Histone H2B Gene Transcription during *Xenopus* Early Development Requires Functional Cooperation between Proteins Bound to the CCAAT and Octamer Motifs

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The ubiquitously expressed transcription factor Oct-1 and several other members of the POU domain protein family bind to a site, termed the octamer motif, that functions in the promoter and enhancer regions of a variety of genes expressed under diverse conditions. An octamer motif present in a conserved histone H2B-specific promoter element is required for S-phase-specific transcription of mammalian histone H2B genes in cultured cells. We have previously shown that the octamer motif in a *Xenopus* histone H2B gene promoter was inactive in nondividing frog oocytes. Here we show that the octamer motif, in addition to regulatory elements (TATAA, CCAAT, and ATF motifs) that are active in oocytes, is required for maximal H2B gene transcription in developing frog embryos. Factors binding to each of the H2B upstream promoter elements are present in oocytes and increase slightly in abundance during early development. The activity of the H2B octamer motif in embryos is not specifically associated with increased binding by Oct-1 or the appearance of novel octamer-binding proteins but requires the presence of an intact CCAAT motif. Our results indicate that synergistic interactions among promoter-bound factors are important for octamer-dependent H2B transcription. We suggest that the activity of the H2B promoter is regulated primarily by changes in the interactions between proteins already bound to the promoter rather than by alterations in their intrinsic abilities to bind DNA.

Many processes in early development require the correct temporal and spatial regulation of gene expression, accomplished in part through the combinatorial activity of multiple DNA-binding proteins that interact with specific cis elements in transcriptional regulatory regions (for reviews, see references 34 and 39). Interactions between multiple DNA-binding regulatory proteins offer not only the potential for finer control of transcriptional activity but also a broader range of responses to environmental cues. Since modulation of the concentration and activity of transcriptional regulatory proteins represents a fundamental method for regulating gene expression, unraveling the mechanisms that control their expression and activity has become an important challenge.

The control of histone gene expression during *Xenopus* development represents an attractive system for the analysis of gene regulation during early embryogenesis. Most vertebrate histone genes are coordinately expressed in dividing cells during S phase as a result of transcriptional and posttranscriptional regulation (2, 9, 16, 19, 38). Several organisms have, in addition, a separate replacement set of constitutively expressed variant histone genes (50, 52). In *Xenopus* cells, histone genes are actively transcribed during oogenesis, inactivated when the germlinal vesicle breaks down during oocyte maturation, and then reactivated in blastula-stage embryos after fertilization. Because the same histone gene sets are replication independent in oocytes and replication dependent in somatic cells and embryos (37, 42), it appears that *Xenopus* histone gene expression undergoes a transition from constitutive or basal activity during oogenesis to cell cycle-regulated expression later in development by a mechanism that is not well understood.

A highly conserved octanucleotide motif present in many vertebrate histone H2B gene promoters (17) plays a central role in the cell cycle regulation of H2B transcription and is an intriguing example of a common regulatory element that mediates the transcription of genes having very different patterns of expression. This regulatory element is present in promoters and enhancers of many eukaryotic genes and is required for S-phase-specific transcription of mammalian histone H2B genes (24, 32), ubiquitous expression of small nuclear RNA genes (1, 33, 41, 46), and lymphoid cell-specific expression of immunoglobulin genes (for a review, see reference 29).

The octamer motif is recognized by several members of the POU domain protein family (20), including the ubiquitous Oct-1 (OTF-1) and the B-cell-specific Oct-2 (OTF-2). Members of this family contain the highly conserved POU domain, which is required for sequence-specific DNA binding and which is a target for several types of regulatory protein-protein interactions (25, 45, 49). The ability of purified Oct-1 to stimulate transcription of a human H2B promoter in vitro provides biochemical evidence for this factor's role in the S-phase-specific activity of the mammalian H2B octamer motif (11). However, conflicting results have been reported regarding the ability of transfected Oct-1 to stimulate the transcription of octamer-containing promoters (28, 47) and concerning a correlation between levels of H2B transcription and the abundance or DNA-binding activity of Oct-1 (7, 26, 31). In addition, recent studies suggest that Oct-1 lacks a C-terminal activation domain necessary to stimulate mRNA synthesis in vivo (47, 48). Consequently, the mechanisms underlying the S-phase specificity of the H2B octamer motif are uncertain but presumably involve cell cycle-dependent changes in (i) the ability of Oct-1 to bind to the H2B promoter, (ii) posttranslational modifications of Oct-1 that result in an increased ability to activate

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H2B transcription, or (iii) interactions of Oct-1 with other factors that supply activation domains.

