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UHF-1, a Factor Required for Maximal Transcription of Early and Late Sea Urchin Histone H4 Genes: Analysis of Promoter-Binding Sites

INSONG J. LEE,† LIN TUNG, DAVID A. BUMCROT, AND ERIC S. WEINBERG*
Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6017

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A protein, denoted UHF-1, was found to bind upstream of the transcriptional start site of both the early and late H4 (EH4 and LH4) histone genes of the sea urchin *Strongylocentrotus purpuratus*. A nuclear extract from hatching blastulae contained proteins that bind to EH4 and LH4 promoter fragments in a band shift assay and produced sharp DNase I footprints upstream of the EH4 gene (from −133 to −106) and the LH4 gene (from −94 to −66). DNase I footprinting performed in the presence of EH4 and LH4 promoter competitor DNAs indicated that UHF-1 binds more strongly to the EH4 site. A sequence match of 11 of 13 nucleotides was found within the two footprinted regions: AGG$^{C}_{C}$GGC$^{C}_{T}$CACTC. Methylation interference and footprinting experiments showed that UHF-1 bound to the two sites somewhat differently. DNA-protein UV cross-linking studies indicated that UHF-1 has an electrophoretic mobility on sodium dodecyl sulfate-acrylamide gels of approximately 85 kDa and suggested that additional proteins, specific to each promoter, bind to each site. In vitro and in vivo assays were used to demonstrate that the UHF-1-binding site is essential for maximal transcription of the H4 genes. Deletion of the EH4 footprinted region resulted in a 3-fold decrease in transcription in a nuclear extract and a 2.6-fold decrease in expression in morulae from templates that had been injected into eggs. In the latter case, deletion of the binding site did not grossly disrupt the temporal program of expression from the injected EH4 genes. LH4 templates containing a 10-bp deletion in the consensus region or base substitutions in the footprinted region were transcribed at 14 to 58% of the level of the wild-type LH4 template. UHF-1 is therefore essential for maximal expression of the early and late H4 genes.

The sea urchin genome contains several histone gene sets which are differentially regulated during development (reviewed in references 23 and 43). The early embryonic genes, organized as a unit containing a gene for each of the five histones, are reiterated several hundred-fold per haploid genome in a tandem array. The late gene set of each haploid genome is composed of 5 to 12 genes for each nucleosomal histone (4, 29, 30, 42) and at least two H1 genes (32, 33, 37), organized in small irregular clusters or found as single genes. The amount of early RNA increases approximately 10-fold from the 16-cell stage to early blastula and then decreases rapidly so that little early histone mRNA remains by the gastrula stage (41, 46, 67). Late gene transcripts are found at low levels in the egg and, depending on the particular gene, increase to maximum levels at the mid-blastula and later stages (2, 3, 18, 24, 29, 31, 32, 37, 42, 49). Nuclear run-on assays indicate that the basis of these changes in mRNA levels is predominantly transcriptional; during blastulation, early gene template activity decreases and late gene transcription increases (31, 62, 70). Measurements of histone RNA synthesis rate and turnover in intact embryos are consistent with changes in the level of transcription of early and late genes (26, 45, 67).

Two approaches have been used to identify *cis*-acting sequences responsible for the regulation of the sea urchin histone genes. Methods developed to follow the embryonic expression of DNA injected into eggs or zygotes (7, 15, 47) have been used to determine that many individual early (8, 12, 13, 66) and late (8, 38, 39) histone genes are closely linked to elements which specify the timing of expression. Specific elements of this type have been found near the *Strongylocentrotus purpuratus* early H3 (EH3) (13) and late H1-β (LH1-β) (38) genes. In addition, the in vivo assay has been used to identify which sequence elements have a role in setting the level of expression of *S. purpuratus* EH3 (13) and H1-β (39) genes and the *Psammechinus miliaris* late H2B-2.1 gene (1). A complementary approach to identify transcriptional sequence elements is possible with the use of an in vitro transcription assay using embryonic nuclear extracts (1, 51, 60, 61). Although the histone gene temporal expression programs seen in vivo were not reproduced when genes were transcribed in nuclear extracts from different embryonic stages (61), the in vitro transcription system does provide a useful assay for positive and negative *cis*-acting transcriptional elements. We have demonstrated with this assay that there are multiple positively acting elements upstream of the *S. purpuratus* early H4 (EH4) gene and that EH4 and late H4 (LH4) templates form a stable transcription complex when incubated with the nuclear extract (61). Since the EH4 and LH4 genes could compete against each other in formation of the complex, we concluded that the two genes shared a requirement for at least one transcription factor. This factor was not required for in vitro transcription of an early H2B (EH2B) gene since the EH4 and LH4 genes did not compete for transcription of the EH2B gene. Competition experiments using EH4 deletion templates placed the binding site for this factor in the EH4 promoter region between positions −436 and −102. In a subsequent series of experiments using the in vitro transcription assay, we demonstrated at least five positive-responding sequence elements and one negative-responding site in the EH4 promoter region (60). The positive-acting elements include a site

* Corresponding author.
† Present address: Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892.

corresponding to the sequence upstream of −102, an H4 gene-specific element (H4SE) located between −62 and −39, a sequence corresponding to a TATA box between −33 and −26, the transcriptional initiation site, and an internal sequence element found between +19 and +50. The negative element is located between −75 and −56.

In the work described here, we present the characterization of a *cis*-acting element in the *S. purpuratus* EH4 and LH4 promoters. This element, corresponding to the site upstream of −102 in the EH4 gene previously identified (60, 61), is shown to be essential for maximal in vitro transcription of both genes in the nuclear extract and for maximal embryonic expression of the EH4 gene after injection into *Lytechinus pictus* eggs. Deletion of the sequence element, however, did not grossly change the in vivo temporal expression pattern. The *trans*-acting factor which interacts with the two gene promoters, designated UHF-1, is most likely a protein of 85 kDa. The factor interacts somewhat differently with the two binding sites, and there may be additional gene-specific factors whose interactions are dependent on the binding of UHF-1 to the *cis* element.

MATERIALS AND METHODS

DNA fragments and templates. The EH4 gene used in these experiments, derived from plasmid pCO2 (53), is the H4 gene of the *S. purpuratus* early repeat unit. A 1.1-kb *Dra*I-*Ava*II fragment consisting of the gene plus 436 bp of sequence upstream from the mRNA cap site and 281 bp of sequence downstream from the 3′ mRNA terminus was cloned into the *Hin*cII site of pUC118 (65) to yield the construct pEH4 as described by Tung et al. (61). This fragment was also subcloned into Bluescript (SK−) (Stratagene) to yield plasmid pEH4(BS).

The LH4 gene used here was derived from the genomic clone λSpL22 (29) and is one of the five to eight LH4 genes of *S. purpuratus*. A 1.6-kb *Sal*I-*Eco*RI fragment, containing the structural gene plus 553 bp of upstream sequence and 626 bp of downstream sequence, was cloned into the *Sal*I and *Eco*RI sites of pBS(−) (Stratagene) to yield plasmid pLH4 (61). This fragment was also recloned by cutting at the *Bam*HI and *Hin*dIII sites in the pBS(−) polylinker and insertion into the corresponding sites of Bluescript (SK−) to yield plasmid pLH4(BS).

Constructs containing both an H4 and an EH2B gene were formed by cloning EH4 or LH4 gene fragments into pEH2B(BK), a plasmid which consists of an *S. purpuratus* *Bam*HI-*Kpn*I EH2B gene fragment (containing 41 bp of upstream sequence and 419 bp of the structural gene) cloned into the *Hin*cII site of Bluescript (SK−). To make a plasmid in which the LH4 and EH2B genes are oppositely oriented (see Fig. 5), pLH4 was cut with *Xho*I, filled in with Klenow fragment, and then cut with *Eco*RI. The resulting 1.6-kb fragment was cloned into the *Eco*RI and *Sma*I sites of pEH2B(BK) to form the construct pLH4-EH2B(BK). When this plasmid is cut with *Nco*I, it serves as a template for a 252-base EH2B transcript and a 292-base LH4 transcript in an in vitro runoff transcription assay. A similar construct, pEH4-EH2B(BK), in which the EH4 and EH2B genes are oppositely oriented, was made by cloning a *Bam*HI-*Hin*dIII fragment of pEH4 (sites in the pUC118 polylinker) into the *Bam*HI and *Hin*dIII sites of pEH2B-BK. When this plasmid is cut with *Nco*I, it serves as a template for the 252-base EH2B transcript and a 322-base EH4 transcript in the in vitro runoff assay.

Probes used for band shift, footprint, methylation interference, and UV cross-linking assays were prepared from subclones of pLH4 and pEH4. A 111-bp *Taq*I fragment from pLH4 (−117 to −7) was cloned into the *Cla*I site of Bluescript (KS+) to form plasmid pLH4-TT, and a 122-bp *Dde*I-*Rsa*I fragment from pLH4 (−173 to −52) was filled in with Klenow enzyme and cloned into the *Eco*RV site of Bluescript (KS−) to form plasmid pLH4-RD. LH4 fragments A and B (Fig. 1) were prepared by digesting these plasmids with enzymes (e.g., *Apa*I and *Xba*I) which cut in the flanking polylinker. The fragments so derived carry sequences corresponding to positions −118 to −6 (fragment A) and −173 to −50 (fragment B), upstream of the LH4 gene. To create EH4 fragment D, a *Bal*31-derived construct of pEH4, which had all sequence deleted upstream of −151, was digested with *Eco*RI and *Acc*I to give a fragment extending from the *Eco*RI site in the pUC118 polylinker to the nucleotide at position +25 in the gene. The fragment was cloned into the *Acc*I site of Bluescript (KS−) to form plasmid pEH4-EA. The fragment obtained when this plasmid is cut with flanking restriction enzymes contains EH4 sequence from −151 to +27. To create EH4 fragment E, a *Bal*31-derived construct of pEH4, which had all sequence deleted upstream of −212, was digested with *Eco*RI and *Nhe*I to give a fragment extending from the *Eco*RI site in the polylinker to position −132 upstream of the gene. This fragment was cloned into the *Eco*RI and *Nhe*I sites of Bluescript (KS−) to form plasmid pEH4-EN.

