by the methods of Colin (10) and McMahon et al. (47). Dejellied eggs were attached to petri dishes treated with 1% protamine sulfate, fertilized, and injected with 10 to 20 pl of linearized plasmid DNA or PCR-amplified DNA (50 μg/ml) in distilled water. PCR-amplified DNA was purified by agarose gel electrophoresis prior to injection. Injected embryos were cultured at 15°C. Mortality by the hatched blastula stage was less than 20%.

RNA extraction and RNase protection assay. For each experiment, equal numbers of embryos (50 to 70) were washed in 0.5 M NaCl-50 mM EDTA-10 mM Tris hydrochloride (pH 7.4) (15,000 × g for 3 min) and dissolved in 200 μl of 0.1 M NaCl-1 mM EDTA-10 mM EGTA-10 mM Tris hydrochloride (pH 7.4)-0.5% sodium dodecyl sulfate. Yeast tRNA (10 μg) was added as a carrier. A portion of this lysate (150 μl) was extracted with 150 μl of phenol-150 μl of chloroform and then precipitated in 0.3 M sodium acetate and 2.5 volumes of ethanol. The remainder of the lysate (50 μl) was analyzed for L1 late H2B DNA as described below. The precipitate was dissolved in water and hybridized with a ³²P-labeled-RNA probe generated by transcription *in vitro* as described previously (12, 48). The probe scanned nucleotides -43 to +62 of the L1 late H2B gene. Hybridization was for 20 h at 47°C in 0.4 M NaCl-40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)-50 mM dithiothreitol-1 mM EDTA-70% (vol/vol) formamide. After hybridization, the RNA was digested with RNase A (40 μg/ml) and RNase T₁ (7.5 U/ml), and the protected fragments were separated on a 6% polyacrylamide-7 M urea gel. The gels were exposed to preflashed X-ray film with an enhancing screen (Dupont Cronex). The autoradiographs were scanned with an LKB Ultrascan XL laser densitometer. Standards of 0.1 and 0.5 μg of late-blastula-stage *S. purpuratus* total RNA were analyzed along with experimental embryos. To calculate the number of L1 late H2B mRNAs transcribed from injected genes, we assumed that late (24-h) *S. purpuratus*

blastulae contain about 4.5×10^7 L1 late H2B transcripts per μg of total RNA, as measured by Ito et al. (28).

DNA extraction and assay. The assay for injected DNA was derived from that of Flytzanis et al. (18). Embryo lysate (50 μl) prepared as described above was treated with proteinase K and then extracted with an equal volume of phenol. The aqueous phase was brought to 0.4 N NaOH and incubated for 1 h at 60°C to hydrolyze RNA and denature DNA. Ammonium acetate (pH 6.6) was then added to 1 M, and the samples were immediately applied to a nitrocellulose membrane, using a Bethesda Research Laboratories Hybris-lot manifold. Filter hybridization was performed by using radioactive probes generated by Sp6 transcription *in vitro*. To determine absolute numbers of L1 H2B genes, known amounts of L1 H2B DNA standards were assayed along with experimental samples, and the autoradiographs were scanned with a densitometer.

Preparation of nuclear protein. Nuclear protein was extracted from *S. purpuratus* embryos at several stages of development as described by Calzone et al. (5). For DNase I footprints, nuclear extract from 24-h embryos was fractionated on a heparin agarose column as described previously (24). The 0.4 M KCl-eluted protein was dialyzed against 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9)-40 mM KCl-0.1 mM EDTA-1.0 mM dithiothreitol-20% glycerol and used for footprint analysis. To determine the number of nuclei represented in a given amount of nuclear extract, chromatin pellets from the final centrifugation step were treated with proteinase K, extracted with phenol, and precipitated with ethanol. The amount of DNA extracted from the pellets was quantitated by measuring its A₂₆₀.

Preparation of radiolabeled DNA probes for gel retardation and footprinting assays. A 600-bp *Bg/III-StuI* fragment that lies 3' of the *S. purpuratus* L1 H2B gene was subcloned into pUC8. The *HindIII* site in the polylinker was labeled at its 3' or 5' terminus by T4 DNA polymerase or T4 polynucleotide kinase, using [α-³²P]dATP or [γ-³²P]ATP (3,000 Ci/mmol; Amersham). An *HhaI* digestion generated a 250-bp fragment spanning the enhancer region. This fragment was purified by gel electrophoresis and used as a probe in gel retardation and footprinting experiments. We synthesized a 35-nucleotide oligonucleotide spanning the L1 H2B abp 1-binding site (see Fig. 4 and 5) and bearing *HindIII* ends. The sequence of this oligonucleotide is given below. When used as a probe in gel mobility shift assays, this oligonucleotide was radiolabeled at its 3' termini by using T4 DNA polymerase and ³²P-dATP.

