Gene Expression, Vol. 7, pp. 133–147, 1998
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1052-2166/98 $10.00 + .00
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Multiple SSAP Binding Sites Constitute the Stage-Specific Enhancer of the Sea Urchin Late *H1*β Gene

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The sea urchin late histone *H1* genes are expressed at low levels up until mid-blastula stage of development when an enhancer element activates transcription to higher levels. Stage-specific activator protein (SSAP) was previously identified as the transcription factor that binds to a sequence motif within the late *H1*-specific enhancer, USE IV, and mediates this stage-specific activation. However, another conserved late *H1*-specific element, USE III, was also shown to contribute to the activated expression of the late *H1* genes. To attain a better understanding of the mechanism of blastula stage activation an extended analysis of the late *H1*-specific DNA sequences of the *SpH1*β gene was performed. Our findings indicate that this region, located between positions −320 and −200, consists of three SSAP binding sites, USE IV, USE III, and another site located between the two, termed Site 2. Although SSAP binds to USE IV in vitro with 10–15-fold higher affinity than to either of the other two sites, multiple sites are necessary for activation. Multimers of either USE IV or USE III activate mid-blastula stage transcription to similar levels in the context of a functional *H1*β basal promoter, but not with a TATA box alone. In addition, multimers of USE IV activate expression of a reporter construct containing an early histone *H1* promoter at an embryonic stage when it is normally repressed. We propose a mechanism for mid-blastula activation of the late histone *H1* genes where SSAP binding sites activate expression, but require the presence of the *cis* sequences of the basal promoter to function.

SSAP binding sites   Sea urchin   Histone genes

DURING the embryonic period of the sea urchin life cycle distinct classes of histone genes are differentially expressed. Among them the early gene family encoding the α histone subtypes, and the late gene family encoding the β and γ subtypes are the best characterized. The distinct expression patterns of these two histone gene families provide an excellent model system for the study of mechanisms that determine temporal regulation.

The genes encoding the early subtypes are present in several hundred tandemly repeated units (40). Active transcription of early genes begins after fertilization and rises rapidly to a peak during the early blastula stage, then steadily declines resulting in repression at the gastrula stage (3,26,34,38,39,51).

In contrast, the members of the late histone gene family are fewer in number and are dispersed throughout the genome with 5–10 copies of each of the core histones (*H2A, H2B, H3, H4*) and only two copies of the late *H1* genes (4,25,26,29,30,41,53). Late histone genes begin to be transcribed during early cleavage stages, but their mRNA levels remain low until the mid-blastula stage, when they undergo a marked activation that increases their rate of transcription between 5- and 15-fold (27,41). Subsequently, they continue to be expressed throughout the remainder of embryogenesis, larval stages, and in actively dividing adult somatic cells (26,34).

Within the regulatory regions of both the early and late *H1* genes the highly conserved upstream sequence elements (USE) 0, I, and II are present in the same relative positions (28). Conservation of se-

Revision received March 31, 1998; revision accepted April 14, 1998.
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quences within these elements has persisted between highly divergent species of sea urchin (31,40). The USE I and USE II elements have also been conserved in *H1* genes of vertebrate species (19,21,47,52).

The late *H1*β and γ genes of the sea urchin, which are co-regulated during embryogenesis (30), contain an additional highly maintained stretch of homology located upstream of the USE II element. This late *H1*-specific region includes two other conserved sequence elements, USE III and USE IV (28–30). Previous studies of the *SpH1*β gene promoter, which included mutagenesis and deletion of the conserved elements, identified several essential sequences. The method of gene transfer by microinjection of cloned DNA into sea urchin eggs and zygotes has greatly facilitated promoter function studies for genes that are temporally as well as spatially regulated (6,15, 22,24,31,42,50). Microinjection of mutant promoter constructs revealed that the USE IV element was indispensable for the activated transcription of the gene at the mid-blastula stage, whereas USE 0 and USE I were requisite parts of the promoter, and together with the TATA box were driving the lower or basal levels of expression observed prior to mid-blastula (31). Concatenated USE IV oligonucleotide sequences conferred the mid-blastula stage activation to *CAT* reporter constructs. USE IV mutations mimic the low-level basal transcription pattern at all developmental stages seen using a −106 deletion construct (10,11,32). Importantly, point mutations in either USE 0 or USE I promoter elements eliminated enhancer activity (31), whereas deletion of either USE II or USE III reduced activated expression by 40%.

Stage-specific activator protein (SSAP) is the only sequence-specific USE IV binding activity found in sea urchin extracts (10,32). Its gene encodes a 44-kDa protein with a DNA binding domain that has homology to the RNA recognition motif (RRM). SSAP binds DNA with high affinity in a sequence-specific manner, but has very low affinity for RNA (11). In addition, SSAP also contains a potent transcriptional activation domain (11,13). SSAP, although present at all developmental stages, undergoes a change in state from monomer to dimer at about the same time in development that the *H1*β gene is activated (10). Evidence that the protein was directly involved in the activation of the *H1*β gene came from experiments in which microinjected SSAP mRNA transactivated co-injected USE IV containing target genes but only at and subsequent to the mid-blastula stage (11).

The high evolutionary conservation of the USE III element among different sea urchin species, and the reduced expression of the *H1*β gene construct that is observed when USE III is deleted, suggest a role for this element in the regulation of the late *H1* genes. In this report we examine the contributions of the individual enhancer elements in the activated expression of the *H1*β gene and establish the role of USE III as another SSAP binding site. In addition, we determine the specificity of binding to USE IV, USE III, and an additional sequence element bound by SSAP, located between these two elements, termed Site 2. The ability of USE III and USE IV to function independently and activate mid-blastula stage transcription in vivo is also assessed. Multimers of either sequence are functional as stage-specific enhancers, but they both require the elements of the *H1*β basal promoter (USE I, USE 0, and the TATA box). In addition, a hybrid early gene/late gene construct, comprised of the *H1*α promoter attached to *H1*β USE IV enhancer sequences, exhibits a four- to sixfold activation above its normal levels at the mid-blastula stage and remains on through the gastrula stage when the wild-type early *H1* promoter is normally repressed. We propose that the stage-specific activation of the late *H1* genes of the sea urchin hinges upon a dual mechanism, where multiple SSAP binding sites are necessary for maximal, temporal activation, and these sites, which constitute the enhancer, require the presence of *cis* sequences contained within histone *H1* promoters.

MATERIALS AND METHODS

Preparation of Nuclear Extracts

Nuclear extract preparations were made from synchronously growing populations of *S. purpuratus* (Marinus, Inc.) embryos. Embryos were grown at 16°C to the desired developmental stage as previously described (15). Crude nuclear extracts were prepared essentially by the method of Morris et al. (45) and exactly as described by Diliberto et al. (15).

Purification of SSAP

SSAP monomer was purified from 12-h nuclear extracts on a Sephacryl S300 column, followed by three passes over a USE IV–DNA affinity column as described (10). The SSAP preparation used in these experiments consisted mostly of monomer activity, but contained dimer activity as well.

Mobility Shift Assays

Mobility shift assays were performed according to methods previously described (10,15). The probes used in the assays were double-stranded oligomers containing either USE III or USE IV and were 31 and 27 bp in length, respectively. Oligomers were

double end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. Approximately 2 fmol of DNA probe was added per reaction, and either 1 μg or 50 ng of poly-(dI-dC) was included as nonspecific competitor with nuclear extracts or purified SSAP, respectively. In the competition assays 50 ng (1200-fold molar excess) of unlabeled competitor was added prior to preincubation with nuclear extract. All double-stranded oligomers were generated by annealing the complementary strands shown in Fig. 1, followed by polyacrylamide gel purification. Relative affinity studies were conducted over a range of competitor concentrations from 1.3 ng to 30 μg.

The binding reactions for the relative affinity mobility shifts were all performed using 2 fmol of USE IV probe, 12 μg of 24-h nuclear extract, and 1 μg of poly(dI-dC). For the list of double-stranded oligonucleotide competitors see Fig. 1. The measurements of percent relative binding were done with an LKB laser-densitometer using preflashed autoradiographic film.