For the footprinting and methylation interference experiments, LH4 fragment A was cut from pLH4-TT with *Apa*I and *Xba*I when the antisense strand was to be labeled and with *Xho*I and *Eco*RV when the sense strand was to be labeled. EH4 fragment D was prepared for the same purposes by digesting pEH4-EA with *Eco*RI and *Apa*I for the labeling the antisense strand and with *Eco*RI and *Xho*I for labeling the sense strand.

We constructed three deletion mutants of EH4 for use in in vitro transcription and in vivo expression assays. To create the Δ−180/−140 mutant, pEH4 was digested with *Xho*I and *Nhe*I, the overhangs were filled in with Klenow enzyme, and the fragment was recircularized by blunt-end ligation. The resulting construct lacked the 41 bp from −180 to −140. To create the Δ−135/−103 and Δ−135/−125 mutants, *Bal*31-produced derivatives of pEH4 which had 5′-flanking sequence removed to base −102 or −124 were digested with *Xba*I and *Hin*dIII, and the fragments containing the EH4 gene (and a small portion of the pUC118 polylinker on each side) were isolated. Intact pEH4 was digested with *Nhe*I and *Hin*dIII, and the 3.25-kb fragment (lacking the EH4 sequence except for the 5′-flanking region upstream of −135) was isolated and ligated to each of the fragments derived from the *Bal*31-derived plasmids. The reaction products were then filled in with Klenow enzyme and blunt-end ligated to circularize the plasmid. The resulting constructs contained a 4-bp sequence from the pUC118 polylinker, AGTC, in place of 33 bp (Δ−135/−103) or 11 bp (Δ−135/−125) of EH4 upstream sequence. The orientation of these constructs was the same as in the original pEH4. These three mutant EH4 genes were also recloned into pEH2B-BK to form constructs containing oppositely oriented EH4 and EH2B genes.

In addition to the constructs and fragments described above, gene fragments from other *S. purpuratus* genes were used as competitors in the band retardation experiments. These include fragments of the EH2B gene (−602 to −43, derived from pEH2B, a subclone of pCO2; 61), of an LH3 gene (−396 to +91, derived from λSpL22), of the EH1 gene

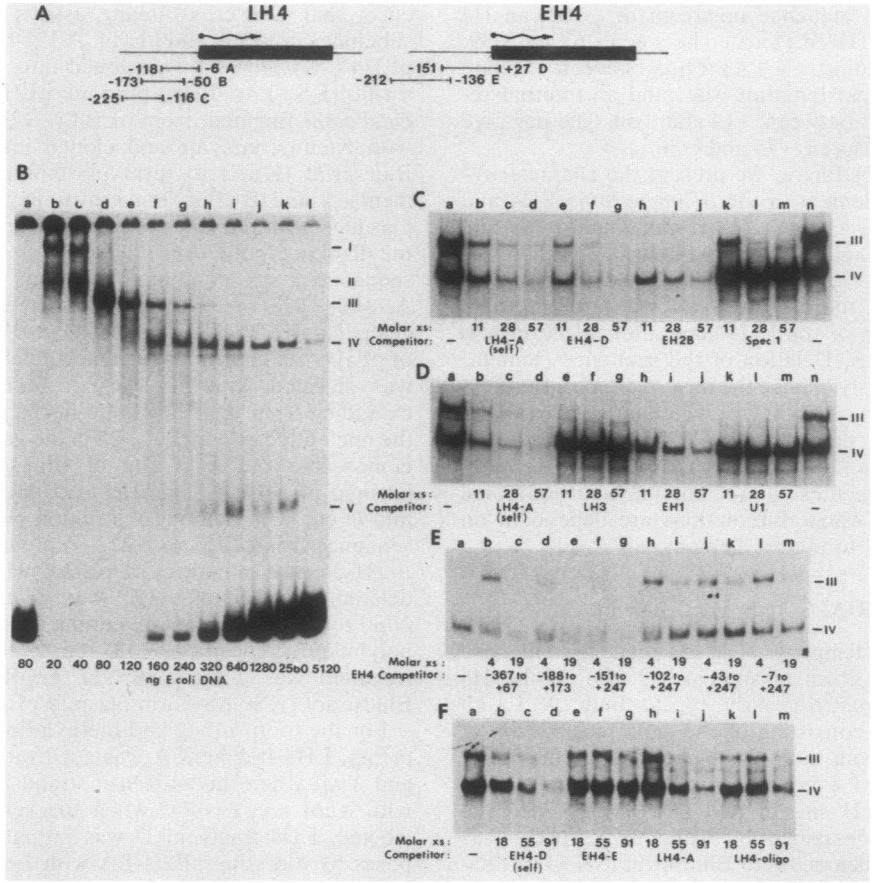


FIG. 1. Detection by gel retardation assays of specific interactions with EH4 and LH4 upstream sequences. Except as noted, reaction mixes contained 1 ng of ^{32}P-labeled DNA fragment and an amount of 0.25DE fraction containing 2 μg of protein. (A) Diagram of the locations of EH4 and LH4 fragments used for the mobility shift experiments. (B) Mobility shift assays using a labeled LH4 fragment B probe with increasing amounts of sonicated *E. coli* nonspecific competitor DNA. Lane a contains the labeled DNA probe and 80 ng of *E. coli* DNA but no extract fraction. (C and D) Mobility shift competition assay using labeled LH4 fragment A. Lanes a and n in each panel contain labeled fragment A and 320 ng of sonicated salmon sperm DNA but no extract fraction. All other lanes contain a combination of specific competitor DNA and sonicated salmon sperm DNA totaling 320 ng per binding reaction. The specific competitors were LH4 fragment A itself (lanes b to d, both panels); EH4 fragment D (panel C, lanes e to g); an early H2B histone gene fragment, derived from pEH2B, extending from −602 to −43 (panel C, lanes h to j); a SpecI gene fragment from −417 to +133 (panel C, lanes k to m); a late H3 histone gene fragment from −396 to +91 (panel D, lanes e to g); an early H1 histone gene fragment from approximately −450 to +550 (panel D, lanes h to j); and a sea urchin U1 snRNA gene (the complete 1,101-bp repeating unit) (panel D, lanes k to m). The competitor DNAs were added at 11-, 28-, or 57-fold molar excess as indicated below each lane. (E) Competition for complex formation with LH4 fragment A by various EH4 fragments. The reaction mix for lane a contained 160 ng of *E. coli* DNA and no EH4 competitor DNA. All other reaction mixes contained an amount of an EH4 competitor fragment equivalent to 4- or 19-fold molar excess of the labeled fragment and 80 ng of sonicated *E. coli* DNA. (F) Competition for complex formation with the EH4 D fragment. Each reaction mix contained labeled EH4 D fragment, an amount of 0.25DE fraction containing 4 μg of protein, and a total of 320 ng of specific competitor and/or sonicated salmon sperm DNA. The reaction mix for lane a contained 320 ng of sonicated salmon sperm DNA and no specific competitor DNA. The other reaction mixes contained unlabeled EH4 D fragment itself (lanes b to d), EH4 fragment E (lanes e to g), LH4 fragment A (lanes h to j), or a fragment containing a single copy of an oligonucleotide, oLH4ftm, extending from −95 to −61 of the LH4 gene (lanes k to m).

(approximately −450 to +550, derived from pEH1, a subclone of pCO2), of the LH1-β gene (−255 to +316, obtained from Geoffrey Childs), of the SpecI gene (−417 to +133, from a *Eco*RI-*Sal*I subclone obtained from William Klein; 21); the complete 1,101-bp U1 small nuclear RNA (snRNA) repeat unit (derived from SpU1-1, from William Marzluff; 52); and a 91-bp fragment of simian virus 40 (SV40) from positions 38 to 128 (59) (from James Alwine).

Oligonucleotides and oligonucleotide-directed mutagenesis. The following single-stranded oligonucleotides were synthesized and used to prepare the double-stranded oligonucleotides used as competitors in binding and UV cross-linking experiments: oLH4ft+, 5′-TCCCTTGACCGTAGGCGGC TCACTCTCAATAG-3′; oLH4ft−, 3′-GGAACTGGCATCC GCCGAGTGAGAGTTATCAGG-5′; oLH4ft+(p), 5′-CCT TGACCGT-3′; oLH4ftm+, 5′-CCCCTTGACCGTAGGTC AATAGAAATTATT-3′, oLH4ftm−, 3′-GAACTGGCATC CAGTTATCTTTAATAAGGG-5′; oEH4ft+, 5′-GGACTA GCGAATACTCGCCACAAGGGGGCGCACTCGAATG G-3′; and oEH4ft−(p), 3′-GAGCTTACC-5′. The shorter oligonucleotides were annealed to the opposite strand and used as primers to synthesize double-stranded molecules containing bromodeoxyuridine for the UV crosslinking studies. For footprint and transcription competitions, the longer complementary strands were annealed and used directly. For the experiment of Fig. 8, a double-stranded oligonucle-

otide consisting of human H4 sequence from −132 to −66 (11) was obtained from Nathaniel Heintz. LH4 mutants A through D and the 10-bp deletion mutant Δ−79/−70 were generated by the method of Kunkel et al. (35), with modifications. All mutant sequences were confirmed by sequencing. The 10-bp deletion of LH4 was generated by using the oligonucleotide, oLH4ftm+.

Preparation and fractionation of nuclear extracts. Nuclei and extracts were prepared by modifications of the methods of Morris and Marzluff (50) and Morris et al. (51) essentially as described by Tung et al. (61). To obtain the fraction used for footprinting and gel retardation assays, the extract was fractionated at 4°C on a DEAE-cellulose (DE52; Whatman) column with HEMG buffer (15% [vol/vol] glycerol, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid [HEPES; pH 7.8], 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride), adjusted to different KCl concentrations. The fractions containing protein obtained after elution with HEMG containing 0.1 KCl were combined and termed the 0.1DE fraction; those obtained after elution with HEMG containing 0.25 KCl were dialyzed against buffer C (15% [vol/vol] glycerol, 25 mM HEPES [pH 7.5], 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride) until the conductivity was less than that of 150 mM KCl. This fraction, termed the 0.25DE fraction, was used for the DNA binding studies.