Oligonucleotides. The sequences of the oligonucleotides used in gel retardation analyses were as follows: H2B abp 1, 5'-AGCTTCCAAATTAAGCTGCCAATCATATTTTGA GAAGCT-3'; and early H2B CCAAT, 5'-GATCCTAAGACCAATGAAAGGATC-3'.

Electrophoretic mobility shift, DNase I protection, and methylation interference assays. Protein-DNA binding reaction mixtures (10 μl) for gel mobility shift assays (19) contained 10 mM Tris (pH 7.4), 1 mM EDTA, 50 mM KCl, 5% glycerol, 4.8 μg of poly(dA-αT) (Pharmacia), 2 μg of nuclear protein extract, and approximately 1 ng of radiolabeled probe DNA. The reaction mixtures were incubated for 15 min at 20°C, and the DNA-protein complexes were separated from free probe by electrophoresis on 20-cm 5% acrylamide gels (5% acrylamide, 0.12% bisacrylamide, 50 mM Tris, 50 mM sodium borate [pH 8.3], 1 mM EDTA, 10% glycerol) at room temperature for 2 h at 200 V. Radiolabeled DNA was visualized by autoradiography, using XAR-5 film and a Dupont Cronex enhancing screen. DNase I (20) and

FIG. 1. Expression of *S. purpuratus* L1 late H2B histone in *L. pictus* embryos. *L. pictus* one-cell zygotes (45) were injected with the wild-type L1 H2B gene (pSpL1), with a 5' deletion mutant (pSpL1H2BΔ5'-491), or with a 3' deletion mutant (pSpL1H2BΔ3'+535). Schematic maps of each of these L1 H2B DNA segments are shown at the top of panels A to C. We quantitated the amounts of L1 H2B mRNA and DNA in the injected embryos by RNase protection (30 embryos) and slot blot assays (15 embryos) after 10, 24, and 34 h of development. Autoradiograms showing the results of these assays are labeled L1 H2B mRNA and H2B DNA. The absolute quantities of L1 H2B mRNA and DNA were obtained by comparing the RNA or DNA signals from injected embryos with standards. The L1 H2B mRNA standard (A and B, lanes G) consisted of 0.5 μg of a preparation of *S. purpuratus* gastrula whole-embryo RNA previously assayed for L1 H2B mRNA content by RNA probe excess titration (28). The L1 H2B DNA standard was 10 to 300 pg of a plasmid consisting of a 1-kb *Bam*HI-*Bgl*II fragment containing the L1 H2B gene cloned in pUC. Samples shown in panels A and C were analyzed on the same gel; those in panel B were analyzed on a separate gel. Lane Bk, Noninjected embryos. Data from panels A to C are summarized graphically in panel D. Symbols: ■, pSpL1; ●, pSpL1H2BΔ5'-491; □, pSpL1Δ3'+535.

methylation interference (54) footprints were performed essentially as described by Heberlein et al. (23) and Hendrickson and Schlieff (25), respectively.

Calculation of transcription rate of the L1 late H2B gene. We calculated the rate of transcription of the L1 late H2B gene (see Fig. 7) from previously published measurements of the absolute number of L1 late H2B molecules at various stages of embryogenesis and the average half-life of late H2B mRNAs (28). The fundamental assumptions in this calculation are that (i) the L1 late H2B mRNA is synthesized with zero-order kinetics and decays with first-order kinetics and (ii) the half-life of the L1 H2B mRNA is similar to the 20-min average half-life of the four to five different late H2B mRNAs (each of which is a product of a distinct late H2B gene). The form of the calculation is described by Anderson and Lengyel (1).

RESULTS

A 3' element is required for the correct ontogenic activation of the L1 late H2B gene. As a first step toward locating the nucleotide sequence elements required for the ontogenic activation of the L1 late H2B gene at the late blastula stage, we asked whether an *S. purpuratus* L1 late H2B gene bearing 3 kb of 5'-flanking sequence and 5 kb of 3'-flanking sequence (Fig. 1A, pSpL1) was transcriptionally activated at

the late blastula stage after injection into *L. pictus* embryos. *L. pictus* was used as the host to provide a means of distinguishing between L1 H2B mRNAs transcribed from injected and endogenous late H2B genes (11). The timing of the early/late histone switch is very similar in *S. purpuratus* and *L. pictus* embryos (R. Maxson, unpublished observations).

We injected approximately 10^4 copies of linearized pSpL1 plasmid DNA into fertilized eggs of *L. pictus* and monitored L1 H2B gene expression during development by hybridization with a radiolabeled RNA probe spanning the 5' terminus of the L1 H2B mRNA (11). The amount of injected DNA in the host embryos was measured on slot blots by using the same probe. Since the half-life of late H2B mRNA is short (20 min) and does not change during embryogenesis (28), the level of late H2B mRNA transcribed from injected genes provides a good measure of transcriptional activity.