Binding reactions with anti-SSAP antisera were incubated for 2 h with 3 μl of preimmune or immune sera, binding buffer, and 10 μg of nuclear extract. Poly(dI-dC) (1 μg) as nonspecific competitor and [γ-^{32}P]ATP end-labeled probe were added to the antisera incubations on ice for an additional 30 min. The generation of anti-SSAP antiserum was performed as described by DeAngelo et al. (9).

DNase I Footprinting

Plasmid pGC375 was created to provide the probe for DNase I footprinting. A 220-bp *Eco*RI, *Xmn*I fragment from M13 deletion construct, *-372H1β* (31), was ligated into the *Eco*RI, *Hinc*II sites in the polylinker of pBluescriptIISK(+). pGC375 (10 μg) was digested with *Bam*HI, and the noncoding strand was end-labeled using Klenow DNA polymerase and [α-^{32}P]dGTP. The labeled 230-bp fragment was released by *Xho*I digestion and purified by polyacrylamide gel electrophoresis.

Approximately 2 fmol of labeled probe (8000 cpm) was incubated with 60–100 μg of 12-h nuclear extract or 50 ng of purified SSAP and reactions were carried out under the conditions described (15).

H1β *Enhancer and* H1α *Enhancer Constructs*

Oligomers containing one or three copies of either USE III, USE IV, or mutant USE IV sequences as shown in Fig. 1 were ligated into the *Eco*RI site of *H1β* promoter deletion constructs –40 or –106 (31). The original plasmids were derived from 5′ promoter deletions of the *Sph1β* gene ligated into the *Sal*I site of M13mp19 (30). The wild-type *H1αLuciferase* construct, p162/163, was pXP2 based (46) and generated as described (16). The corresponding *H1α*-USE IV enhancer-containing construct, p162/163(USEIVx3), was derived by subcloning a 123-bp *Bam*HI fragment containing three copies of USE IV from the plasmid, pGC355A (11), into the *Bam*HI site just upstream of the *H1α* gene in the polylinker of pXP2. The identity and orientation of inserts in all constructs was confirmed by DNA sequencing using the Sequenase version 2.0 kit (U.S. Biochemical) and CsCl purified DNA.

Microinjection of the Sea Urchin Fertilized Eggs

Lytechinus pictus fertilized eggs were injected according to the procedures of McMahon et al. (43) and Colin (5) and exactly as described (31). In the case of the M13 derivatives all plasmids were linearized at the *Sal*I site downstream of the *H1β* gene. In order to internally control for expression, all constructs were coinjected with a *CAT* reporter construct containing the basal promoter of the *H1β* gene, –106*CAT* (pGC355) (11). Both –40 and –106 derivatives were injected at concentrations of 30 μg/ml and co-injected with either 30 or 90 μg/ml of internal *CAT* control plasmid, respectively. *Luciferase* constructs were linearized downstream of the *Luciferase* gene by *Bsp*I digestion and injected at concentrations of 30 μg/ml. In addition 30 μg/ml of the plasmid, pGC355A (11), was co-injected with the *Luciferase* constructs as an internal control for expression. DNA titrations were previously performed with injected M13, *CAT*, and *Luciferase* constructs, so that the concentrations of DNA are saturating with respect to their levels of expression. Expression levels of all injected constructs were normalized with respect to expression of the co-injected control constructs.

Preparation of RNA and DNA From Embryos

Typically, 250 embryos were collected from either 10- or 20-h injected or control samples. The extraction of RNA from these samples was performed as described (31). Aliquots of 50 embryo equivalents were removed from ethanol for analysis in RNase protection assays.

DNA from 20 injected or control embryos was prepared and applied to nitrocellulose using a Minifold II apparatus (Schleicher and Schuell) as previously described (17,31). Either M13mp19 or pXP2 was used as probe in slot blot hybridizations.

RNase Protection Assays

The methods and materials for preparation and gel purification of RNA probes, hybridization conditions,

RNase digestion, and analysis of protected fragments were obtained from Ambion Corp. using the T3/Sp6 Maxiscript and RPAII kits. RNA probes were labeled with [α-^{32}P]GTP. Fragments were analyzed on 6% polyacrylamide, 7 M urea gels using Long Ranger acrylamide solution (FMC Bioproducts). The *SpH1β* gene antisense probe was synthesized from a pSp64 derived plasmid (31), linearized by *Sac*II digestion to produce a 370-base probe that protected a 317-base RNA fragment. The antisense *CAT* probe, which was 317 bases, protected an RNA fragment of 257 bases and was generated from the T3 promoter of the *Sal*I linearized plasmid Bsc*CAT* (generously provided by Dr. Andrew Ransick). The activity of the injected genes was quantitated using PhosphorImager analysis (Molecular Dynamics).

Luciferase *Assays*

Injected or control embryos were collected in duplicate for each experiment and immediately assayed for luciferase activity. The expression of the *Luciferase* gene was measured by enzymatic activity (14) and as described (16). One hundred embryos were collected, pelleted, and resuspended gently in 100 μl Buffer I (25 mM glycyl-glycine, pH 7.8, 15 mM magnesium sulfate, 4 mM EGTA) containing 1 mM dithiothreitol (DTT), 1% Triton X-100, and 1% bovine serum albumin (BSA). The lysed cells were microfuged at 4°C for 5 min at high speed to pellet the cellular debris. The supernatant was then transferred and added to 360 ml of Buffer II (Buffer I, 15 mM potassium phosphate, pH 7.8) containing 1 mM DTT and 2 mM ATP. Separately, 4 ml of Buffer I containing 2 mM DTT and 0.2 mM D-luciferin was prepared as substrate. Light output from each sample was measured in 40-s intervals with a luminescence photometer.

Chloramphenicol Acetyl Transferase (CAT) Assays

Thirty injected embryos were collected in duplicate at 16 and 40 h postfertilization. Embryos were washed in 250 mM Tris-HCl (pH 7.5) and resuspended in 100 μl of the same buffer. Following three freeze–thaw cycles (43), *CAT* enzyme activity was assayed as described (20). *CAT* activity was quantitated by PhosphorImager analysis (Molecular Dynamics).

RESULTS

Characterization of USE III Binding Activities

The USE III sequence element is highly conserved within the late gene-specific regions of the late *H1β* and γ genes in both *S. purpuratus* and *L. pictus* (28–30), and mutations in this element result in approximate twofold decreases in transcriptional activity (31). These results suggested that this *cis*-regulatory element is a recognition sequence for a transcription factor. We examined the developmental profile of factor(s) binding to the USE III sequence with a series of staged nuclear extracts (15), using a double-stranded 31 mer (Fig. 1) containing the conserved region of USE III as the probe in a mobility shift assay. With cleavage stage extracts from 8–16 cell (4 h), and 64 cell (8 h) as well as early blastula stage extracts (12 h), one major complex and a few minor complexes were formed with the USE III probe (Fig. 2A). The major complex (arrow 1) and a minor complex (arrow 2) were also present with extracts from hatching (17 h) and mesenchyme (24 h) blastula stages, but in addition a third, slower migrating complex (arrow 3) appeared, which was the major USE III binding activity in 24-h extracts.

The distinct pattern of factors recognizing the USE III probe in Fig. 2A was strikingly similar to the previously characterized pattern of SSAP binding to USE IV (10,32). Previous sequence comparisons of the conserved regions of USE III and USE IV yielded no obvious extended homologies between these two elements (28,31). To determine whether USE III is bound by the same protein as USE IV, a mobility shift competition assay was performed. In Fig. 2B double-stranded, USE IV oligomer (Fig. 1) was used as a probe and incubated with 24-h extracts in binding reactions using 50 ng (1200-fold molar excess) of different double-stranded competitors (Fig. 1). In lane 1, which contains no competitor, three distinct complexes were observed. The two faster migrating species corresponded to the 44-kDa SSAP monomer bound to the labeled probe (10), and the slower migrating complex was the bound SSAP homodimer (12). All three complexes were eliminated and/or supershifted in binding assays by addition of polyclonal anti-SSAP antisera to the reactions (10). The different mobilities of the two species of monomer in mobility shift assays are presumably due to posttranslational modification by phosphorylation (Z. Li and G. Childs, unpublished data). Both USE IV and USE III specifically competed (lanes 2 and 5) all three SSAP complexes, whereas the *H1* nonspecific (ns) and the H2b (ns) sequences did not (lanes 3 and 4).