In vitro transcription assay. Transcription in nuclear extracts was done as described by Tung et al. (61) except that up to 10 μl of nuclear extract was added per reaction and in some cases the final reaction volume was 40 μl. Template DNAs were cut with restriction enzymes, and transcription in the presence of α-^{32}P-labeled nucleoside triphosphates resulted in runoff transcripts of a size expected from correct initiation of EH4 and LH4 RNAs. The template plasmids also contained an EH2B gene so that a reference transcript was always produced. Quantitation of transcription was performed by scanning X-ray films of the gels with an LKB UltraScan laser-enhanced densitometer, and normalizing the amount of H4 transcript produced in each reaction by the level of EH2B transcript. Films with exposures in the linear response range were used for scanning.

Analysis of gene expression by injection of DNA into eggs. Injections into unfertilized *L. pictus* eggs were performed essentially as described elsewhere (8). A continuously flowing micropipette was used to deliver approximately 2 pl (0.2% egg volume) into the cytoplasm of each egg. Following injection, eggs were fertilized and development was allowed to proceed for 8 or 24 h at 17°C. For each time point, RNA was extracted from 30 eggs and assayed by RNase protection (as described in reference 8) after hybridization to 2 × 10^5 cpm of antisense riboprobe derived from pEH4(BS) (which contains the *S. purpuratus* EH4 gene), as diagrammed in Fig. 6. The protected RNA fragments were purified by phenol extraction and separated on an 8% acrylamide–7 M urea gel. At each time point, DNA prepared from 15 embryos was applied to a slot blot and assayed by hybridization to nick-translated Bluescript DNA [the vector for pEH4(BS)]. Quantitation was carried out by scanning the films of the RNA gel and the DNA slot blot and normalizing the amount of protected RNA fragment by the amount of DNA in that particular sample.

Gel retardation assays. We adapted methods of Fried and Crothers (16) and Strauss and Varshavsky (57) for electrophoretic band shift analysis. DNA fragments were labeled by using Klenow enzyme to fill in with the appropriate α-^{32}P-labeled nucleotide. Binding reactions were carried out in a buffer composed of 12% (vol/vol) glycerol, 20 mM HEPES (pH 7.8), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 75 mM KCl in a total volume of 20 μl. The reaction mixes contained 1 to 2 ng of labeled DNA fragment, an amount of 0.25DE fraction containing 2 to 4 μg of protein, nonspecific DNA (sonicated salmon sperm DNA or *Escherichia coli* DNA; Sigma), and in many cases specific DNA competitor fragments. The labeled probe was added last, 10 min after addition of the nonspecific DNA. Reaction mixes were incubated for 30 min at 16°C and then for 5 min on ice. In the case of competitions with specific DNA fragments, the competitor DNAs were preincubated with the 0.25DE fraction on ice for 5 min prior to addition of the nonspecific DNA. The mix was then incubated for 10 min on ice, and finally the labeled probe was added. Each reaction mix was electrophoresed on a 4% native polyacrylamide gel (acrylamide-methylene bisacrylamide; 60:1) in running buffer (15 mM Tris hydrochloride [pH 7.8], 7 mM sodium acetate, 2 mM EDTA) for 4 h at 30 mA at 4°C. The gel was then dried and exposed to X-ray film (RX Fuji).

DNase I footprinting. The conditions for protein binding were identical to those for the band shift assay, and the footprinting protocol was a modification of the method of Galas and Schmitz (17). After the 30-min binding reaction at 16°C and 5 min on ice, 2 μl of 10× DNase I buffer (10 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM DTT) containing 0.002 μg of DNase I per ml was added. The digestion was allowed to proceed for 1 min on ice and then stopped by the addition of 150 μl of Sarkosyl buffer (1% Sarkosyl, 0.1 M NaCl, 0.1 M Tris [pH 8.0], 10 mM EDTA). Nucleic acids were extracted with phenol-chloroform, precipitated with ethanol, and analyzed on 8% acrylamide gels containing 7 M urea and 1× TBE (3:1).

Methylation interference assay. DNA probes were methylated for 3 min at 20°C in a 308-μl reaction mix containing 50 ng of labeled probe (approximately 2.5 × 10^6 cpm), 1 μg of sonicated salmon sperm DNA, 300 μl of cacodylate buffer (0.05 M sodium cacodylate [pH 8.0], 10 mM $MgCl_2$, 0.1 mM EDTA), and 2 μl of dimethyl sulfate. The reaction was terminated by the addition of 50 μl of purine stop buffer (2.5 M β-mercaptoethanol, 3.0 M sodium acetate [pH 6.0], 0.1 mM EDTA, 0.5 mg of tRNA per ml). Nucleic acids were precipitated twice with ice-cold 95% ethanol, and the pellet was resuspended in 5 μl of 1× TE buffer. The methylated probe was then incubated with 26 μl of the 0.25DE fraction (containing 52 μl of protein), in a total volume of 50 μl, in the presence of sonicated salmon sperm DNA at a concentration of 350 μg/ml for 30 min at 16°C and electrophoresed exactly as for the gel mobility shift assay. The unshifted and shifted probes were electroeluted from the gel into 1× TBE buffer in dialysis tubing, purified by phenol-chloroform extraction and ethanol precipitation, and treated with 10% (vol/vol) piperidine at 90°C for 30 min. The samples were freeze-dried, washed several times, and then electrophoresed on an 8% polyacrylamide sequencing gel.

UV DNA-protein cross-linking. Procedures for cross-linking were adaptations of those of Chodosh et al. (5), Wu et al. (69), and Dailey et al. (11). Oligonucleotides containing the UHF-1-binding sites of EH4 and LH4 (oEH4ft+ and oLH4ft+), as well as an LH4 oligonucleotide carrying a 10-bp deletion (oLH4ftm+), were annealed with their respective primers by heating at 80°C and subsequent slow cooling. An extension reaction with Klenow fragment was carried out in a 50-μl volume containing 50 mM Tris hydrochloride (pH 7.8), 10 mM $MgCl_2$, 50 mM NaCl, 4 mM DTT,

0.1 mg of bovine serum albumin per ml, 0.2 mM dATP, 0.2 mM 5-bromo-2'-dUTP (Pharmacia), 4 mM dCTP and dGTP, and 120 μCi of [α-^{32}P]dCTP and −dGTP (800 Ci/mmol). After phenol-chloroform extraction, passage through a Sephadex G-25 quick-spin column, and precipitation with ethanol in the presence of tRNA (100 μg per sample), the labeled probe was resuspended in 1× TE. Conditions of incubation with the 0.25DE fraction were identical to those of the band shift assay except that 26 μl of the 0.25DE fraction (containing 80 μg of protein), 20 ng of probe, and 9.6 μg of sonicated salmon sperm DNA were added to the binding mix, and the final reaction volume was 50 μl. The reaction was carried out in an Eppendorf tube at 16°C for 30 min, followed by 5 min on ice. The tube cap was opened, and the tube was exposed to a UV lamp (Chromato-vue transilluminator model C63) at a distance of 4 cm for 60 min with gentle mixing every 10 min. After UV illumination, 1 μl of DNase (2 μg/μl in 200 mM $CaCl_2$) and 2 μl of micrococcal nuclease (1.6 U/μl) were added. The reaction was incubated at 37°C for 15 min and stopped by the addition of 51 μg of 2× sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris hydrochloride [pH 6.8], 20% [vol/vol] glycerol, 5.6% SDS, 0.004% bromphenol blue, 0.004% xylene cyanol, 10% β-mercaptoethanol). The sample was then boiled for 5 min and electrophoresed in a 6% polyacrylamide gel containing 0.375 M Tris hydrochloride (pH 8.8) in a Tris-glycine running buffer (0.025 M Tris [pH 8.3], 0.192 M glycine, 0.1% SDS).

RESULTS

Upstream regions of the EH4 and LH4 genes bind a common factor. A sea urchin 18-h embryo nuclear extract, active in transcribing both early and late H4 histone genes (61), was chromatographed on a DEAE-cellulose (DE52) column to obtain fractions that would yield simple patterns in electrophoretic mobility shift experiments. Three fractions were obtained: a 0.1 M KCl flowthrough fraction (0.1DE), which contained 60 to 65% of the starting amount of protein and 0.5 to 10% of the RNA polymerase II; a 0.25 M KCl fraction (0.25DE), which contained 35 to 40% of the protein and 32 to 100% of the RNA polymerase II; and a 0.6 M KCl fraction (0.6DE), which contained 1 to 2% of the protein and no detectable RNA polymerase II. Although the individual fractions were not competent to transcribe the LH4 or EH4 gene, a combination of the 0.1DE and 0.25DE fractions was sufficient to obtain as high a level of transcription as with the total extract (data not shown). When EH4 and LH4 promoter regions were incubated with either of these fractions, protein binding activity was detected with an electrophoretic gel retardation assay. We demonstrate below that the 0.25DE fraction contained a positively acting transcription factor that is required for high levels of expression of both the EH4 and LH4 genes. Specific promoter-binding activities have also been found in the 0.1DE fraction, and these will be described in a future publication.