A representative RNase protection assay for the expression of pSpL1 in *L. pictus* embryos is shown in Fig. 1A. RNA from embryos injected with pSpL1 protected probe fragments of 61 and 64 nucleotides, as expected for the heterogeneous start of the L1 H2B transcript (49). RNA from noninjected embryos did not protect these fragments (Fig. 1A, lane Bk). Using L1 H2B DNA and L1 H2B mRNA standards, we measured the absolute amounts of L1 H2B genes and L1 H2B mRNA in embryos aged 10 and 24 h after

FIG. 2. Influence of 3'-flanking sequence on expression of the L1 late H2B gene in 10- and 24-h embryos. Linearized L1 late H2B gene constructs were injected into *L. pictus* embryos. The embryos were cultured, and L1 late H2B transcripts and genes were quantitated for Fig. 1. The plots show levels of L1 late H2B mRNA versus L1 late H2B DNA at 10 h (A) and 24 h (B) after fertilization. Symbols: ●, Constructs bearing the 5-kb *Bgl*/II fragment 3' of the gene; ○, constructs lacking this fragment.

fertilization. (The L1 H2B mRNA standard was a preparation of whole-cell RNA from 24-h embryos previously assayed for L1 H2B mRNA by probe excess titration [28].) Between 10 and 24 h, the quantity of L1 H2B mRNA increased 22-fold, from 3.9×10^4 to 8.4×10^5 molecules per embryo. However, the number of L1 H2B genes increased only sixfold, from 3.1×10^5 to 1.8×10^6 genes per embryo during this same interval. The ratio of L1 H2B transcripts to pSpL1 DNA molecules thus increased from 0.12 to 0.45 between 10 and 24 h (Fig. 1D). Expressing the transcriptional activity of the L1 H2B gene as a ratio of L1H2B mRNA to L1 H2B genes is justified because the quantity of L1 H2B DNA was not saturating in this experiment (see Fig. 2). These results suggest that the injected *S. purpuratus* L1 late H2B gene undergoes transcriptional activation during development as does the L1 H2B gene in *S. purpuratus* embryos. Such activation is not merely a general property of genes injected into sea urchin zygotes, since injected early H2B genes exhibit a peak of expression prior to the 10- to 12-h stage (11, 57).

Having demonstrated that the expression (transcripts per gene) of the L1 H2B gene in pSpL1 increases in late-stage host embryos, we tested the effect of gross deletions of L1 H2B flanking DNA on this increase. An L1 late H2B gene lacking 5'-flanking sequences to position -491 (designated pSpL1H2BΔ5'-491; Fig. 1B) was transcriptionally activated similarly to the L1 late H2B gene possessing 3 kb of 5'-flanking sequences (pSpL1; Fig. 1A): the ratio of L1 H2B transcripts to L1 H2B genes increased 4.2-fold between 10 and 24 h after fertilization. In contrast, the transcriptional activity (transcripts per gene) of a mutant L1 late H2B gene in which the 3'-flanking sequence was deleted to position +535 (designated pSpL1H2BΔ3'+535; Fig. 1C) did not increase between 10 and 24 h, suggesting that the deleted region contains a developmental activator element (Fig. 1D).

To further confirm the existence of an activator element 3' of the L1 H2B gene, we examined the relationship between the copy number of injected L1 late H2B genes and their level of expression. The level of L1 H2B expression should reach a saturation value as the level of L1 H2B genes increases, and this value should provide a measure of the transcriptional capability of a given construct. We performed such titration analyses on L1 H2B genes that contain the 3' element and mutant genes that do not. Figure 2 shows the relationship between the measured amounts of L1 late H2B mRNA and L1 late H2B DNA in injected embryos at 10

and 24 h of development. At 10 h, the level of expression of the control L1 H2B genes reached a maximum value of approximately 1.1×10^5 transcripts per embryo at 6×10^5 DNA molecules per embryo; higher levels of DNA up to 1.5×10^6 molecules per embryo did not produce a significant increase in L1 H2B mRNA levels. The mutant L1 H2B genes lacking the putative 3' activator element were expressed at a maximum level of approximately 30 to 40% of the wild-type value at 10 h. At 24 h of development, the wild-type constructs were expressed at a maximum level of 1.6×10^6 transcripts per embryo at 2×10^6 DNA molecules per embryo. The mutant constructs, in contrast, were expressed at 12 to 15% of the wild-type value; even very high levels of mutant DNA, up to 7×10^6 molecules per embryo, did not result in an increase in L1 H2B mRNA. The relative transcriptional activity of these L1 H2B constructs was the same at saturating amounts of DNA as at subsaturating amounts (Fig. 2 versus Fig. 1). The results presented in Fig. 1 and 2 show that the DNA sequence 3' of the L1 late H2B gene contains an activator element whose activity increases during development. We demonstrate below that this 3' activator element is not an artifact of altered RNA processing caused by deletion of processing signals but rather is an enhancer of L1 H2B transcription.