To directly examine the identity of the factor(s) binding to USE III from nuclear extract preparations, polyclonal anti-SSAP antisera generated against purified SSAP (9) was added to binding reactions. The mobility supershift assay was performed with 24-h nuclear extract as shown in Fig. 3C. The 24-h extract (10 μg) was preincubated without antisera (–), with anti-SSAP antisera (I), or with preimmune sera (PI)

USE III: 5'(+) AATTTTGAAGCTTGAAAGGTTCTCTGTCTAA
AACTTCGAAC TTTCCAAGAGACAGATTTTAA 5'

USE IV: 5'(+) AATTCAGAATCAGATTTAAAACTTGTC
GTCTTAGTCT AAATTTTGAACAGTTAA 5'

H1(ns): 5'(+) GATCCTAAGAGAAGTTTGA
ATTCTCTTCAAACTCTAG 5'

H2B(ns) : 5'(+) GATCCCAAATTAAGCTGCCAATCATATTTTGA
GGTTTAATTCGACGGTTAGTATAAAACTCTAG 5'

Site 2: 5'(+) AATTAATACTTTATTAATTATAAAGAA
TTATGAAATAATTAATATTTCTTTTAA 5'

USE IIIx3:
5'(+) AATTCTGAAGCTTGAAAGGTTCTCTGTCTAAAATTTTGAAGCTTGAAAG
GTTCTCTGTCTAAAATTTTGAAGCTTGAAAGGTTCTCTGTCTA

5'(-) AATTCTAGACAGAGAACCTTTCAAGCTTCAAAATTTTAGACAGAGAACC
TTTCAAGCTTCAAAATTTTAGACAGAGAACCTTTCAAGCTTCAG

USE IVx3:
5'(+) AATTCACAAGTTTTAAATCTGATTCTGAATTGACAAGTTTTAAATAATC
TGATTCTGAATTGACAAGTTTTAAATCTGATTCTG

5'(-) AATTCAGAATCAGATTTAAAACTTGTCAATTCAGAATCAGATTTAAAAC
TTGTCAATTCAGAATCAGATTTAAAACTTGTG

Mutant(USE IV)x3: Eco RI ends
5'(+) AATTCTGAAGCTGGCCAGGTTCTCTGTCTAAAATTTTGAAGCTGGCCAG
GTTCTCTGTCTAAAATTTTGAAGCTGGCCAGGTTCTCTGTCTAG

5'(-) AATTCTAGACAGAGAACCTGGCCAGCTTCAAAATTTTAGACAGAGAACC
TGGCCAGCTTCAAAATTTTAGACAGAGAACCTGGCCAGCTTCAG

Mutant(USE IV)x3: Bam HI ends
5'(+) AGCTCTGAAGCTGGCCAGGTTCTCTGTCTAAAATTTTGAAGCTGGCCAG
GTTCTCTGTCTAAAATTTTGAAGCTGGCCAGGTTCTCTGTCTAG

5'(-) AGCTCTAGACAGAGAACCTGGCCAGCTTCAAAATTTTAGACAGAGAACC
TGGCCAGCTTCAAAATTTTAGACAGAGAACCTGGCCAGCTTCAG

FIG. 1. Double-stranded oligomers.

before adding either USE III or USE IV probes. With both the USE III and USE IV probes the SSAP dimer complex is present in control lanes (−) and PI as indicated by the arrow. However, in the lanes with immune sera (I) the dimer complex was recognized by the SSAP antibodies and a supershifted slower migrating complex resulted, which is also indicated by an arrow. Taken together the results of Fig. 2 indicated that SSAP also bound the USE III sequence and that it was the major protein from crude extracts recognizing the binding site.

Examination of SSAP Binding to USE III

The availability of authentic, affinity column-purified SSAP from blastula stage nuclear extracts (10) allowed us to directly test the possibility that USE III was an SSAP binding site. Figure 3A shows a mobility shift assay with both the USE III (lanes 1, 2, and 3) and USE IV (lanes 4, 5, and 6) probes, side by side, incubated with 12-h extracts, 24-h extracts, and SSAP purified from 12-h nuclear extracts, respectively. Both probes were bound by SSAP monomer and dimer from the 12- and 24-h extracts, as indicated with arrows. The purified SSAP preparations used in this study consisted predominately of the two monomer species, but small quantities of the dimer were also present (9). Comparison of the mobility of the complexes formed with the nuclear extract to those formed with the purified factor clearly indicated that USE IV and USE III were both recognized by SSAP.

A DNase I footprinting assay was used to define the boundaries of USE III that were recognized by SSAP. The DNA binding site for SSAP within the USE IV sequence was previously mapped on both the coding and noncoding strands, to an AT-rich 9-bp protected segment, 5′-GTTTTAAAT-3′ (10,32). Here we generated a DNA fragment extending from −372 to −152, which contained all of the conserved late

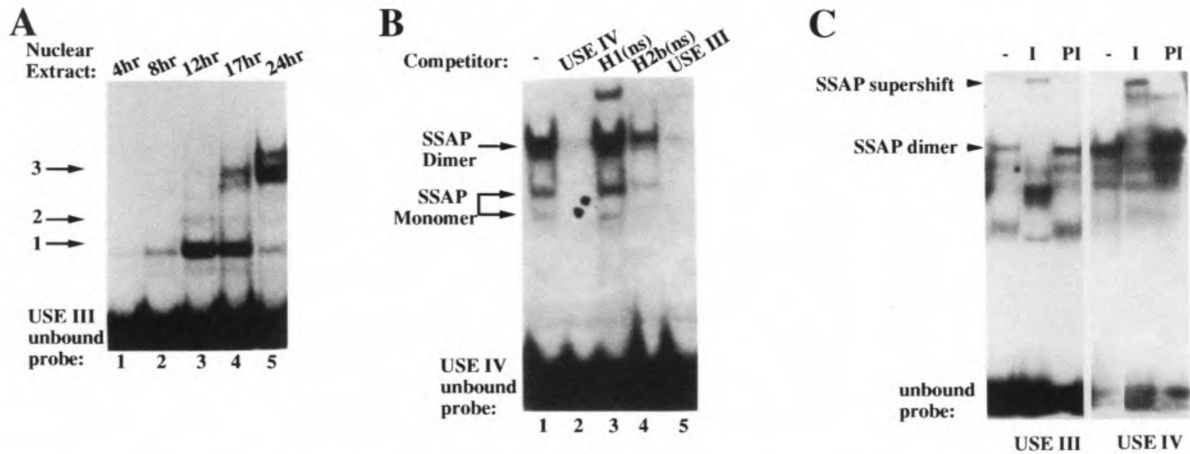


FIG. 2. Developmental profile of USE III binding activities. (A) Mobility shift analysis was performed using a [γ-^{32}P]ATP end-labeled, 31-bp, double-stranded, synthetic oligonucleotide containing the conserved sequences of USE III (Fig. 1) as a probe. Nuclear extract (NE, 10 μg) prepared from 4-h (16 cell), 8-h (64 cell), 12-h (early blastula), 17-h (hatching blastula), and 24-h (mesenchyme blastula), shown in lanes 1–5, respectively, were incubated with probe. Complexes formed are indicated by arrows 1, 2, and 3. (B) A [γ-^{32}P]ATP end-labeled, 27-bp, double-stranded USE IV oligonucleotide (Fig. 1) was used as a probe in a competition mobility shift analysis. The 12-h NE (10 μg) was incubated with USE IV probe and 50 ng of unlabeled, competitor oligonucleotides: lane 1, no competitor; lane 2, USE IV; lane 3, H1 nonspecific (ns); lane 4, H2b (ns); lane 5, USE III. SSAP monomer and dimer complexes, indicated by arrows, were previously characterized (10).