We prepared three overlapping fragments from a region just upstream of the site of LH4 transcriptional initiation (Fig. 1A) and tested their gel mobilities after incubation with the 0.25DE fraction. Fragments A (−118 to −6) and B (−173 to −50) gave very similar patterns of retardation, whereas fragment C (−225 to −116) did not bind proteins under any of the conditions used. Figure 1B shows a gel of fragment A incubated in the presence of different amounts of nonspecific competitor DNAs. Of the five discrete bands (I to V) observed, only one (band IV) was competed for specifically by particular gene fragments and represents a specific DNA-protein interaction. A collection of promoter regions of various *S. purpuratus* genes was tested for the ability to compete for bands III and IV formed with the LH4 A fragment (Fig. 1C and D). The specific binding to form band IV was competed for by fragments containing the EH4 (−151 to +27), EH2B (−602 to −43), and EH1 (approximately −450 to +550) promoters but not by fragments of the Spec1 gene (−417 to +133), LH3 gene (−396 to +91), and U1 snRNA gene (the complete 1,101-bp repeating unit). All of these fragments, however, did compete for formation of the nonspecific band III complex.

We then determined which region of the EH4 fragment binds the factor (Fig. 1E). Competitor fragments were prepared from a series of 5' *Bal*31-generated deletion mutants. Band IV was clearly competed for by EH4 fragments containing 367, 188, and 151 bp of upstream sequence but not by EH4 fragments containing 102, 43, or 7 bp of upstream sequence. The binding site in the EH4 gene is therefore between −151 and −102, and the site in the LH4 gene is between −118 and −50 (because of the overlap in the identically shifted A and B LH4 fragments and because these two fragments competed with each other for the formation of band IV). To confirm the presence of a binding site near the EH4 gene, a mobility shift experiment was performed with a labeled EH4 fragment from −151 to +27 (EH4-D). The same pattern of bands was obtained as with LH4 fragment A (Fig. 1F). The EH4 fragment D itself and LH4 fragment A (lanes h to j) readily competed for band IV, but an upstream EH4 fragment (fragment E, −212 to −136) did not compete at all (lanes e to g). A fragment containing a single cloned copy of a 33-bp synthetic oligonucleotide of LH4 gene sequence (−95 to −63) also competed, but somewhat less effectively than the larger LH4 fragment.

To summarize these results, it appears that upstream segments of DNA of the EH4 and LH4 genes bind a common factor, which we will designate UHF-1. The site of interaction is between −118 and −50 near the LH4 gene and between −151 and −102 near the EH4 gene. There is a binding site for this factor also near the EH2B gene and near the EH1 gene but not near the LH3, Spec1, or U1 snRNA gene. The binding site in the EH2B gene has been localized to a region between −441 and −112 by competition experiments using upstream H2B fragments (data not shown), but nothing more is known about the location of the binding site in the H1 gene.

Precise determination of the UHF-1 site by footprinting and methylation interference. To confirm and extend the results of the mobility shift experiments, we performed a series of DNase I footprints. The antisense strand of the EH4 gene showed a strong footprint between −133 and −106 (Fig. 2A), and the EH4 sense strand exhibited a strong footprint between −130 and −108 as well as protection of bases −103 and −102 (Fig. 2B). When increasing amounts of EH4 (lanes d to f) and LH4 (lanes g to i) competitor DNAs were added, both footprints were weakened. The EH4 DNA was two to three times as good a competitor as the LH4 DNA. The whole footprint was relieved by addition of either competitor, indicating that the maintenance of the footprint is dependent on a factor (or factors) which can bind to either gene. The sense strand was protected somewhat differently from the antisense strand in that the footprint was extended slightly downstream and that hypersensitive sites flanked the major protected region. Curiously, the hypersensitive sites formed on both strands were not abolished when the EH4 and LH4 competitor fragments were added.

A very similar footprint was obtained from a gel-purified

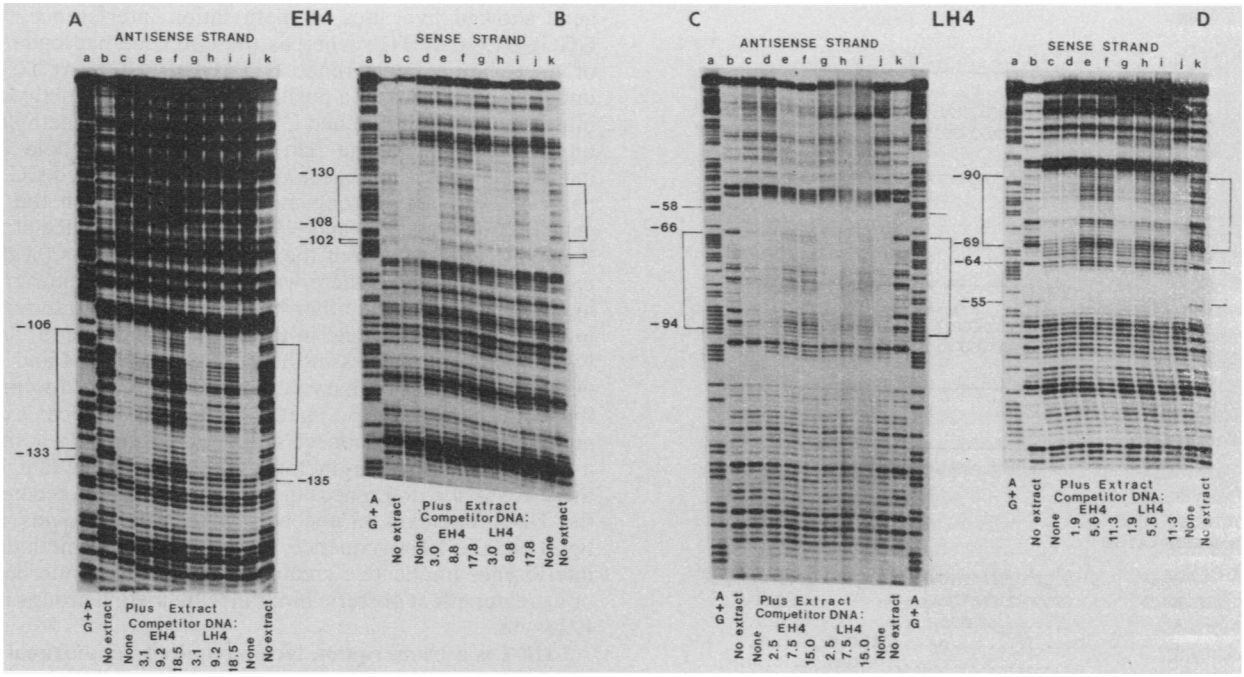


FIG. 2. DNase I footprints of the EH4 and LH4 promoter regions formed with the 0.25DE fraction. In each of the panels, lane a contains a Maxam-Gilbert A+G sequence ladder of the probe. No extract fraction was added to the reaction mixes of lanes b and k in each panel. In the reaction mixes of lanes c and j, 2 ng of labeled probe was first incubated with an amount of 0.25DE fraction containing 2 μg of protein for 30 min at 16°C prior to addition of DNase I. The reaction conditions for lanes (d to i) were identical to those for lanes c and j except that EH4 fragment D (−151 to +27) or LH4 fragment A (−118 to −6) was added in the molar excess amount indicated below each lane. In each case, an amount of sonicated salmon sperm DNA was added to bring the total amount of DNA to 320 ng per reaction.

EH4-protein complex. After the binding reaction, the EH4 fragment, labeled on the antisense strand, was treated with DNase I and electrophoresed under the conditions used for the gel retardation assay (as for Fig. 1F). The DNA-protein complex of band IV was eluted, deproteinized, and run on a sequencing gel. The area of DNase I protection was very similar to that shown in the directly footprinted EH4 fragment (data not shown). UHF-1, therefore, is responsible for the formation of band IV as well as for the footprint of the EH4 gene.

The LH4 footprint patterns were very similar to those obtained with the EH4 fragments. A strong footprint was seen on the antisense strand between −94 and −66 and at base −58 (Fig. 2C), and the sense strand was protected from −90 to −69 and at bases −64 and −55 (Fig. 2D). The base at −55 directly abuts a stretch of sequence (−63 to −53) at which the DNase I does not cut in the control DNA (Fig. 2D, lanes b and j); thus, it is impossible to say whether there is another extended protected region here. The fact that there is also a protected base at −58 on the antisense strand, however, does hint at either an interaction with another factor in this region or a continuation of protection due to the major factor or factor complex. Like the EH4 sites, the LH4 hypersensitive sites were not competed for by EH4 and LH4 fragments at concentrations that were effective in relieving the footprint.

As with the EH4 footprints, LH4 sense and antisense strand footprints were competed for by the EH4 and LH4 fragments, and the EH4 fragment was a better competitor. This result is revealing, since one possible interpretation of the initial binding experiments could have been that there is a family of factors which bind to the H4 genes, with each factor binding somewhat specifically to one gene. The footprint competition results, however, appear to rule this out. The data indicate that there is a common factor which binds to both the EH4 and LH4 genes and that the factor binds more tightly to the EH4 gene.

The footprints are summarized in the diagram of Fig. 3. Protected regions are shown by the brackets, ambiguous regions in which DNase I does not cut the unprotected DNA are indicated by dots above the bases, and hypersensitive sites are shown by arrowheads. The footprinted regions of the EH4 and LH4 genes have remarkably similar topologies. Both footprints extend for at least 28 uninterrupted bases on the antisense strand and for 22 to 27 uninterrupted bases on the sense strand. The sense strand is also protected at an additional 1 to 2 bases downstream of an unprotected 4-base stretch. Hypersensitive sites are found only on the sense strands of the two genes (except for a weak site at −135 on the EH4 antisense strand). Within the two footprints, closer to the downstream boundary, there is a conserved sequence with an 11-of-13-bp match: AGGNGGCNCACTC (sense-strand sequence). The position of the common sequence relative to the footprint is not precisely identical in the two genes. The protected region is shifted 1 to 2 bp downstream in the LH4 gene, compared with the EH4 gene, when the boundaries of the footprints are matched with reference to the conserved sequence.