Location of the 3' activator element. To determine the approximate location of the L1 H2B activator element, we prepared a series of deletions in the 3'-flanking region and tested their expression relative to that of control pSpL1H2BΔ5'-491 DNA after injection into host embryos. Since the effect of deleting 3'-flanking sequences was greater at 24 h of development than at 10 h (approximately eightfold versus approximately threefold; Fig. 2), we assayed the mutant H2B genes in 24-h embryos. We measured the activity of the mutant DNAs at subsaturating DNA levels because it is sometimes difficult to inject enough DNA to achieve saturation without toxic effects on the embryos. Thus, we measured the amounts of both L1 H2B mRNA and L1 H2B DNA in injected embryos and used the ratio of L1 H2B RNA to L1 H2B DNA as a measure of the transcriptional activity of the mutants. To compare accurately the activities of the mutant genes, we chose experiments in which the amount of control and mutant DNA was 2×10^6 molecules per embryo or less; thus, the number of transcripts per gene is an accurate reflection of the transcriptional activity of the injected DNA. Furthermore, we se-

FIG. 3. Expression in 24-h embryos of L1 late H2B deletion mutants lacking segments of 3'-flanking sequence. (A) Diagrams of the mutant H2B genes. Using the assay described in the legend to Fig. 1, we compared the levels of expression (transcripts per gene) of five deletion mutant constructs with that of a wild-type L1 late H2B gene (pSpL1H2BΔ5'-491; Fig. 1B). (B) Summary of results ●, Average transcriptional activity of a set of four BAL 31 mutants whose breakpoints are indicated on the x axis relative to the mRNA start site of the L1 late H2B gene. (The H2B mRNA 3' terminus maps to position +445). Expression of the wild-type construct at 24 h postfertilization was arbitrarily set to 100%, and the expression of deletion mutants at 24 h was plotted relative to this value. □, Average transcriptional activity of an internal deletion mutant in which the *StuI* fragments between +1135 and +3900 were deleted (A, construct 6). The error bars represent 1 standard deviation ($n = 3$ or 4). The *HhaI-StuI* fragment (—) was used as a probe in studies on DNA-protein interactions in the activator region (Fig. 4 and 5).

lected experiments in which the amounts of control and mutant DNA were within a factor of 2 of each other.

Figure 3 shows a plot the relative levels of expression (transcripts per gene) of various 3' deletion constructs relative to the expression of the control construct at 24 h postfertilization. Little or no loss of activity occurred when sequences to +1204 were deleted. An internal deletion removing two *StuI* fragments located between +3900 and +1133 behaved substantially the same as the +1204 deletion. A significant (greater than 1 standard deviation) decrease in H2B gene expression, 8- to 10-fold in magnitude, was evident when sequences were deleted to +933. Further deletion of sequences to +533 resulted in only a slight (not

statistically significant) reduction of activity. Thus, the majority of the loss of H2B transcriptional activity occurred in the 200 bp between +933 and +1133 (489 to 689 bp downstream of the mRNA termination site). Sequence analysis of approximately 2 kb of DNA 3' of the L1 H2B gene has failed to provide any evidence of another gene (A. Zhao, M. Baker, and R. Maxson, unpublished observations), supporting the view that the 3' activator is not merely a part of a downstream promoter.

To locate the 3' activator element more precisely, we assumed that it would consist of one or more protein-binding sites, as do many enhancer elements in viruses and in genes expressed in terminally differentiated cells (52, 53). We

FIG. 4. DNase I protection analysis of the L1 late H2B enhancer region. Heparin agarose-enriched nuclear protein from 24-h embryos was incubated with a radiolabeled *HhaI-StuI* fragment that spans the H2B enhancer region. Following treatment with DNase I, the labeled DNA was electrophoresed on a 6% acrylamide-urea gel and visualized by autoradiography. G and G+A chemical cleavage reactions performed on each of the labeled DNAs were used as markers (40). The location of the protected region on each fragment is indicated by open boxes. The location of the footprint on the nucleotide sequence of the enhancer region is shown in Fig. 5C (bold type, underlines).

decided to map such sites and test their activities by mutational analysis *in vivo*. Gel mobility shift experiments with a 242-bp *HhaI-StuI* DNA fragment (coordinates +892 to +1133) encompassing the DNA region shown previously to contain the majority of enhancer activity (Fig. 3) revealed that this fragment formed one prominent complex with nuclear protein prepared from 24-h embryos (data not shown). This complex was not affected by a vast molar excess of a control nonhomologous DNA fragment but was reduced in amount by an excess of a nonradioactive homologous (*HhaI-StuI*) fragment; thus, it was the result of a specific DNA-protein interaction (data not shown).