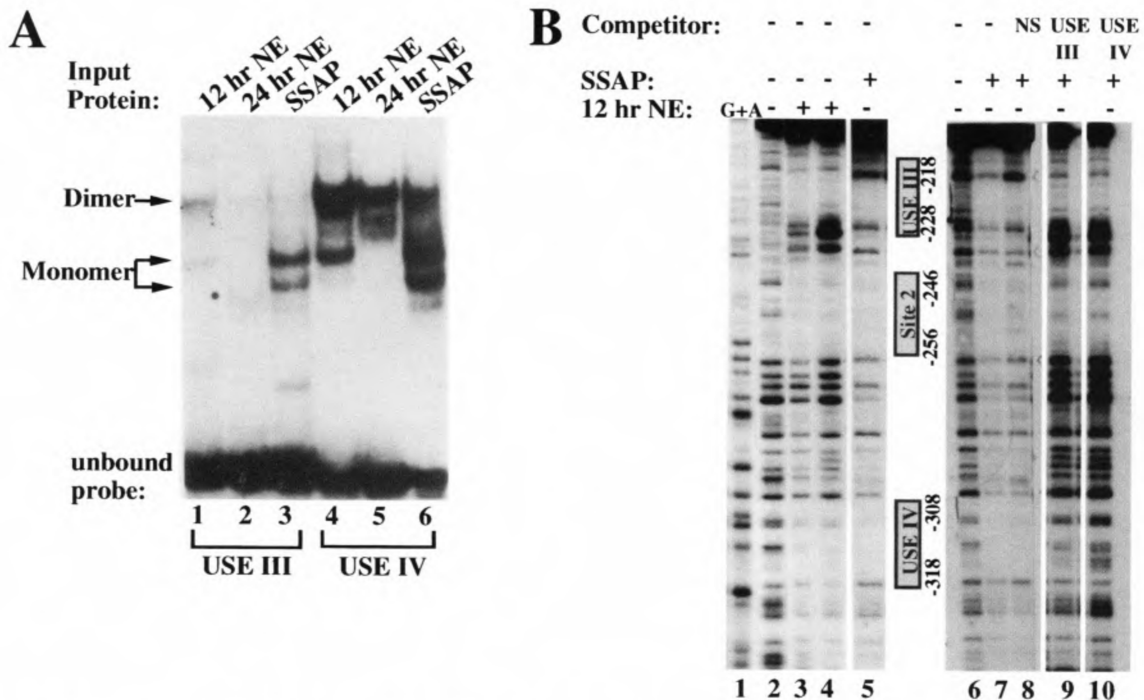


FIG. 3. Characterization of SSAP binding to USE III and USE IV. (A) Mobility shift analysis was performed with the USE III and USE IV probes, identical to those used in Fig. 2. Probes were incubated with 10 μg of 12-h nuclear extract (NE), 10 μg of 24-h NE, and 10 ng of SSAP purified from 12-h NE (USE III: lanes 1, 2, 3; USE IV: lanes 4, 5, 6). SSAP monomer and dimer complexes are indicated by the arrows. (B) DNAse I protection of the 230-bp probe on the noncoding strand incubated with 12-h nuclear extract (NE) and purified SSAP: lane 1, Maxam Gilbert G+A sequencing reactions used as a marker for lanes 2–10; lanes 2 and 6, DNAse I digestion of the probe in the absence of input protein; lanes 3 and 4, probe incubated with 60 and 100 μg of 12-h NE, respectively; lanes 5 and 7, probe incubated with 50 ng of purified SSAP; lanes 8, 9, and 10, probe incubated with 50 ng of purified SSAP and 100 ng of competitor oligonucleotides (Fig. 1) as indicated above the lanes: H1(ns), USE III, and USE IV, respectively. Protected areas are marked by gray rectangles: USE III, Site 2, and USE IV. (C) Mobility shift assay was performed with 24-h nuclear extract and USE III and USE IV [γ-^{32}P]ATP end-labeled probes. Extract was preincubated without antisera (–), with anti-SSAP antisera (I), or with preimmune sera (PI). Arrows at the left of the figure refer to the SSAP dimer and SSAP supershifted antibody complexes.

gene-specific sequences including USE IV and USE III, to assay DNase I protection on the noncoding strand. Three protected areas were detected when 12-h nuclear extract was the source of input protein corresponding to USE IV, USE III, and an additional site between the two, termed Site 2 (Fig. 3B, lanes 3 and 4). The extra site was also identified in DNase I footprints using a shorter probe (10). Incubation of authentic sequence-specific DNA–affinity column-purified SSAP with probe (lanes 5 and 7) yielded the same three cleavage-resistant sites that were found with 12-h nuclear extract. In addition, these three areas, recognized and protected from DNAse I cleavage (shown as gray rectangular areas) by SSAP in lane 7, were eliminated by the addition of 2400-fold molar excess of either USE III or USE IV oligonucleotide competitor (lanes 9 and 10, respectively), but not by nonspecific H1 competitor oligonucleotide (lane 8).

The bases protected in the USE III element and Site 2 were 5′-GCTTGAAAG-3′ and 5′-ATTATA AAG-3′, respectively. An alignment of the protected sequences of all three sites within the *SpH1*β gene and of the corresponding USE IV and USE III sequences from the *Lp* and *SpH1*γ genes is diagrammed in Fig. 4. Site 2 was not as well conserved between the β and γ genes as were the USE III and USE IV sites. From the sequences shown in Fig. 4, a consensus sequence for SSAP binding is inferred, which is a string of T residues followed by three invariant A residues, G/A T/A T T T/G A A A G.

Relative Affinity of SSAP for the Three Sites Within the H1β *Late Gene-Specific Region*

The sequence divergence within the three binding sites suggested that SSAP could recognize them with

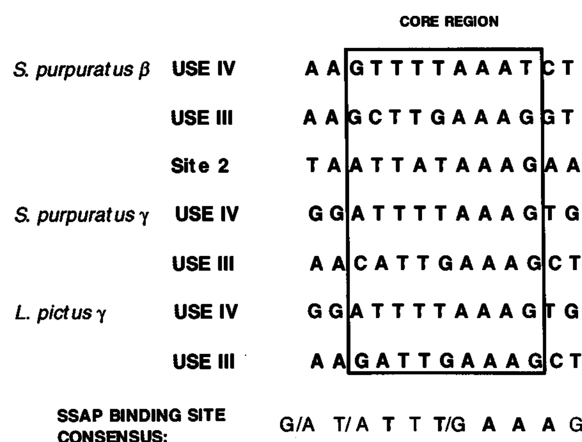


FIG. 4. Sequence alignment of the protected sequences of the identified SSAP binding sites of the *SpH1*β gene and the homologous sequences of the *SpH1*γ and *LpH1*γ genes. A 9-bp consensus sequence is inferred as shown at the bottom.

different affinities. In addition, qualitative comparison of the binding assays in Fig. 3B indicated that SSAP might not be binding the USE III probe with as high an affinity as the USE IV probe. The relative affinity of SSAP for specific and nonspecific DNA was determined by incubating 24-h nuclear extract with a ^{32}P-labeled, USE IV, 27 mer in the presence of increasing amounts of unlabeled specific and nonspecific competitor (Fig. 5B). The equilibrium dissociation constant, K_d, of SSAP binding to double-stranded USE IV was previously determined to be 3×10^{-9} M (11). The relative K_ds of the different competitor DNAs used in the binding assays should be proportional to the ratio of competitor to probe concentrations necessary to compete binding by 50% (48). Figure 5A shows the results of one of several mobility shift assays, where 2 fmol of USE IV probe was incubated with 10 μg of 24-h nuclear extracts and increasing amounts (1.3 ng to 1.3 μg) of unlabeled Site 2 as competitor DNA. The same assay was performed with the following double-stranded competitors; USE IV, USE III, poly(dA-dT) and poly(dI-dC). The 24-h extract preparation used in these assays contained predominantly SSAP dimer such that the monomer, if present, was undetectable.