To characterize the UHF-1-binding sites further, we performed methylation interference experiments in which fragments were subjected to mobility shifts similar to those shown in Fig. 1. The G sequence of the DNA in the shifted band was compared with that of the unshifted band as well as with unreacted control DNA. For both strands of each gene,

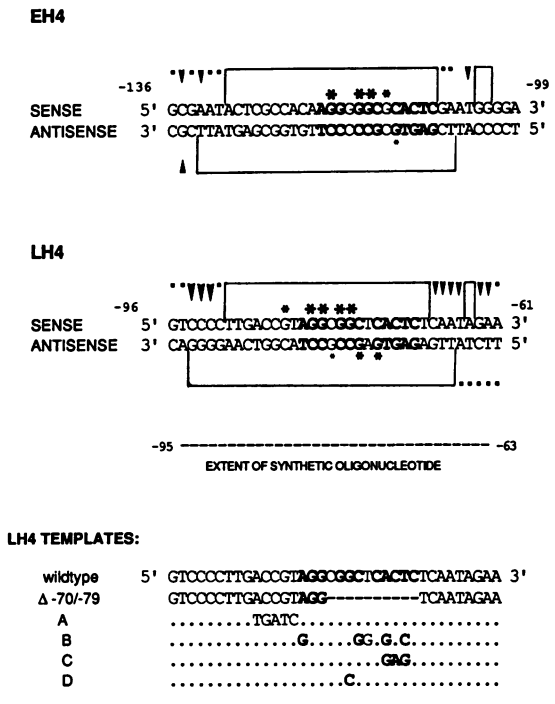


FIG. 3. Comparison of UHF-1-binding sites upstream of the EH4 and LH4 genes. The sequence of the footprinted regions of the two genes is displayed. The antisense strand is the template for mRNA synthesis. The numbering is relative to the 5' end of the most abundant mRNA species. Brackets indicate the positions of protected bases. Dots above and below the bases are positions which are not cleaved by DNase I in the naked DNA. Arrowheads indicate the positions of hypersensitive sites (i.e., sites at which the DNA is cleaved more effectively in the presence of the extract fraction than in its absence). The large arrowheads signify a great enhancement of cleavage, and the small arrowheads indicate a smaller increase in DNase I activity at these positions. Asterisks indicate G's at which methylation inhibits binding to the protein factor, with the size of the asterisk indicating the relative interference effect. The bases printed in bold letters comprise the sequence conserved at the sites in the two genes. The extent of the synthetic oligonucleotide used in the UV cross-linking experiment of Fig. 8 is shown below the LH4 sequence. The sequences of the various LH4 templates used in the transcription experiments of Fig. 5C are given at the bottom. The bases printed in bold letters are those at the positions conserved between the LH4 and EH4 genes. In the deletion mutant, dashes indicate the deleted bases. In mutants A to D, the dots indicate the bases which are unchanged from the wild-type, and the bases indicated are those which are substituted in the mutants.

the decrease in a particular G in the shifted DNA fragment ladder (Fig. 4, lanes c) was accompanied by an enrichment of that G in the unshifted DNA fragment (lanes d). Interference with binding was imparted by methylation of one G on the EH4 antisense strand (−112), four G's on the EH4 sense strand (−119, −116, −115, and −113), three G's on the LH4 antisense strand (−79, −76, and −74), and five G's on the LH4 sense strand (−84, −81, −80, −78, and −77). These G's are marked in Fig. 3 by asterisks of different sizes, indicating their relative importance in the binding of the factor. Contacts with the sense strand appear to be more critical than contacts with the antisense strand in both genes. Although the important G's generally were found in the region of common sequence, there are a number of surprising differences between the EH4 and LH4 results. The early gene showed five sites of methylation interference (CAA GGGGGCGCACTC), whereas the late gene had eight sites of methylation interference (GTAGGCGGCTCACTC) (an underlined G indicates a position which showed methylation interference, an underlined C indicates that a G methylation on the opposite strand had an effect, and a bold letter indicates a conserved base). Two of the conserved GC base pairs showed very strong methylation effects in the LH4 gene (at positions −80 and −76) but no interference at all in the EH4 gene. At two of the conserved positions (at the 3' end of the sequence) there was no methylation interference in either gene. On the other hand, methylation interference in one nonconserved site in the EH4 gene (at −113) and at two nonconserved sites in the LH4 gene (at −84 and −79) was observed. In summary, although the DNase I footprints, footprint competitions, methylation interference experiments, and conservation of sequence all indicate a similar interaction of a protein factor UHF-1 with the binding site, there are subtle differences in the way the protein recognizes the DNA duplexes at the two sites. UHF-1 binds more tightly to the EH4 sequence, but the effects of methylation interference implicate a greater number of base interactions or a greater effect of steric hindrance by methyl groups in the LH4 site.

UHF-1 is a transcription factor required for maximal EH4 and LH4 expression. To determine whether UHF-1 is really a transcription factor, we tested deletions and base substitution mutants for transcriptional activity in an embryo nuclear extract. Figure 5 shows the results of transcription of wild-type and mutant EH4 and LH4 templates. In these experiments, we used constructs containing an EH4 and an EH2B gene or an LH4 and an EH2B gene as templates (Fig. 5A). Cutting the DNA in either case with *Nco*I results in the oppositely oriented genes being situated at the ends of the linear DNA, and runoff transcripts of different sizes initiated at the two promoters can be easily distinguished by gel electrophoresis. The H2B transcript, although weaker than the H4 transcripts, serves as a standard to normalize the results of various H4 mutant templates.

We first tested the effects of deletion of the UHF-1-binding site on transcription in the nuclear extract (Fig. 5B). An EH4 template lacking nucleotides −135 to −103 was transcribed only 37% as well as the wild-type gene (compare lanes a and d). A deletion removing nucleotides −135 to −125, however, resulted in no decrease in EH4 transcription (lane c). This latter deletion covers a region at the upstream side of the footprint but does not include the sequence conserved between the EH4 and LH4 genes or any of the G's which interfere with UHF-1 binding when methylated. A template containing a deletion extending from −180 to −140 (lane b) was also transcribed as well as the wild-type gene. The result of transcription of the 10-bp LH4 deletion is shown in Fig. 5C (compare lanes a and c). The LH4 deletion template was transcribed at only 14% of the level of the intact LH4 gene. These comparisons have now been done over 10 times for the EH4 gene and 5 times for the LH4 gene with a range of decrease of 2- to 5-fold for the EH4 gene and 2- to 10-fold for the LH4 gene, depending on the extract used and the conditions of the experiment. The sequences from −124 to −103 of the EH4 gene and from −79 to −70 of the LH4 gene, therefore, are required to bind a positively acting transcription factor.

Templates containing base substitutions in the footprinted region of the LH4 gene (indicated in the lower part of Fig. 3) were also transcribed in the nuclear extract (Fig. 5C, lanes d to g). The percentage of wild-type level of transcription for

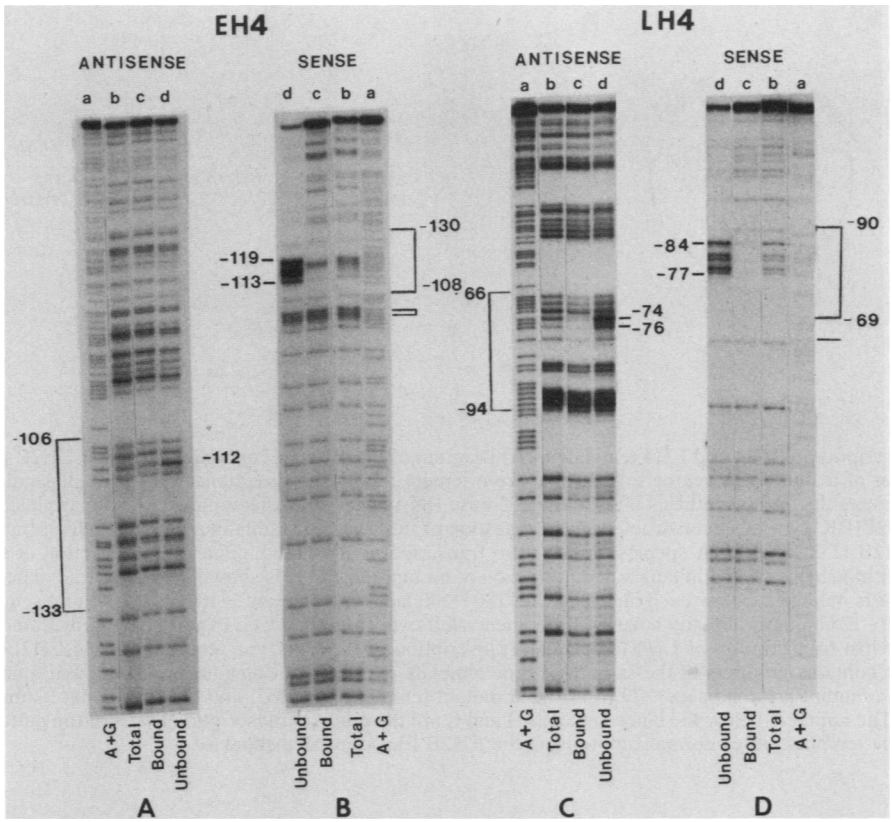


FIG. 4. Methylation interference assay of UHF-1 binding to EH4 and LH4 promoter regions. Labeled DNA fragments were identical to those used in the DNase I footprinting experiments. Lane a in each of the four panels is a Maxam-Gilbert A+G reaction of the labeled fragment. DNA fragments methylated with dimethyl sulfate and cleaved with piperidine were run in lane b of each panel (marked Total). After incubation with the 0.25DE fraction, the fragment was electrophoresed as for Fig. 1, the specific band with retarded mobility and the unbound fragment were cut out of the gel, and the DNA was eluted, purified, and then cleaved with piperidine. Lane c in each panel shows the cleavage pattern for the band with retarded mobility (bound DNA), and lane d in each panel shows the pattern for the unshifted band (unbound DNA). The four panels are labeled exactly as in Fig. 2 for the four DNA strands analyzed. The bracket adjacent to lane a in each panel indicates the extent of the fragment protected by DNase I in the footprint experiment of Fig. 2.

each mutant template, normalized for EH2B transcription, is indicated below each lane of Fig. 5C. Interestingly, the most dramatic decrease in transcription was obtained with mutant A (lane d), in which the base changes were not even in the conserved sequence region (although the G at position −84 of the late H4 gene did show a moderate effect of methylation interference). This mutation was as deleterious to transcription as the 10-bp deletion (compare lanes c and d). The other three mutants also resulted in lower levels of transcription, indicating that the sequence of the whole region under study is important for high activity. The change from G to C at −77 resulted in only a threefold reduction of transcription (lane g), although in vitro methylation of this G resulted in a complete prevention of UHF-1 binding (Fig. 4). Similar effects of the mutations were obtained when the DNAs were cut with *Sca*I in addition to *Nco*I (data not shown), a treatment which places the LH4 test gene and the EH2B control gene on separate fragments.