We recently analyzed regulatory elements and factors that mediate transcription of a Xenopus histone H2B gene (21). The regulatory elements required for maximal transcription in oocytes, which are naturally arrested in G2, before the first meiotic division, are remarkably similar to those required for basal expression of a human histone H2B gene in cells arrested at the G1/S phase boundary (32). Although the sequences of these vertebrate histone gene promoters are quite divergent, the functional promoter in each case consists of TATA, CCAAT, and ATF (CREB)-like motifs in nearly identical configurations. These structural and functional similarities strongly suggest that the accumulation of histone mRNA and protein during amphibian oogenesis is accomplished through basal transcription, accompanied by an increased stability of histone mRNAs, rather than through the activation of a set of germ cell-specific transcriptional regulatory elements.

To further extend these studies, we examined the regulation of Xenopus histone H2B gene transcription in embryos. We report here that the octamer motif in the H2B promoter is a functional promoter element during early embryogenesis. Octamer-dependent H2B gene transcription in embryos was not due to increased binding by Oct-1 but was strictly dependent on the presence of a CCAAT motif. These results demonstrate for the first time that the octamer motif is required for maximal H2B transcription in developing vertebrate embryos and suggest that synergistic interactions between Oct-1 and other factors bound to the Xenopus H2B promoter are important for octamer-dependent H2B transcription. Because the regulatory sequences that control amphibian histone H2B transcription in developing embryos are the same as those required for S-phase-specific transcription of mammalian histone H2B genes in cultured cells, these results support the hypothesis that histone gene transcription is coupled to the cell cycle early in amphibian development.

MATERIALS AND METHODS

Plasmids. The histone genes used in these studies contain synthetic in-frame linkers that allow transcripts from the exogenous genes to be distinguished from those of the endogenous genes (18). All of the H2B mutant templates except the double mutants have been previously described (21). The A'-C'O-'(ATF'-CCAAT' octamer') double mutant was generated by oligonucleotide site-directed mutagenesis with a single-stranded M13/H2B LS(-50/-44) template and previously described methods (21). The A'-C'O and A'-C'O- mutants were generated by the polymerase chain reaction, using pLS(-79/-74), pLS(-50/-44), and mutagenic oligonucleotide primers. The resulting products were cloned into pUC19, and the plasmids were confirmed by restriction enzyme digests andideoxynucleotide sequence analysis.

Preparation of Xenopus oocytes and embryos. Adult Xenopus laevis frogs were obtained from Xenopus I (Ann Arbor, Mich.). Ovaries were removed from adult female frogs, washed several times in modified Barth's solution (MBS) (6), and then swirled in MBS containing 2 mg of collagenase (type IA; Sigma Chemical Co., St. Louis, Mo.) per ml at room temperature for 2 to 4 h to remove follicle cells. Egg laying was induced by injecting 100 U of pregnant mare serum gonadotropin into the dorsal lymph sac on day 1 and 500 U of human chorionic gonadotropin on day 2. On day 3, eggs were manually stripped into petri dishes. Testes were surgically removed from adult male frogs, and a sperm suspension was then prepared in MBS by teasing apart the testes with forceps. Eggs were incubated for 15 min in a minimal volume of sperm suspension to allow sperm to adhere prior to fertilization, which was then initiated by flooding the eggs with 0.1X MBS. After 15 min, eggs were dejellied for 5 min in 2.5% L-cysteine-0.6% Tris (pH 7.9), after which they were washed extensively in MBS. Oocytes and embryos at desired stages (36) were transferred to microcentrifuge tubes (30 oocytes or embryos per tube), and the excess fluid was removed; then they were quickly frozen on dry ice. Frozen embryos were stored at -80°C until further use.

Embryo injections and analysis of RNA. Dejellied embryos were transferred to MBS containing 4% Ficoll (Sigma) and injected at the two-cell stage with 20 nl of a solution containing 100 pg of a supercoiled test H2B template and 100 pg of a supercoiled reference (histone H1 or H2A) template in 10 mM Tris (pH 7.4)-0.5 mM EDTA. Sets of 20 embryos were injected for each template tested. Three to four hours after fertilization, embryos were transferred to 0.1X MBS and maintained there until gastrulation (stage 10.5). In preliminary studies, transcription of exogenous Xenopus histone H2B genes was first detected shortly after the midblastula stage and increased to high levels by the midgastrula stage approximately 11 h after fertilization; therefore, injected embryos were allowed to reach gastrulation (stages 10 to 11) before collection. Nucleic acid extractions and S1 nuclease protection assays were performed as previously described (21). Gene-specific transcripts were quantitated by scanning autoradiographs with a Bio-Rad model 620 densitometer (Bio-Rad Laboratories, Richmond, Calif.) and by analyzing dried gels with a Betascope model 603 blot analyzer (Betagen, Waltham, Mass.). Transcription of each H2B promoter tested was normalized to that of the injected reference gene (H1 or H2A) and expressed relative to the transcription of the wild-type H2B promoter. The replication of each test template relative to that of the reference template in injected embryos was examined by wavease chain reaction amplification of plasmid sequences recovered from injected embryos, using primers specific to the injected plasmids. Amplified DNA was subjected to electrophoresis on agarose gels, transferred to nylon membranes, and hybridized with probes specific to the amplified region of the test and reference templates. Specific hybridization was directly quantitated with the Betascaner.