We carried out DNase I protection and methylation interference footprint analyses to locate the factor-binding sites on the *HhaI-StuI* fragment (Fig. 4 and 5). We observed clear DNase I protection over the sequence GCTGCCAATCATATT (Fig. 4). The location of this sequence within the 242-bp *HhaI-StuI* fragment and the nucleotide sequence of

this fragment are shown in Fig. 5C. A methylation interference analysis of the major DNA-protein complex seen in band shift experiments showed that methylation of several G residues within the DNase I-protected region substantially reduced the binding of a factor to the DNA (Fig. 5A and C). The results of both DNase I protection and methylation interference analyses revealed that H2B enhancer-binding protein contacted the lower strand more closely than the upper (Fig. 4; Fig. 5A and B). The coincidence of the methylation interference and DNase I footprints demonstrates that the mobility shift and DNase I footprint assays detect the same factor. We designate this factor H2B activator-binding protein 1, or H2B abp 1.

Functional importance of the binding of H2B abp 1 to the activator element. To determine whether the H2B abp 1 site influences expression of the L1 late H2B gene *in vivo*, we prepared mutant L1 H2B genes in which various amounts of DNA bearing the site was either deleted or transferred to an

C

HhaI +900 Δ+933

CTTATGAAATCGTGAAGTAAGGTTAAAAACAGTTAACTCTATATTCCAAATTAAAGCTGCCAATCATA
GAATACCTTTAGCACTTCATTCCAATTTTTGTCAATTTGAGATATAAGGTTTAAATTCGACGGTTAGTAT

+1000 Δ Δ Δ

TTTGTAGAGAACTTTTTTACTGCCTTCAAGTTTAAACAACGCTCTAAGAATGATGGGAGATGTCTT
AAACTCTCTTTGAAAAATGACTTAAGTTCAATTTTGTTCAGATTCTTACTACCCCTCTACAGAA

TCTTATTGTGTATAAAGCTCTACAAAAAGGTTGTATTCTGATTTTGTCTATAGAAAGTTGGCAGG
AGAATAACACATATTTGAGATGTTTTTCCACACATAAAGACTAAAAACAGTATCTTCCAAACCGTCC

+1100 StuI

CCAGTGAGACATCTTCATATACGGCATTTCAAATTAGG
GGTCACTCTGTAGAAGTATATGCCGTAAGTTTAATCC

FIG. 5. (A and B) Methylation interference footprint analysis of the H2B activator region. Nuclear protein from 24-h embryos was incubated with a radiolabeled, methylated *HhaI*-*StuI* fragment that spans the enhancer element and electrophoresed on a 5% acrylamide gel. The major DNA-protein complex was electroeluted, and the DNA was cleaved with piperidine. The cleavage products were subjected to electrophoresis on an 8% polyacrylamide-7 M urea gel and visualized by autoradiography. We observed a methylation interference footprint only on the lower strand (open box). The corresponding region on the upper strand is indicated by a black box. (C) Nucleotide sequence of the enhancer region. Deletion analysis (Fig. 3) located the enhancer between the *StuI* site and the 3' breakpoint of the BAl 31 deletion construct Δ+933. The position of the DNase I footprint is shown in bold type with horizontal lines above and below. The bases whose methylation interfered with activator factor binding are indicated by triangles.

was inserted 5' of the L1 H2B gene (actΔ+921), transcription of the gene increased 10.2-fold, similar to the increase observed when the entire 358-bp activator fragment was inserted [compare the difference between lanes -act and +act (+888-+1245) with the difference between lanes -act and actΔ+921]. These results place the majority of the L1 H2B 3' activator element in the 50 bp between Δ+921 and Δ+972, a region that includes the H2B abp 1-binding site.

To confirm this finding, we replaced sequences 3' of the H2B abp 1-binding site with a 500-bp pBR fragment and deleted sequences 5' of the H2B abp 1-binding site to position +936. The resultant mutant DNA thus contained only 32 bp of the activator element, including the 17-bp footprinted region, in a location 222 bp further 5' than in the mutant L1 H2B-5' ACT. While the 500-bp pBR fragment had little effect on the expression of the L1 H2B gene (-act versus -act+pBR; Fig. 6D), the 32-bp H2B abp 1-binding site activated expression of the L1 H2B gene 15-fold relative to the -act control [compare lanes -act and act (+936-+967)]. Reversing the orientation of the 32-bp fragment had no discernible effect on its activity [-act+pBR versus act (+936-+967) reverse]. This experiment both confirms that a small DNA fragment that bears the H2B abp 1-binding site also includes the majority of the L1 H2B activator element and provides additional evidence that the L1 H2B activator is an enhancer.

Developmental timing of H2B abp 1 DNA-binding activity.

A simple model that explains how the 3' activator element causes the ontogenic increase in L1 H2B histone gene expression is that a positive-acting factor binds this element, and the concentration of this factor increases during development, resulting in an increase in L1 H2B gene expression. H2B abp 1 is clearly a candidate for such a factor. We tested the prediction that the DNA-binding activity of H2B abp 1 increases during development in parallel with the activity of the L1 H2B gene by means of a gel shift assay on nuclear extracts prepared from sea urchin embryos at different stages of development. Radiolabeled H2B abp 1 oligonucleotide formed a major and a minor complex with protein

L1 late H2B gene lacking the 3' activator element (Fig. 6A and C). To be certain that any potential effects of these mutations on L1 H2B expression were transcriptional, we performed the deletion analysis on a construct in which a 358-bp fragment bearing the activator element was placed 5' of the L1 H2B gene (Fig. 6A, L1H2B-5' ACT). These mutant genes were injected into zygotes, and their activities were measured in 24-h embryos.