SSAP bound to USE IV with approximately 10- to 15-fold higher affinity than either Site 2 or USE III. Both the SSAP monomer and dimer bind to USE IV with apparently the same dissociation constant as well as on and off rates (10). In Fig. 5B the amount of oligonucleotide necessary to compete 50% of specific binding is 9×10^{-9}, 1.3×10^{-7}, and 8×10^{-8} M for USE IV, USE III, and Site 2, respectively. As expected from the AT-rich nature of all the identified binding sites, SSAP had a slightly increased affinity, almost 10-fold higher, for the nonspecific sequences of poly(dA-dT) than for those of poly(dI-dC). However, the interactions of SSAP with the elements of the *H1*β late gene-specific region were between 100,000- and 5,000-fold stronger than those with nonspecific DNA sequences.

Assessment of USE III Function In Vivo

The in vitro binding data established that USE III was also a site where SSAP could specifically bind to DNA. However, the apparent lower affinity that SSAP exhibited for the USE III sequence raised questions regarding its capacity to function in vivo. A series of *H1*β promoter constructs was designed to test the functional potential of USE III and to directly compare it with that of USE IV. One or three copies of either USE III or USE IV was placed upstream of a –40 late *H1* TATA box or a –106 basal promoter construct (Fig. 6A). The TATA box construct is tran-

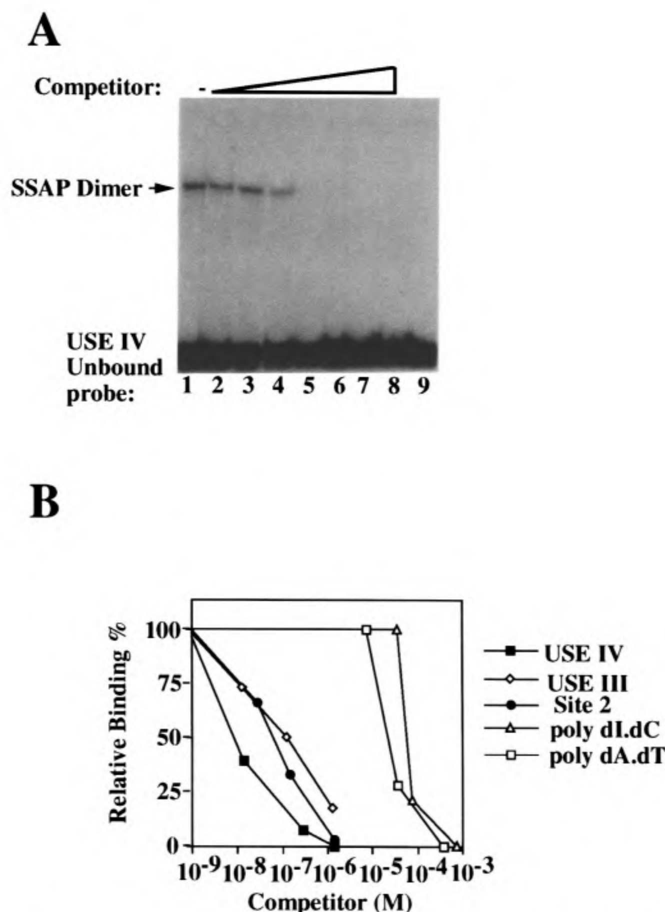


FIG. 5. Relative affinity of SSAP for specific and nonspecific DNA sequences. Mobility shift analysis was performed with a [γ-^{32}P]ATP end-labeled USE IV probe (identical to probe in Fig. 2B) incubated with 10 μg of 24-h nuclear extract (NE) in the presence of increasing amounts of competitor oligonucleotides. (A) Mobility shift assay with increasing amounts of Site 2: lane 1, no competitor; lane 2, 0.5 ng; lanes 3 and 5 ng; lanes 4 and 10 ng; lane 5, 50 ng; lane 6, 100 ng; lane 7, 250 ng; lane 8, 500 ng; lane 9, probe only. SSAP dimer complex is indicated with an arrow. (B) Graphed results of mobility shift assays with percent specific binding plotted against the molar amount of competitor used: USE IV (■), USE III (◇), Site 2 (●), poly(dA-dT) (□), poly(dI-dC) (△). 100% binding is assumed with 0 M competitor.

scriptionally inactive by itself with < 1% of wild-type promoter activity (31). The −106 construct has been characterized as the basal promoter because it constitutively drives the low-level or nonactivated expression of the *H1*β gene, which corresponds to approximately 20% of wild-type promoter activity (31).

Because the results of the microinjections were meant to be quantitative with respect to control plasmids, it was imperative that measurements could be normalized for expression levels. Therefore, a −106*CAT* construct was co-injected with all *H1*β constructs to provide an internal control for levels of expression. The promoter on the control plasmid is identical to the −106*H1*β construct used to test the activity of the USE III and USE IV sequences (11). Expression levels of injected plasmids were directly compared by RNase protection assays using both an *H1*β probe and a *CAT* probe (Materials and Methods).

For the −40 construct derivatives, 30 μg/ml of *H1*β experimental DNA and 30 μg/ml of −106*CAT* control DNA were coinjected into sibling zygotes. The experimental results of the 10-h (early blastula) embryo collection are graphed at the top of Fig. 6B. At this time point, which corresponds to the early blastula stage, activation by either USE IV or USE III was not expected because this precedes the activation of the late *H1* genes. The results indicated that none of the constructs containing SSAP binding sites showed any increase in activity over the −40 control. The activity of −40 was designated 0.5% of wild-type as measured in a separate experiment (data not shown) and the activity of the −40 derived constructs was calculated relative to the control. At the top of Fig. 6C are the graphed results and in Fig. 6E, lanes 2–6, an assay is shown for the same −40 constructs at 20 h postfertilization, which is the mesenchyme blastula stage, when the *H1*β gene is fully activated.

Again, there was no observed increase in activity for constructs containing USE III or USE IV. Neither of the SSAP binding sites was capable of activating transcription with only a homologous, variant TATA box.

The second set of constructs tested was the −106 derivatives (Fig. 6A). In these experiments the activities of all microinjected constructs were normalized with respect to the original −106 promoter construct injected into the same batch of embryos. The concentration of injected plasmids was 30 μg/ml for the −106 derivatives and 90 μg/ml for the −106*CAT* control construct. At 10 h postfertilization all the constructs exhibited basal levels of expression. The graphed data for these constructs, shown in Fig. 6B (bottom), indicate that at this time in development the −106 construct, designated as onefold activation, was driving the expression from all the constructs. The USE III and USE IV elements did not contribute to the activity of the artificial constructs or the authentic enhancer of the −469*H1*β wild-type construct.