In summary, the essential sequence for maximal transcription of the EH4 gene is located between −124 and −103, a region which includes the 13-bp consensus element. A 10-bp segment of the consensus sequence of the LH4 promoter (−79 to −70) was also shown to be essential. However, substantial changes in the sequence of the LH4 consensus region did not completely abolish UHF-1 transcriptional activity even when the base substitutions were at sites at which G methylation completely inhibited UHF-1 binding. Surprisingly, the strongest effect of the four substitution mutants was seen with the mutant (D) containing changes outside of the consensus sequence, indicating that the region upstream of the consensus sequence is important as well.

The UHF-1 site is required for maximal EH4 in vivo expression but not for appropriate temporal regulation. The effect of a deletion of the UHF-1 site was also tested with an in vivo transcription assay. We injected the wild-type *S. purpuratus* EH4 gene (as plasmid pEH4; Fig. 6A) or the EH4 gene carrying the deletion Δ−135/−103 into eggs of *L. pictus*. Eggs were fertilized after injection, and RNA was prepared from 8-h morulae and 24-h mesenchyme blastulae. The *S. purpuratus* EH4 transcript is expected to protect a 120-base fragment of a riboprobe prepared from plasmid pEH4(BS) (Fig. 6A). As a control, we used total RNA from *S. purpuratus* morulae in the protection assay, and a 120-nucleotide fragment was in fact seen (Fig. 6B, lane c). Two smaller protected fragments, which may represent naturally occurring EH4 RNAs with shorter 3′ untranslated regions, were also evident. RNA from embryos derived from uninjected *L. pictus* eggs did not protect a 120-base riboprobe fragment, but much shorter fragments were seen (Fig. 6B, lanes a and b). This finding is not unexpected, since the one

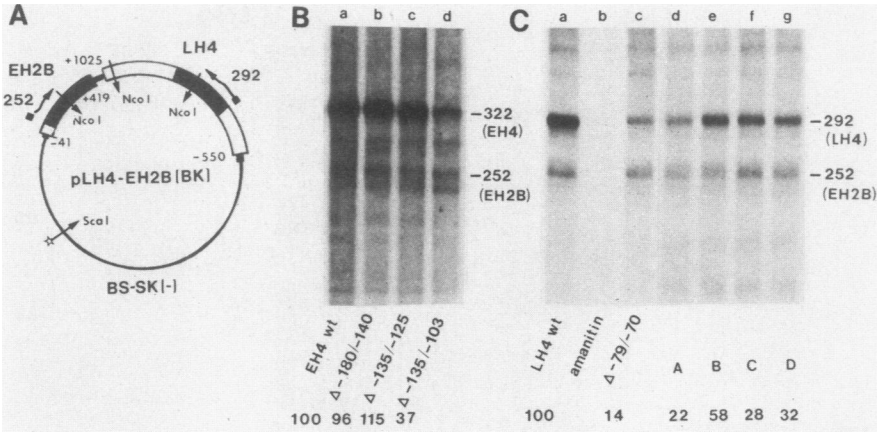


FIG. 5. In vitro transcription of EH4 and LH4 templates. (A) Diagram of the plasmid containing LH4 and EH2B genes. Both genes were inserted in the polylinker of a Bluescript vector so that they are oriented in opposite directions. When the plasmid is digested with *Nco*I, transcription of the genes results in the synthesis of 292- and 252-base RNA species, as diagrammed. A similar plasmid containing EH4 and EH2B genes, pEH4-EH2B(BK), was also constructed; *Nco*I digestion of this plasmid results in a template which transcribes distinguishable EH4 (322-base) and EH2B (252-base) RNA species. (B) In vitro transcription of EH4 templates. Transcription of the wild-type template, pEH4-EH2B(BK) cut with *Nco*I, is shown in lane a. Transcription of mutant templates is shown in lanes b to d. The missing EH4 upstream sequence of each mutant is indicated below each lane (e.g., Δ−180/−140 lacks the bases at −180 and −140 and sequence in between). The Δ−135/−103 and Δ−135/−125 mutants actually have a 4-bp sequence left over from the pUC118 polylinker substituted for the deleted 33- and 11-bp stretches. (C) In vitro transcription of LH4 templates. Transcription of the wild-type template, pLH4-EH2B(BK) cut with *Nco*I, is shown in lane a. Lane b contains products of the same wild-type template except that α-amanitin was added at a final concentration of 2.5 μg/ml. Transcription of a mutant lacking bases −79 to −70 and mutant templates A, B, C, and D carrying base substitutions (see Fig. 3) is shown in lanes (c to g). The numbers below the lanes in panels B and C are the relative transcription levels of the mutant templates compared with that of the wild-type template, after normalization using the EH2B transcript as a standard.

published early gene sequence of *L. pictus* (EH3) (55) shares little homology to the *S. purpuratus* H3 gene (58) in the sequence between the coding region and the conserved 3′ stem-loop structure, and even in the coding region, the sequences of these genes differ at 10.5% of the nucleotide positions.

Embryos injected with the wild-type pEH4 construct made abundant transcript at the morula stage (Fig. 6B, lane d), but very little transcript derived from the injected gene was found at the mesenchyme blastula stage (lane e). Since transcription of the injected *S. purpuratus* EH4 gene clearly followed the appropriate temporal program (a more extensive time course has been performed; data not shown), we conclude that all of the information for correct timing of expression is contained within a fragment extending from −436 to +720. Embryos injected with the mutant template lacking the UHF-1-binding site also were transcribed with appropriate timing (lanes f and g), but the level of expression in the morula was 2.6-fold lower than in the wild-type template. (Quantitation was performed densitometrically by normalizing the amount of protected fragment by the amount of plasmid DNA detected in the embryos.) The UHF-1 site is therefore important for maximal expression of the gene in vivo as well as in vitro. It is interesting that the magnitudes of decrease in transcription of the deleted template were quite similar in this assay (Fig. 6) and the in vitro transcription system (Fig. 5). The in vivo experiment indicates, in addition, that the UHF-1 site is not essential for the shutoff of transcription which occurs by the mesenchyme blastula stage.

UHF-1 is not Sp1 and is distinct from human H4TF-1. A factor, termed H4TF-1, has been found to bind to a GC-rich site upstream of a human H4 histone gene (10, 11). This site is essential for maximal transcription of the human H4 gene (11, 20). The sea urchin UHF-1 and human H4TF-1 sites superficially resemble an ideal Sp1 site (Fig. 7). In the case of the H4TF-1 site, a lack of competition for binding by oligonucleotides containing Sp1 sites indicates that Sp1 is not the binding factor (11). Adjacent to the H4TF-1 site upstream of the human H4 gene is a perfect Sp1 consensus site which has also been shown to be incapable of competing for H4TF-1 binding (11). We were therefore interested in determining whether the sea urchin UHF-1 could bind to the human H4-binding site and whether authentic Sp1-binding sites could compete for UHF-1 binding.

A gel mobility shift competition assay very much like those shown in Fig. 1 was performed with oligonucleotide and DNA fragment competitors (Fig. 7) except that in this case, a labeled oligonucleotide containing the EH4 binding site for UHF-1 was used as a probe. Incubation with the 0.25DE fraction resulted in two shifted bands, indicated as III and IV, because of their similarity to the shifted bands formed when larger DNA fragments were subjected to the assay (Fig. 1). Although self-competition with an EH4 gene fragment was effective (lanes b to d), competition was not observed when we used comparable molar equivalents of a 75-bp oligonucleotide extending from −132 to −66 of the human H4 gene (a region which includes the H4TF-1 site and the putative Sp1 site) (lanes e to g) or a 91-bp fragment of the SV40 genome containing all six GC boxes (bases 38 to 128, Tooze nomenclature) (lanes l to n). The sea urchin late embryonic EH1-β gene contains an element termed USE1 which differs from an Sp1 site by one base and, when changed into a perfect Sp1 sequence, causes transcripts to accumulate at an earlier developmental time (39). When a fragment of the H1-β gene (−255 to +316, which includes the USE1 site) was tested for competition for UHF-1 binding, no effect was observed (lanes h to j).