Placing the 358-bp activator fragment 491 bp 5' of the L1 H2B gene resulted in a 10-fold increase in the L1 H2B RNA/L1 H2B DNA ratio [Fig. 6B, lanes -act and +act (+888-+1245)], demonstrating that the activator functions independently of position 5' or 3' of the gene and acts at a transcriptional level. Placement of a smaller segment lacking the H2B abp 1-binding site (Δ+972; Fig. 6A) in this same location in an insignificant (1.5-fold) increase in the L1 H2B RNA/L1H2B DNA ratio relative to that of the -act control (Fig. 6B). Thus, a deletion to position +972 eliminated virtually all of the activator element. When a fragment bearing an additional 50 bp of the 358-bp activator fragment

FIG. 6. Activity of portions of the L1 H2B 3' activator element when placed 5' of the L1 H2B gene. (A) Schematic map of the activator region showing deletion mutations. See Materials and Methods for a detailed description of the construction of mutant H2B genes. We used PCR amplification to prepare a 358-bp DNA fragment (coordinates +888 to +1245) spanning the 3' activator region. This fragment was cloned in the forward orientation in a *Hind*III site 5' of the L1 H2B gene (position -491) in the mutant pSpL1H2BΔ5'-491Δ3'+535 (Fig. 3A), which lacks the 3' activator element. The resultant construct is designated L1 H2B-5' ACT. We deleted DNA 5' of positions +921 and +972 by means of PCR. The location of the H2B abp 1 DNase I footprint (Fig. 4) is indicated by horizontal lines above and below the nucleotide sequence. (B) Autoradiograms showing expression of L1 H2B-5' ACT, Δ+921, and Δ+972 after injection into sea urchin zygotes. Injections were as described in the legend to Fig. 1. Amounts of L1 H2B mRNA and DNA in 24-h embryos were measured by RNase protection and DNA slot blots (see Materials and Methods). Lane Bk, Noninjected control embryos. Other lanes show analyses of mutant DNAs depicted in panel A. (C) Mutant constructs. We deleted DNA 5' of +936 and replaced 278 bp of DNA 3' of +967 with a 0.5-kb *Hinf*I fragment derived from pBR322, creating mutant act (+936-+967), which bears only 32 bp of the 3' activator. Construct act (+936-+967) reverse is identical to act (+936-+967) except that the 32-bp H2B abp 1-binding site is in the opposite orientation. Construct -act+pBR contains the 0.5-kb pBR fragment but lacks the H2B activator 32-bp fragment. (D) Autoradiograms showing RNase protection and DNA slot blot analysis of -act (pSpL1H2BΔ5'-491Δ3'+535; A), act (+936-+967), and act (+936-+967) reverse after injection into sea urchin zygotes. Mutant DNAs depicted in panel C were injected into 150 zygotes, and their expression was analyzed as described for panel B.

extracts prepared from 24-h embryos (Fig. 7). To confirm that the H2B abp 1 binds specifically to this radiolabeled oligonucleotide, we compared the ability of nonradiolabeled, homologous and heterologous oligonucleotides to compete with the radiolabeled oligonucleotide for binding. We chose an oligonucleotide bearing the CCAAT element of the 5'-flanking region of the early H2B gene as the heterologous competitor because the H2B abp 1-binding site contains a sequence resembling a canonical CCAAT element (9) (Fig. 5C) and we wished to test the possibility that a CCAAT factor with wide binding specificity interacts with the H2B enhancer. While small amounts of the homologous oligonucleotide greatly reduced binding, the heterologous (early H2B CCAAT) oligonucleotide had no discernible effect on binding (Fig. 7A). The early H2B CCAAT oligonucleotide was capable of binding protein in the 24-h extract (Fig. 7B); thus, its failure to compete with the labeled H2B enhancer-binding site for protein binding is not an intrinsic property. These results show that (i) a factor(s) binds specifically to a 35-bp oligonucleotide containing a DNA sequence defined by footprinting to be an H2B abp 1-binding site; (ii) H2B abp 1 may be heterogeneous, since two complexes are evident in the mobility shift assay; and (iii) H2B abp 1 is distinct from a factor that binds the CCAAT motif of the 5'-flanking region of the early H2B gene.