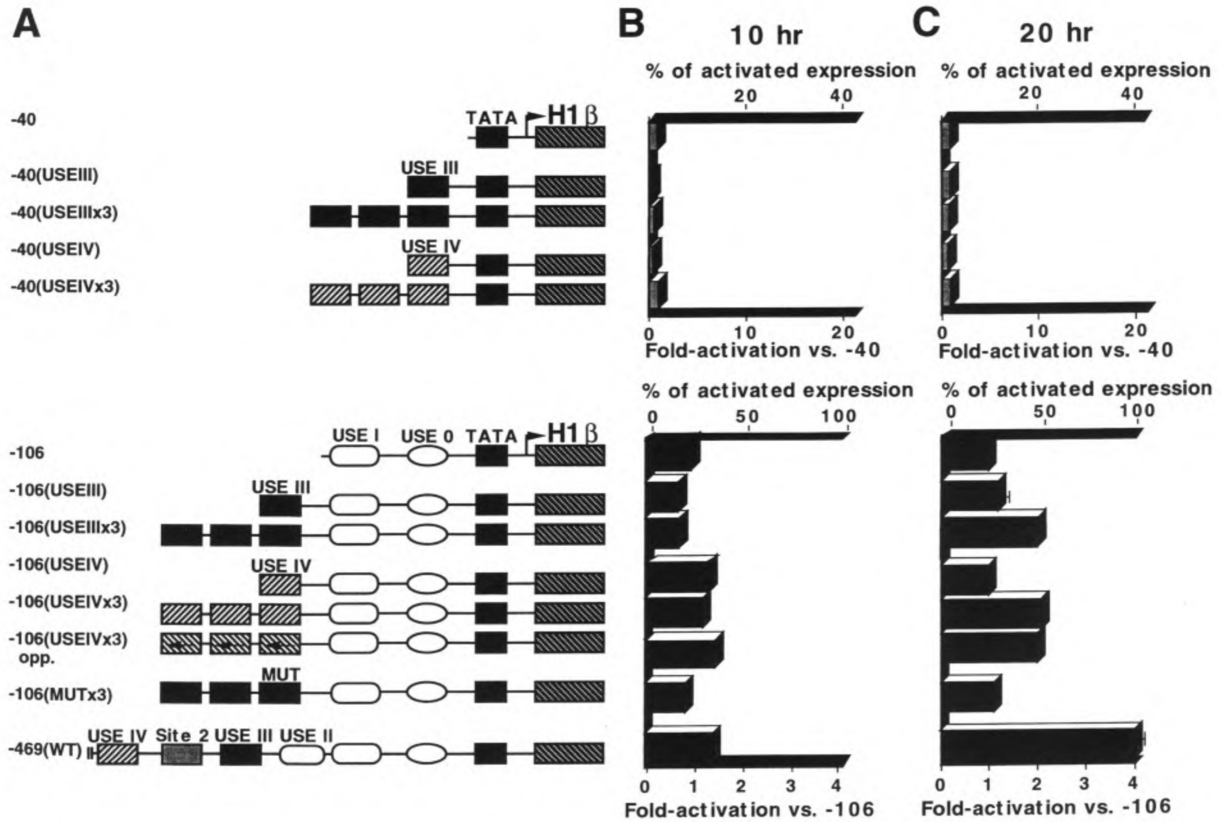
The results for the same constructs at 20 h postfertilization are graphed at the bottom of Fig. 6C. The −106 promoter is the reference point and is expressed as 25% of wild-type activity in these experiments, which is in agreement with previous results (31). One copy of USE III did not activate over basal levels of expression; however, three copies exhibited a reproducible twofold stimulation of the basal promoter (Fig. 6F, lanes 2–4). Similar results were obtained with the USE IV constructs. Although one copy of USE IV showed no appreciable effect on expression, three copies also activated the basal promoter to twice its normal level (Fig. 6F, lanes 5–7). The distance-independent nature of USE IV has been previously demonstrated by its conveyance of mid-blastula stage activation to reporter constructs when placed downstream of the reporter gene (10,11,32). To determine whether orientation had any discernible effect on activation, a construct with three USE IV sites in the opposite direction (Fig. 6A) was tested. The reverse orientation multimer also exhibited a twofold activated expression with respect to the basal promoter (Fig. 6F, lanes 11–13). A construct with three mutant binding sites (Fig. 1) was included as an additional control (Fig. 6A). When the mutant, double-stranded oligomer was used as a probe in mobility shift assays, no complexes were formed with 8- or 24-h nuclear extracts (data not shown). As expected, these mutant sites were not able to activate transcription over basal levels (Fig. 6F, lanes 8 and 9). The −469*H1*β construct (Fig. 6A) contains the entire upstream region necessary to activate the gene to maximal levels (31). In these experiments the −469*H1*β wild-type construct, containing the authentic enhancer, activated the basal promoter approximately fourfold (Fig. 6F, lanes 8 and 10; 12 and 14). Therefore, both the artificial USE III and USE IV multimer constructs enhanced the activity of the basal promoter in a stage-specific manner at approximately 50% of the maximal or wild-type levels.

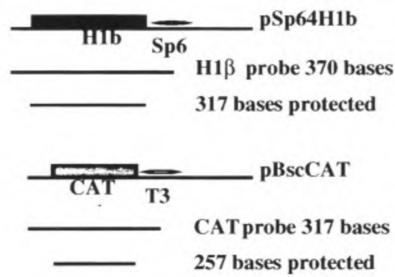
H1α/β *Hybrid Promoter Constructs*

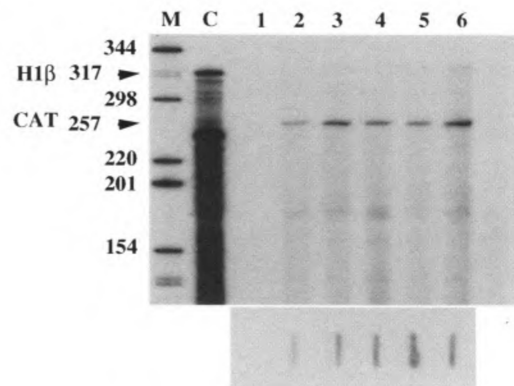
The results in Fig. 6 indicated that either of the SSAP binding sites, USE IV or USE III, could mediate stage-specific activated expression in the context of the basal promoter but not with the TATA box alone. These results were consistent with previous studies, which indicated that mutation of either USE 0 or USE I abolished promoter activity to < 1% of wild-type levels, and that both of these elements were essential for *H1* gene activity (31). Point mutations that abolish basal promoter activity early in development also prevent activated transcription later in development. An *H1*α/β hybrid promoter construct was created to determine whether late gene enhancer sequences could confer blastula stage activation to the *H1*α promoter, which utilizes similar conserved sequence motifs as the *H1*β gene but in addition is subject to repression at the mid-blastula stage of development (16).

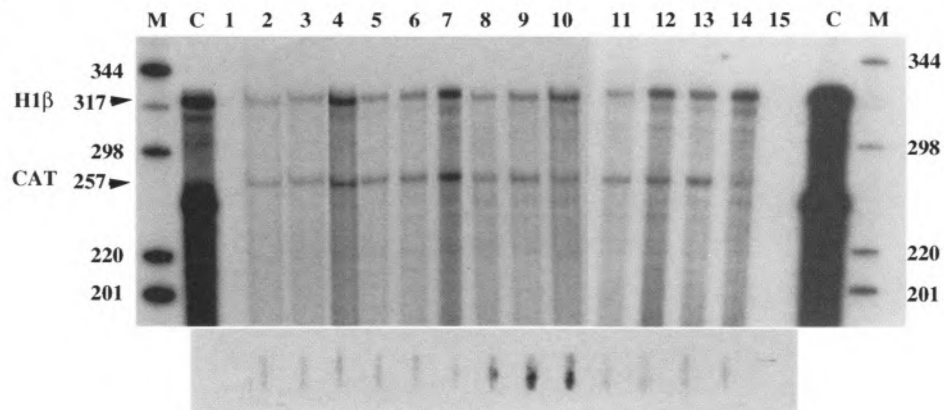
The plasmid p162/163 (Fig. 7A) has the functional promoter of the *S. purpuratus* early *H1* gene from position −65 to +39, which includes USE 0, a TATA box, an initiator, and an additional internal element located in the 5′ leader region. These sequences convey the proper early gene temporal pattern of expression to the *Luciferase* reporter genes. Expression levels were highest at early blastula stages between 9 and 12 h postfertilization, declined at the 20-h mesenchyme blastula stage, and were very low by the 40-h gastrula stage (16). Although all four of the early *H1* promoter elements present in p162/163 functioned in positive regulation of the gene, only USE 0 was also implicated as a negative control element (16).

The hybrid early/late *H1* promoter of p162/163(USE IVx3) shown in Fig. 7A contains three copies of USE IV immediately upstream of USE 0. Both p162/163 and p162/163(USE IVx3) were microinjected at concentrations of 30 μg/ml and were coinjected with 30 μg/ml of pGC355A as an internal control for expression. pGC355A is essentially a late *H1*β–*CAT* fusion with the homologous basal promoter of the *H1*β gene upstream of the *CAT* gene and three copies of USE IV downstream. The expression of the construct parallels the pattern of expression of the late *H1*β gene and has been previously used as an example of the activity of USE IV stage-specific, enhancer sequences (11).