The lack of competition with the human H4, SV40, and sea urchin H1-β fragments and oligonucleotides makes it

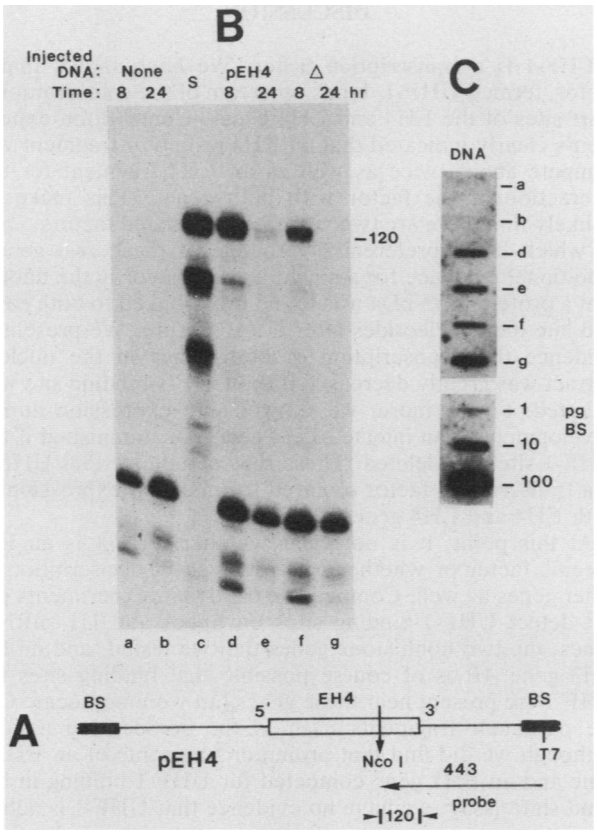
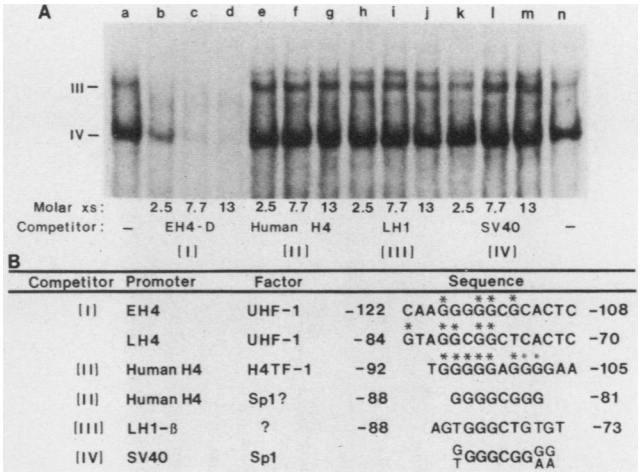


FIG. 6. Demonstration that UHF-1 functions as a positive transcription factor in vivo. (A) Probe used for the RNase protection assay. The *S. purpuratus* EH4 gene including 436 bp of upstream sequence and 282 bp of downstream sequence, cloned in Bluescript [pEH4(BS)], was cut with *Nco*I so that T7 RNA polymerase would produce an antisense transcript of 443 bases complementary to the 3′ 120 bases of EH4 mRNA. (B) Analysis of EH4 gene expression. Thirty *L. pictus* uninjected eggs (lanes a and b) or eggs injected with either pEH4(BS) (lanes d and e) or the Δ−135/−103 derivative (lanes f and g) were fertilized and allowed to develop for 8 (lanes a, d, and f) or 24 (lanes b, e, and g) h. Total RNA from *S. purpuratus* morulae was also subjected to the protection assay and run in lane c as a standard. (C) Slot blot analysis of DNA. The blot is labeled so that the letters correspond to the samples run on the gel shown in panel B. Each slot contains DNA from 15 embryos probed with labeled Bluescript DNA. Standards consisting of 1, 10, and 100 pg of Bluescript DNA plus carrier DNA from 15 uninjected mesenchyme blastula embryos were also probed with the labeled vector.



B

Competitor	Promoter	Factor		Sequence	
[I]	EH4	UHF-1	-122	CAAGGGGGCGCACTC	-108
	LH4	UHF-1	-84	GTAGGCGGCTCACTC	-70
[II]	Human H4	H4TF-1	-92	TGGGGGAGGGGAA	-105
[II]	Human H4	Sp1?	-88	GGGGCGGG	-81
[III]	LH1-β	?	-88	AGTGGGCTGTGT	-73
[IV]	SV40	Sp1		GGGCGGGG (T; AA)	

FIG. 7. (A) Test for competition for UHF-1 binding by DNA containing Sp1 and human H4TF-1 sites. Binding reaction mixes contained 1 ng of an oligonucleotide containing the intact UHF-1 site of the EH4 gene (oEH4ft) labeled with [α-^{32}P]dGTP, 2 μl of the 0.25DE fraction (4 μg of protein), and a total of 320 ng of DNA. Control binding reaction mixes contained 320 ng of sonicated salmon sperm DNA but no specific competitor DNA (lanes a and n). The other reaction mixes contained the EH4-D fragment which extends from −151 to +22 (lanes b to d); a double-stranded synthetic oligonucleotide containing the human H4 histone gene sequence from −66 to −132, a region which contains binding sites for both Sp1 and human H4TF-1 factors (11) (lanes e to g); a fragment of the sea urchin LH1-β gene stretching from −255 to +316 (lanes h to j); or a 91-bp fragment of the SV40 genome (between positions 38 and 128) (lanes k to m). The competitors were added at a 2.5-, 7.7, or 13-fold molar excess, as indicated below each lane. In these reactions, the total DNA level was brought up to 320 ng with the addition of salmon sperm DNA. (B) Sequence similarities between factor-binding sites in the competitor DNAs (note that the H4TF-1 sequence is from the antisense strand, whereas the other sequences are those of the sense strands). Asterisks above the sequence show G's which interfere with binding when methylated (Fig. 4 and reference 11). The competitor fragment containing the particular binding site is indicated in the first column.

very unlikely that UHF-1 is Sp1. Furthermore, UHF-1 appears to be distinct from human H4TF-1, since the human H4 oligonucleotide containing the H4TF-1 site does not compete for UHF-1 binding. The H4TF-1 and UHF-1 sites are also distinct when the G patterns of methylation interference are compared (Fig. 7). Additional evidence that UHF-1 is neither Sp1 nor H4TF-1 is that the EH4 fragment containing the UHF-1 site shows no mobility shift in extracts from HeLa and Nomalwa cells (data not shown). A final piece of evidence comes from the transcription experiments of Fig. 5, in which mutant B, which contains an ideal Sp1 site (28), is actually transcribed with lower efficiency than the wild-type template.

Protein-DNA interactions studied by UV cross-linking. To gain an idea of the molecular weight of the UHF-1 protein, we carried out UV cross-linking exp iments with bromodeoxyuridine-substituted oligonucleotides. Figure 8 shows the results with an LH4 oligonucleotide (lanes a to d) and an EH4 oligonucleotide (lanes e to f). In both cases, the predominant UV-cross-linked protein had a molecular size of approximately 85 kDa (lanes a and e). The specificity of the interaction was demonstrated by the ability of EH4 (lanes b and f) and LH4 (lanes c and g) fragments containing UHF-1-binding sites to compete for binding and cross-linking with this protein species. On the other hand, a fragment of the LH4 gene which lacks the UHF-1-binding site failed to compete for interaction with the 85-kDa protein (lanes d and h). Interestingly, in addition to this protein, two other proteins (of approximately 115 and 170 kDa) were found cross-linked to the LH4 oligonucleotide (lanes e and h) and were specifically competed for by the EH4 and LH4 gene fragments (lanes f and g). These two proteins did not appear to interact with the EH4 oligonucleotide in the cross-linking experiment (lanes a to d), yet their interaction with the LH4 oligonucleotide was subject to competition by the EH4 fragment. Similarly, a protein of approximately 58 kDa was cross-linked to the LH4 oligonucleotide (lane a) but not to the EH4 oligonucleotide (lane e). As with the specific proteins cross-linked to the EH4 oligonucleotide, the EH4

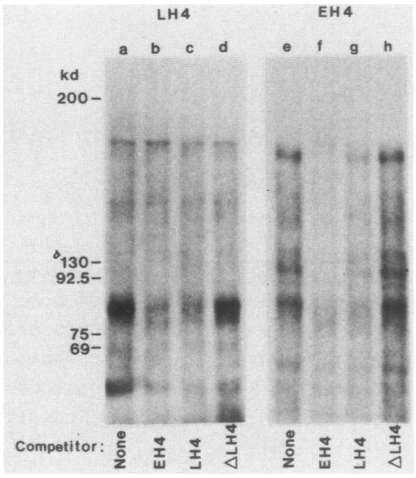


FIG. 8. Cross-linking of protein to LH4 and EH4 oligonucleotides. Oligonucleotides corresponding to the UHF-1-binding site of the EH4 gene (oEH4ft) or the LH4 gene (oLH4ft) were incubated with the 0.25DE fraction. The remaining lanes contained similar reaction mixes except that a 100-fold molar excess of unlabeled oEH4ft (lanes b and f), oLH4ft (lanes c and g), or oLH4ftm (lanes d and h) oligonucleotide was included. The latter oligonucleotide contains a 10-bp deletion in the UHF-1-binding site. After a 30-min preincubation at 16°C and a 60-min period of UV irradiation done on ice, a combination of DNase I and micrococcal nuclease was added and the reaction mix was incubated for 15 min at 36°C. Prestained Amersham Rainbow and Bio-Rad protein markers (molecular sizes indicated in kilodaltons [kd] on the left) were electrophoresed alongside the lanes containing the reaction mixes.

and LH4 gene fragments were both able to compete for the 58-kDa protein interaction (lanes b and c), but in this case, even the LH4 fragment carrying the deletion was an effective competitor (lane d).

These results are consistent with there being a common 85-kDa UHF-1 factor that binds to the two promoters. In addition, there appear to be 115- and 170-kDa proteins which cross-link to the EH4 sequence and a 58-kDa protein which cross-links to the LH4 sequence. The interactions with the 115- and 170-kDa proteins are specific, since unlabeled EH4 and LH4 oligonucleotides carrying the wild-type sequence competed for binding, but the mutant LH4 oligonucleotide did not. Although the unlabeled LH4 oligonucleotide was an effective competitor for binding of these proteins to the EH4 sequence, the two proteins did not bind to the labeled LH4 oligonucleotide in the assay. This apparent paradox could be explained if the binding to the EH4 promoter of the 115- and 170-kDa proteins is dependent on binding of the common 85-kDa UHF-1 protein. In this model, if the 85-kDa protein is sequestered by prior incubation with unlabeled LH4 DNA, the 115- and 170-kDa proteins would not be able to bind to the labeled EH4 oligonucleotide. There are now ample precedents for factors which bind weakly or not at all to DNA but which bind to other proteins to enhance transcription (e.g., leucine zipper proteins; reviewed in references 27 and 48). The specificity of binding of the 58-kDa protein to be labeled LH4 oligonucleotide, however, is not easily explained since unlabeled EH4 and mutant LH4 oligonucleotides both competed for the interaction.