We tested extracts prepared from embryos aged 9, 18, and 24 h for enhancer-binding activity. Such activity was

scarcely detectable in the 9-h extract, increased substantially in the 18-h extract, and then declined in the 24-h extract (Fig. 7B and C). This temporal pattern of binding activity is probably not a general property of our nuclear extracts, since an oligonucleotide bearing the CCAAT element of the early H2B gene bound protein in the 9-h extract as well as the 18-h extract (Fig. 7B). Furthermore, using the identical procedure to prepare nuclear extracts, Calzone et al. (5) identified several factors that bind the sea urchin *CyIIIa* actin gene and have distinct temporal patterns during development. When we normalized the amount of H2B abp 1 DNA-binding activity to the quantity of embryonic DNA and compared these normalized DNA-binding activities with estimates of the transcription rate of the L1 late H2B gene at 9, 18, and 24 h of development (Fig. 7C), we found that H2B abp 1 DNA-binding activity per nucleus increased ninefold between 9 and 18 h of development, closely paralleling the changes in the transcription rate per gene of the L1 late H2B histone gene in *S. purpuratus* embryos. These results strongly suggest that the binding of H2B abp 1 to the L1 late H2B enhancer region plays a role in the transcriptional activation of the L1 late H2B gene in late-blastula-stage embryos.

DISCUSSION

Using the expression of microinjected late H2B genes in *L. pictus* embryos as an assay for *cis*-regulatory sequences,

FIG. 7. Developmental regulation of H2B abp 1. A 35-bp double-stranded oligonucleotide bearing the H2B abp 1-binding site was labeled at its 3' termini and incubated with nuclear extract from 24-h embryos. The resultant protein-DNA complexes were subjected to electrophoresis on 5% acrylamide gels and visualized by autoradiography. To assess the specificity of protein binding to this oligonucleotide, we compared the competitive ability of a nonlabeled, homologous, double-stranded oligonucleotide with that of an oligonucleotide bearing a CCAAT element present in the 5'-flanking region of the early H2B histone gene (A). The molar excess of these competitor DNAs over the amount of labeled oligonucleotide is indicated. To examine the developmental timing of H2B abp 1-binding activity, we performed gel shift assays on nuclear extracts prepared from 9-, 18-, and 24-h embryos (B). The probes were either the H2B abp 1 oligonucleotide or the early H2B CCAAT oligonucleotide. The protein-DNA complexes (B) were scanned with a densitometer and normalized to the number of nuclei from which the extract was derived. We plot the relative DNA-binding activity per nucleus at 9, 18, and 24 h of development with the relative transcription rate per gene of the L1 late H2B gene (C). The transcription rate of the L1 late H2B gene was calculated from the rate of accumulation and half-life of the L1 late H2B mRNA (28) as described in Materials and Methods.

we found that a DNA segment located 3' of the L1 H2B gene is necessary for the ontogenic increase in L1 late H2B gene expression. Quantitative measurements of L1 late H2B DNA and mRNA in injected 10- or 24-h embryos showed that the amount of L1 H2B transcript is directly proportional to the amount of L1 H2B DNA at low levels of DNA and reaches a saturation value at higher levels. This saturation

value is substantially higher in late-stage than in early-stage embryos. That L1 H2B gene expression is saturable with increasing amounts of L1 H2B DNA and that deletion of the activator region causes a decrease in the L1 H2B DNA concentration at which maximal L1 H2B expression is reached are consistent with the view that positive-acting factors interact with the L1 H2B activator region and the amounts of these factors increase during development, resulting in the ontogenic increase in L1 H2B gene expression.

Footprinting experiments showed that a nuclear factor, designated H2B abp 1, binds a 15- to 20-bp sequence in the activator region, and deletion of a 32-bp DNA segment containing this region reduced expression of the L1 H2B gene to a level comparable to that observed when the majority of the 3'-flanking sequence was removed. These results demonstrate that most if not all of the transcription-stimulatory activity of the 3' activator element is contained in the 32-bp fragment that also contains the H2B abp 1-binding site. Indeed, our mutagenesis experiments provide no evidence for transcription effector elements in the 3'-flanking region of the L1 H2B gene other than the 32-bp segment containing the H2B abp 1-binding site. Also supporting a role for the H2B abp 1-binding site in L1 H2B expression are competition experiments demonstrating that a 35-bp oligonucleotide bearing this site can compete *in vivo* for a positive-acting effector of L1 H2B transcription (data not shown).

The L1 H2B abp 1-binding site functions when placed 5' of the L1 H2B gene, even when 500 bp of pBR sequence is interposed between the H2B abp 1 site and the L1 H2B 5'-flanking sequence. Moreover, it works equally well in either orientation; thus, it is a transcriptional enhancer. That the L1 H2B 3' enhancer element seemingly consists of only a single protein-binding site makes it unusual among enhancers, which generally comprise several protein-binding DNA elements (34, 50, 58). Since H2B abp 1 apparently functions without the cooperation of other adjacently bound proteins, it is likely to be a strong activator of transcription. Although we do not yet know which promoter elements the 3' enhancer interacts with, higher-resolution deletion analysis of the promoter has shown that proper regulation of the L1 H2B gene requires at most 170 bp of 5'-flanking sequence, a region that includes octamer and CCAAT elements (A. Zhao and R. Maxson, unpublished observations) in addition to the TATA box.