A
B
10 hr
C
20 hr
% of activated expression
-40
-40(USEIII)
-40(USEIIIx3)
-40(USEIV)
-40(USEIVx3)
TATA
H1β
USE III
USE IV
Fold-activation vs. -40
-106
-106(USEIII)
-106(USEIIIx3)
-106(USEIV)
-106(USEIVx3)
-106(USEIVx3) opp.
-106(MUTx3)
-469(WT)
USE I
USE 0
MUT
USE IV Site 2 USE III USE II
Fold-activation vs. -106


D
pSp64H1b
H1b
Sp6
H1β probe 370 bases
317 bases protected
pBscCAT
CAT
T3
CAT probe 317 bases
257 bases protected


E
M C 1 2 3 4 5 6
344
H1β 317
298
CAT 257
220
201
154


F
M C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 C M
344
H1β 317
298
CAT 257
220
201

The results of two independent microinjection experiments each assayed in duplicate at two developmental intervals are graphed in Fig. 7B. The choice of collection times reflected the need for a time point when the enhancer was active, and the early gene promoter also retained activity (16 h), as well as a time point when the late gene enhancer was still active and the early gene was almost completely repressed (40 h). The 16-h time points indicate the activity of the construct containing USE IV sites was four- to sixfold greater than the *H1α* promoter alone.

Similar results from 40-h embryos also confirm the positive effect of USE IV sites on the activity of the early *H1* promoter. The activity of the *H1α-Luciferase* construct was lower in gastrula stage embryos, which is in agreement with repression of wild-type early *H1* transcription. However, when the USE IV multimer was present, *Luciferase* expression levels were stimulated approximately four- to sixfold. In the experiments shown in Fig. 7B, the USE IV sequences were able to activate the *H1α* promoter to about four to six times the normal level at both blastula and gastrula stages.

DISCUSSION

The Mid-Blastula Stage Enhancer of the H1β *Gene Consists of Three SSAP Binding Sites*

The late histone *H1* genes of the sea urchin are expressed in a characteristic temporal pattern during embryogenesis, in that they undergo an activation at the mid-blastula stage of development (29,30). Thus, they provide a model system in which to examine a mechanism of temporally regulated transcriptional activation. In this article we extend our study of the mechanism of *SpH1β* gene activation by analyzing the conserved nucleotide sequences that are present in the late but not the early *H1* genes (27).

USE IV was initially examined because of the dramatic loss of activated transcription at the mid-blastula stage, when it was deleted from the wild-type promoter (31). SSAP was subsequently identified as the transcription factor that binds to USE IV and directs mid-blastula stage activation of target genes (10,11). The mobility shift assays in Figs. 2 and 3 clearly indicate that USE III is also a binding site for SSAP. The DNase I footprint of the entire late *H1β* gene-specific region in Fig. 3B detected an additional SSAP binding site, Site 2, located between USE III and USE IV. There is a distance of exactly 90 bp between USE III and USE IV in the *SpH1β*, *SpH1γ*, and *LpH1γ* genes, and Site 2 is 28 bp upstream of USE III. Site 2 was not previously identified by sequence conservation analysis; however, further comparison of the *Lp* and *Spγ* genes indicated that another conserved sequence stretch, NTTTTAAGN, fit the SSAP binding consensus and was in the same relative position, 29 bp upstream of USE III at −255 in both genes. Whether these sites are functional elements of the enhancer in sea urchins is presently unknown. The spacing of the conserved USE III and USE IV elements with respect to each other as well as to the other prospective sites positions them all on the same side of the DNA helix.

Our findings demonstrate that the late *H1* gene-

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FIG. 6. In vivo activity of USE III and USE IV *H1β* M13 constructs. (A) Schematic of constructs tested for mid-blastula stage activation, −40 is the *H1β* gene up to the TATA box; the −40 derivative constructs are shown directly below it. −106 is the *H1β* gene basal promoter including the TATA box, USE 0 and USE I; the −106 derivative constructs are shown below it. −469 contains the entire *H1β* regulatory region and is designated the wild-type (WT) *H1β* construct. (B and C) Bar graphs indicate the activity of microinjected constructs from (A) at 10 and 20 h postfertilization (pf), respectively, as determined by RNAse protection assay and quantitated by PhosphorImager analysis. Shown at the top of each graph is the percent of activated expression, and at the bottom is the fold activation with respect to control construct. The −40 constructs were all injected into *L. pictus* sibling zygotes and their activity was directly compared with that of the −40 control construct, designated as 0.5% of WT activity (data not shown). The −106 constructs were divided into four groups: group 1, −106, 106(USE III), −106(USE IIIx3); group 2, −106, −106(USE IV), −106(USE IVx3); group 3, −106, 106(MUTx3), −469; group 4, −106, −106(USE IVx3), −106(USE IVx3)opp., −469 with each group injected into *L. pictus* sibling zygotes and compared to the activity of the −106 control construct injected with that group. In the 10-h experiment $n = 1$, and in the 20-h experiment $n = 2$ for all constructs. The activity of all constructs was normalized with respect to the activity of the co-injected control construct, −106*CAT* (see Materials and Methods). (D) Schematic of the constructs used and probes synthesized for RNAse protection of transcripts from microinjected constructs (see Materials and Methods); *H1β* probe protects 317 bases of *SpH1β* message, *CAT* probe protects 257 bases of *CAT* message. (E) RNAse protection assay of −40 constructs with RNA extracted from 50 embryo equivalents at 20-h pf; lane M, [γ-^{32}P]ATP end-labeled, *Hin*dIII digested pBR322; lane C, 20 μg of *S. purpuratus* total 24-h control RNA; lane 1, uninjected embryos; lane 2, −40; lane 3, −40(USE III); lane 4, −40(USE IIIx3); lane 5, −40(USE IV); lane 6, −40(USE IVx3). *H1β* and *CAT* probe RNA fragments are indicated with arrows at the left. Below each lane are the slot blot hybridizations of *H1β* DNA extracted from 20 injected embryos at 20-h pf and hybridized to M13mp19 probe. (F) RNAse protection assay of −106 constructs from 50 embryo equivalents at 20-h pf; lanes M and C (same as E); lanes 1 and 15, uninjected embryos; lanes 2, 3, and 4 (group 1), −106, −106(USE III), −106(USE IIIx3); lanes 5, 6, and 7 (group 2), −106, −106(USE IV), −106(USE IVx3); lanes 8, 9, and 10 (group 3), −106, −106(MUTx3), −469; lanes 11, 12, 13, and 14 (group 4), −106, −106(USE IVx3), −106(USE IVx3)opp., −469. Protected probe fragments are indicated as described in (E). Below each lane are slot blot hybridizations as described in (E).