DISCUSSION

UHF-1 is a transcription factor. We have shown that a factor, termed UHF-1, binds upstream of the transcriptional start sites of the EH4 and LH4 genes. Competition experiments clearly indicated that an EH4 promoter fragment will compete about twice as well as an LH4 fragment for the interaction of the factor with both genes. This makes it unlikely that there are two separate but related factors, each of which binds preferentially to one of these two genes. Additional evidence for a single UHF-1 factor is the finding that a protein of 85 kDa was found cross-linked to both early and late oligonucleotides after UV exposure. We presented evidence that transcription of both genes in the nuclear extract was greatly decreased if the UHF-1-binding site was mutated. Furthermore, we showed that expression during development of an injected EH4 gene was diminished if the UHF-1 site was deleted. These results indicate that UHF-1 is a transcription factor required for maximal expression of both EH4 and LH4 genes.

At this point, it is not clear whether UHF-1 is an H4-specific factor or whether it is involved in transcription of other genes as well. Competition band shift experiments did not detect UHF-1-binding sites on Spec1 and U1 snRNA genes, the two nonhistone genes that we tested, and on the LH3 gene. (It is of course possible that binding sites for UHF-1 are present near these genes but were not located on the particular fragments used in the competition assay.) Although we did find that promoter fragments of an EH2B gene and an EH1 gene competed for UHF-1 binding in the band shift assay, we have no evidence that UHF-1 is acting as a transcription factor for these two genes. In fact, although the UHF-1-binding site in the EH2B gene is between −441 and −112 (from band shift competition experiments), an EH2B construct with only 41 bp of 5′ sequence was transcribed in the nuclear extract as efficiently as a construct with over 1 kb of upstream DNA (61a). Consistent with a lack of a role for UHF-1 in EH2B transcription, at least in vitro, was the observation that depletion of the factor by preincubation with the EH4 or LH4 gene (61) or with a double-stranded oligonucleotide containing the LH4 footprinted region (unpublished results) had no effect on the transcription of an EH2B template with over 1 kb of 5′-flanking sequence. The detection of binding activity by the band shift competition assay is therefore not sufficient to claim a functional role for the factor. Further work on the EH2B and EH1 genes is necessary to clarify the role of UHF-1 in their transcription.

Comparison of sea urchin and human H4 gene promoters. The sea urchin and human H4 promoters are organized quite similarly (Fig. 9). The two human H4 genes that have been studied in detail both contain upstream elements in which one strand is purine rich. The Hu4A gene contains a GC-rich region located between −113 and −92 which is required for efficient transcription in nuclear extracts from S-phase cells (11, 20). As mentioned above, a factor termed H4TF-1, which binds to this element, has been purified and stimulates the transcription of H4 genes in a nuclear extract depleted of the factor (11). As we have shown here (Fig. 7), the binding specificities of sea urchin UHF-1 and human H4TF-1 are distinct from each other and from that of Sp1, although all three factors bind to GC-rich sequences (the antisense strand is G rich in the Hu4A gene, whereas the sense strand is G rich in the UHF-1 sites). The human H4TF-1 preparation contains polypeptides of 105 and 110 kDa (11), somewhat larger than the 85-kDa protein which binds to the UHF-1

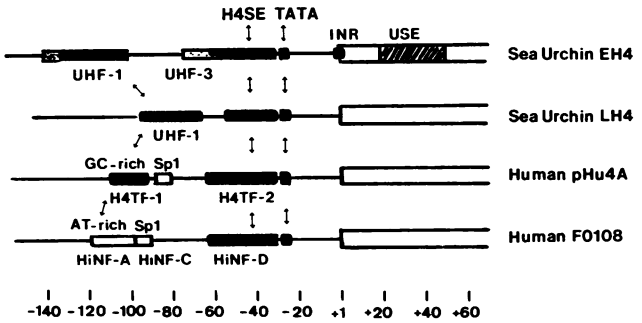


FIG. 9. Comparison of sea urchin and human H4 gene promoters. Sequence elements in the promoter regions of the sea urchin EH4 and LH4 genes and the human Hu4A (10, 11) and F0108 (63, 64) genes are indicated as black boxes (positive elements), stippled boxes (negative elements), or open boxes (without demonstrated function). Factors that form footprints are denoted directly below the appropriate site. Each gene contains a TATA box and an H4SE site. In the case of the EH4 gene, the initiator region (INR) and an internal sequence region (ISE) are required for maximal transcription in vitro (60), and two negative sites, the UHF-3 site (60) and a second site further upstream (1a), are adjacent to the positive H4SE and UHF-1 elements. The internal sequence region is located somewhere within the region indicated by the hatched box (its boundaries are not defined by footprints or sequence homologies, as are the other sequence elements).

sites. If the UHF-1 protein is the sea urchin homolog of human H4TF-1, the binding specificities of the factors have diverged. Another human H4 gene, F0108, contains an AT-rich site located between −115 and −100 which binds a protein factor termed HiNF-A (63, 64). This binding activity is present throughout the cell cycle of HeLa and other transformed cells (64) but is cell cycle regulated in nontransformed diploid tissue culture cells (25). Just downstream of these purine-rich sites in each of the two human H4 genes is an Sp1 consensus sequence which (for one of the two H4 genes) is shown to bind an Sp1-like factor (HiNF-C) (64). Functional roles for HiNF-A or HiNF-C have not yet been reported, however.

In addition to the purine-rich sequence elements, human and sea urchin H4 genes (as well as all known vertebrate and echinoderm H4 genes) contain a conserved sequence just upstream from the TATA box. This region contains sequence homologies previously identified as typical of echinoderm and vertebrate H4 genes (references 6, 23, 54, 60, and 68 and references therein). We have termed this region the H4SE and have pointed out that it consists of two regions (60). Echinoderm, mammalian, and avian H4SEs contain a GTCCG located 5 to 15 bases upstream from the TATA element. Sea urchin EH4 and LH4 genes have an even more extensive homology in this proximal region of the H4SE ($GTCCGC^{A}_{T}$). The more distal region of the H4SE is conserved in most vertebrates, but the sea urchin EH4 genes have a distinct sequence in this region. A different consensus sequence can be written for the H4SE distal region of the sea urchin LH4 genes (4, 29, 44, 55). Sea urchin H1 genes also contain a very similar element with an identical proximal domain but a distinct H1-specific distal domain (39). The H4SE has been shown to be required for maximal transcription in those cases in which it has been tested. Deletions of the region containing the H4SE or base substitutions in the element resulted in decreases in transcription of the sea urchin EH4 gene in the nuclear extract (60), in expression of a transfected human F0108 H4 gene (34), and in expression of a *Xenopus laevis* H4 gene after introduction into *Xenopus* oocytes (6). A protein factor, designated H4TF-2, was found to bind to the H4SE site of the human H4 gene Hu4a (10, 11), and a factor, designated HiNF-D, was found to bind to the H4SE sequence of the nonallelic human H4 gene F0108 (64). The exact relationship between these two factors is not yet established. Although we have not yet demonstrated a sea urchin H4SE binding factor, we assume that a protein similar to H4TF-2 or HiNF-D does exist (the term UHF-2 is reserved for this factor).

In addition to the UHF-1 and H4SE elements found in both the EH4 and LH4 genes, we have recently demonstrated a number of other *cis*-acting regions which appear to be restricted to the EH4 gene (60). In vitro transcription of the EH4 gene requires a specific sequence at the site of transcriptional initiation and an internal sequence element located between +19 and +50. We have also found at least two negatively responding sites. One, termed the UHF-3 site, is adjacent to the H4SE. Mutation of this site results in increased template activity in the nuclear extract (60). Deletion of the second negative element, located just upstream of the UHF-1 site, results in a lack of repression in the late blastula of an injected EH4 gene (1a). Repression of the EH4 gene in the late embryo, therefore, could be due in part to an interference with UHF-1 binding. Since transcription of a template lacking a UHF-1 site is only lowered two- to five-fold, however, the shutoff of transcription would have to involve inhibition of activation caused by other factors as well.

Histone regulons in the sea urchin. The regulation of early and late histone genes in the sea urchin is apparently not as simple as one might have predicted. Since the five early genes are all present in a 6- to 7-kb repeat unit, temporal expression could theoretically be controlled by a single stage-specific enhancer. This appears not to be the case, however, since individual genes of the cluster, injected into eggs of related sea urchin species, are regulated properly during development. This has previously been shown for the EH2A (12, 66), EH2B (8), and EH3 (13) genes, and we have demonstrated here that it is also true for the EH4 gene. Each early gene promoter appears to be distinct, and there are currently no candidates for common factors which could interact with all five promoters to induce or repress transcription. In contrast to the early genes, the late genes might be expected to have gene-specific *cis*-acting elements. The timing of expression of the different late genes coding for a particular histone type is not coordinate (2, 26, 29–31, 49), and in some cases there is specificity of expression of subtypes in particular adult tissues (19, 30, 40). The promoters of such genes would be expected to be complex, and it is not surprising that the promoter of the one late gene analyzed in detail thus far, the H1-β gene of *S. purpuratus*, contains a variety of 5′ elements essential for maximal activity (39), one of which appears to be a stage-specific enhancer (38). It is possible that some of the proteins which interact with the late H1-β promoter elements are also involved in the transcription of other histone genes, both late and early.

In addition to UHF-1, there are two other examples of a factor which binds to two histone genes with distinct developmental programs. A factor, termed TSAP, binds to the four promoters of the two *P. miliaris* late H2B and H2A nonallelic gene pairs (1), shown to have different developmental programs (30). The TSAP-binding site is essential for maximal expression of at least one of these genes (H2B-2.1)

(1). Another example of a factor which is specific for a class of histone genes is the octamer-binding protein, OTF-1 (also called Oct-1). This transcription factor mediates the cell cycle regulation of a human histone H2B gene (14, 36) and is found in most H2B genes (22, 54), including early (56, 58) and late (1, 9) H2B genes of the sea urchin. UHF-1, therefore, may not be unique in acting as a transcription factor for two differentially programmed genes of a particular histone class.

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