A survey of a large number of factor-binding sites revealed that the H2B abp 1 target sequence closely resembles several binding sites recognized by both fushi tarazu (*ftz*) and engrailed (*en*) proteins of *Drosophila melanogaster* (14) (Fig. 8). *en* is the prototype of the engrailed class of homeobox genes, and *ftz* is a member of antennapedia class. Both classes of genes encode homeodomain proteins that recognize a highly degenerate set of sequences having the loose consensus TCAATTAAAT, and both classes include members that are expressed in the early *Drosophila* zygote (6, 17, 22, 35, 60). At least one of these, the product of the *ftz* gene, is known to function as a transcriptional enhancer (29, 61). The similarity of the H2B abp 1 to several *en/ftz*-binding sites (Fig. 8) suggested to us that H2B abp 1 may be a member of the antennapedia or engrailed class of proteins.

Consistent with this conjecture, we have demonstrated recently that both the *Drosophila ftz* protein and a sea urchin antennapedia class homeodomain protein known as Tghbox4 (16) are able to bind with high affinity to the H2B abp 1 target sequence (A. Zhao, G. Vansant, T. Humphreys, and R. Maxson, submitted for publication). Moreover, an antibody

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H2B abp 1 AGCTGCCAATCATATATTTTG
          * * * * *
EN6       AGTCGCTAATTAGAGAG TTG
          * * * * *
EN5       CGTTGCCAATTAGCCG
          * * * * *
EN4       GCGGTCGAATTAAATTAT
          * * * * *
ENVIR5    AACTGCCAATTAGCTA
          * * * * *
EN1"      AAATATCAATCAATTTCGTC

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FIG. 8. Comparison of nucleotide sequences of the L1 H2B abp 1-binding site with *ftz* and *en* sites. Symbols: *, matches; ', nucleotide transitions. The *ftz/en*-binding sites were taken from Desplan et al. (14).

directed against the sea urchin Tghbox4 protein prevents binding of H2B abp 1 to its target sequence but does not prevent binding of *ftz*, strongly suggesting that H2B abp 1 is an antennapedia class homeodomain protein closely related to Tghbox4. Curiously, a portion of the H2B abp 1 site (GC CAATCA) also resembles a canonical CCAAT element (ACCAATCA [9]). However, neither a CCAAT element from the promoter of the *S. purpuratus* early H2B gene (ACCAATGA) nor one from the L1 late H2B gene (AC CAATCA) is able to compete with the activator 35-mer fragment for binding of H2B abp 1 (Fig. 7; A. Zhao, and R. Maxson, unpublished observations). Thus, H2B abp 1 is distinct from the factors that bind CCAAT elements in early and late H2B promoters, and its binding specificity must depend, at least in part, on sequences flanking the CCAATCA motif.

Are all late histone genes regulated by a common mechanism involving H2B abp 1? We think not. Barberis et al. have identified a unique factor designated TSAP that binds a degenerate element in the 5'-flanking region of the tissue-specific late variants H2A-2 and H2B-2 (4). The H2A-2 and H2B-2 genes are expressed in gastrula-stage and older embryos and exclusively in tube feet in the adult, coincident with the DNA-binding activity of TSAP. The correspondence of TSAP DNA-binding activity and H2A-2 and H2B-2 expression suggests that TSAP has a role in the regulation of these genes. Although TSAP may also participate in the regulation of L1 somatic late H2B gene expression (it could, for example, act in conjunction with H2B abp 1), three observations suggest that this is not likely to be the case: (i) we have been unable to identify TSAP-binding sites anywhere in the 5'- or 3'-flanking sequence of the L1 late H2B gene; (ii) the developmental profile shared by the H2A-2 and H2B-2 genes is distinct from that of the L1 late H2B gene (32); and (iii) the L1 late H2B gene is expressed in all adult tissues, whereas TSAP is tube foot specific. The apparent lack of TSAP-binding sites in the L1 H2B gene as well as the contrasting patterns of expression of the tissue-specific and somatic late histone variants suggest that distinct mechanisms account for the regulation of these two classes of genes.

Furthermore, our data suggest that even among the somatic late histone genes there is a diversity of temporal control mechanisms. Lai et al. have characterized an enhancer element responsible for the stage-specific activation of a late H1 histone gene of *S. purpuratus* (36, 37). We have found recently that the H2B abp 1 protein does not bind this element, contrary to the hypothesis that H2B abp 1 is a common regulator of somatic late histone genes. It appears,

rather, that evolution has produced at least three distinct genetic mechanisms, one causing selective expression of tissue-specific late histone genes in late-stage embryos and specific adult tissues, a second activating somatic late H1 genes in late-stage embryos, and a third activating somatic late H2B genes. This panoply of temporal controls shows that mechanisms conferring "lateness" in histone gene expression have arisen independently several times during the course of evolution, though probably in response to the same selection pressure, as yet unknown.

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