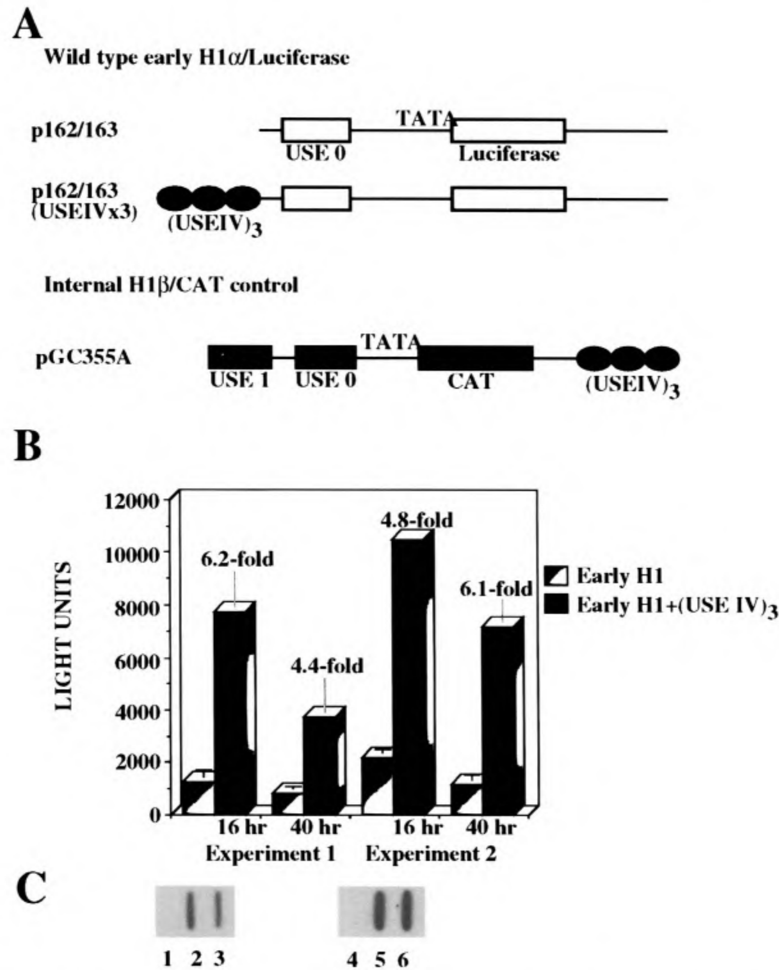

FIG. 7. Enhancer activity with early *H1* promoter. (A) Schematic of constructs tested for activity; p162/163 contains the *H1α* minimal functional promoter fused to a *Luciferase* reporter gene; p162/163(USE IVx3) is identical to p162/163 with three copies of USE IV enhancer sequences upstream. pGC355A contains the basal promoter of the *H1β* gene upstream of a *CAT* reporter gene and three downstream copies of USE IV. (B) Bar graph of results of two separate microinjection experiments assayed in duplicate. p162/163 and p162/163(USE IVx3) were co-injected with pGC355A into *L. pictus* sibling zygotes; *Luciferase* activity and *CAT* activity were assayed in duplicate at 16- and 40-h postfertilization. The *Luciferase* activity, indicated as light units, of p162/163 (light bars) and p162/163(USE IVx3) (dark bars) were normalized to expression of the internal *CAT* control; background activity as measured with equal numbers of uninjected embryos was subtracted from all values. (C) Slot blot hybridizations of radiolabeled pXP2 to DNA extracted from 20 embryos at 16-h pf: lanes 1, 2, and 3, uninjected embryos, p162/163, and p162/163(USE IVx3) from Experiment 1; lanes 4, 5, and 6, uninjected embryos, p162/163, and p162/163(USE IVx3) from Experiment 2.

specific DNA sequences comprise a mid-blastula stage enhancer with at least three degenerate binding sites for the transcription factor, SSAP. The three binding sites we examined were bound specifically by SSAP but with a discernible difference in affinity. The differential effect produced by deletion of USE IV versus deletion of USE III from the upstream regulatory region of the wild-type *H1β* gene (31) correlates very well with the relative affinities data in Fig. 5. The binding of SSAP to USE IV was approximately 10- to 15-fold greater than it was to either Site 2 or USE III.

It is interesting that 3 mers of USE III, which are lower affinity SSAP binding sites, had the same activation potential as 3 mers of USE IV when placed upstream of the basal *H1β* promoter. Perhaps placing the USE III sites in close proximity to one another increases their ability to recruit SSAP. The spacing of individual elements within an enhancer can alter their cooperative interactions (18). There is a strong footprint over the USE III site in Fig. 3B, which suggests that the close proximity of Site 2 may induce a cooperative interaction. Although we did not address the issue of cooperativity between the binding sites, the requirement of multiple sites for maximal activation was clearly demonstrated. Binding sites for the well-characterized transcription factor, Sp1, when present in multiple copies, interact in a synergistic

manner to activate transcription (1). In many other systems multiple enhancer elements can synergistically activate transcription (33,37,44). In the sea urchin embryo the positive regulation of the aboral ectoderm-specific gene, *SpSpec2a*, is controlled by multiple Otx binding sites (36).

SSAP Enhancer Binding Sites Require Promoter Elements to Function

In the context of the wild-type regulatory region of the late *H1* genes the presence of the high-affinity USE IV sequence is clearly essential but not solely responsible for activated expression. USE III is necessary for maximal levels of activated expression but requires the presence of USE IV to function (31). In these previous studies the deletion of USE III from the wild-type enhancer resulted in a 40% reduction in activated expression in blastula stage embryos. Because this deletion left Site 2 intact, there were potentially two SSAP binding sites remaining in the enhancer, USE IV and Site 2. It was unknown whether a single high-affinity site could direct activated expression. We decided to conduct a systematic addition of SSAP binding sites immediately upstream of the inactive homologous TATA box (–40 construct) or the homologous basal promoter (–106 construct) to compare the potential enhancer activity of USE IV and USE III. Previous results with USE IV on a variety of homologous promoter combinations (10,11) showed that six tandem copies of USE IV could stimulate transcription as much as 14-fold. In Fig. 6 the data from the microinjection of the artificial *H1*β constructs indicated that 3 mers of either USE IV or USE III, but not single copies, could activate mid-blastula stage-specific transcription to similar levels. This activation required the elements up to –106, but could not function in the context of the *H1*β TATA box alone. In addition, the functional promoter of the *H1*α gene fused to a *Luciferase* reporter gene, as defined by Fei and Childs (16), was activated four- to sixfold at the mid-blastula by the presence of three USE IV sites. In gastrula stage embryos the *Luciferase* activity of the early gene promoter construct without USE IV sequences decreased, but the hybrid construct was again expressed at levels four- to sixfold above the early *H1* promoter. Therefore, this activation imparted by the blastula stage-specific enhancer also resulted from the combined activities of the early promoter and late enhancer.

Under conditions where the wild-type authentic promoter construct, –469*H1*β, activated expression to fourfold above basal levels, the multimers of USE IV and USE III were only capable of a twofold activation. We believe the explanation for this is the absence of USE II from the artificial constructs. Deletion of USE II from the wild-type promoter resulted in a 40% decrease in activated expression (31), which would account for why artificial multimer constructs could activate to only 50% of wild-type levels. This *cis*-acting element, also present in the early *H1* genes and in all *H1* replication-type genes, is implicated as a positive acting element involved in S-phase cell cycle regulated transcription (7,8). Interestingly, deletion of USE II from the *SpH1*α promoter did not alter expression (16), which may reflect the dissociation of early gene expression from cell cycle control during rapid cleavage in the embryo (23).

SSAP is the transcription factor that mediates the mid-blastula activation of the *H1*β gene by binding to multiple sites in the late *H1* enhancer region. The activity of the protein itself is regulated in a temporal manner. The monomer binds to DNA and persists until approximately mid-blastula stage, when the homodimer becomes the prevalent DNA binding species. The appearance of the dimer correlates with the activated expression of the *H1*β gene (10). Recently, we presented evidence that in mammalian cells the activation domain of SSAP can homo-synergize when multiple binding sites are present in reporter constructs (13). We ascribe this property of SSAP to its ability to interact with multiple components of the general transcription machinery such as TBP, TFIIB, TFIIF74, and the coactivator, $TAF_{II}110$ (13). Our experiments show that in the early sea urchin embryo SSAP cannot function efficiently when its binding sites are placed in the context of the *H1*β TATA box alone. Thus, SSAP function requires the activity of these yet to be fully characterized, promoter-specific, transcription factors.

It is tempting to speculate that multiple binding sites function to mediate several heterogeneous interactions between SSAP and the basal transcription machinery. This model for simultaneous contact was put forth initially to explain the synergy observed with multiple binding sites for the yeast activator, GAL 4 (2,35). In embryos it is likely SSAP does not mediate the initial recruitment of general transcription factors because it requires the basal promoter elements and does not function efficiently with just a TATA box. Instead, SSAP may stabilize the existing preinitiation complexes of the basal promoter. In addition, the interaction of TFIIF74 with the activation domain of SSAP implies a possible role in elongation, because TFIIF can suppress the transient pausing of RNA polymerase during transcription in vitro (49). The future identification of which interactions are pertinent in the activation of the late *H1* genes and how they are regulated temporally in the sea urchin will expand our current model.

ACKNOWLEDGEMENTS

We thank all members of our laboratory for helpful discussions that improved this manuscript. We especially thank Dr. Daniel DeAngelo for contributing the purified SSAP used in these experiments as well as his invaluable technical guidance. We also thank Dr. Andrew Ransick for the plasmid, Bsc*CAT*. This work was supported by NIH grant GM-30333. L.E. was supported in part by NIH training grant T32 GM07491 from NIGMS.

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