Extract preparation. Thirty oocytes or embryos in a 1.5-ml microcentrifuge tube were placed in 0.45 ml of a homogenization buffer (10 mM Tris [pH 7.8], 10 mM 1,4-dithiothreitol, 0.1% Triton X-100, 5 mM EDTA) and homogenized in an OMNI 2000 homogenizer with a 5-mm-diameter generator (OMNI International, Waterbury, Conn.) at 700 to 800 rpm for 30 to 60 s. Homogenates were mixed for 15 min at 4°C on a rotating platform and then centrifuged at 12,000 × g for 10 s. The supernatant was transferred to a clean 1.5-ml microcentrifuge tube, melting-point bath oil (Sigma) was layered over the supernatant, and the supernatant was recentrifuged for 30 min. Aliquots of the second supernatant were then transferred to clean tubes and stored at -80°C. Protein concentrations, measured by the Bradford protein assay (Bio-Rad), were typically 1 to 5 mg/ml. The use of a low-ionic-strength buffer with a nonionic detergent resulted in a more efficient extraction of factors binding to the H2B gene.
promoter with less proteolysis and fewer nonspecific DNA-binding proteins than in our previous method (21).

**DNA-binding studies.** DNA-binding studies were performed with 5 to 10 μl of oocyte and staged embryo extracts. Synthetic oligonucleotides containing the consensus octamer-binding site (OCTA), the variant octamer motif (H2B-OCTA), an ATF (CREB)-binding site (13, 15, 23), a TEF-1-binding site (8, 51), and an ATF/TEF-1-binding site have been previously described (21). The oligonucleotide containing a CCAAT-binding site had the sequence 5'-GCATT AGCCAGGCAATGGGAATAGC-3' (the underlined region denotes sequences derived from the H2B promoter). Oligonucleotides were labeled at their 3' ends by using the Klenow fragment of DNA polymerase I and [α-32P]dATP. Standard binding reactions were carried out in 30 μl of 10 mM Tris (pH 7.8)-5 mM MgCl2-0.5 μg of poly(dl-dC)-10 mM 1,4-dithiothreitol-1% Ficoll-50,000 cpn (0.2 to 0.5 ng) of the labeled oligonucleotide; the mixture was incubated for 5 min at 4°C prior to addition of labeled oligonucleotide. Antisera and oligonucleotide competitors were added prior to addition of labeled probe. After incubation for 30 min at 4°C, the mixture was subjected to electrophoresis on a 6% polyacrylamide gel in 0.5× TBE (45 mM Tris, 45 mM boric acid, 0.5 mM EDTA). Autoradiography on dried gels was performed at −20°C for 1 to 2 h, using Kodak XAR5 film and intensifying screens.

A rabbit polyclonal antiserum directed against bacterially expressed Xenopus Oct-1 and Oct-91 was prepared as previously described for the Oct-60 antiserum (22). For DNA-binding reactions, 1 μl of either preimmune serum or immune serum was used. The Oct-60 antiserum was diluted 1:10 in binding buffer, and the Oct-91 antiserum was diluted 1:3.

**RESULTS**

A variant octamer motif is required for H2B gene transcription in embryos. It is not known whether histone gene transcription during early amphibian development is coupled to the cell cycle, as described for somatic cells, or independent of DNA replication, as in oocytes. This issue was addressed by examining the cis-acting regulatory sequences required for Xenopus H2B gene transcription in developing embryos. Our hypothesis, based on previous studies using synchronized cell cultures, was that S-phase-specific H2B transcription should be dependent on the octamer motif in the H2B promoter. Initial studies performed with 5'-deletion mutants showed that sequences upstream of −100 were not required for H2B transcription in embryos (data not shown). Therefore, we tested the expression in embryos of a set of linker scanning mutants that span the H2B promoter. Plasmid templates were injected into dividing embryos at the two-cell stage, and the accumulation of accurately initiated transcripts was analyzed in gastrula-stage embryos after the activation of zygotic transcription. The relative abundance of the test and reference templates recovered from the embryos was measured to control for possible effects of specific mutations on replication of the H2B templates; however, differential replication due to altered promoter sequences was not observed (data not shown).

Three main observations emerged from these results. The first is that regulatory sequences required for maximal H2B transcription in oocytes were also functional in developing embryos. Nucleotide substitutions that abolished the TATA element, LS(−32/−25), and the CCAAT box, LS(−79/−74), each resulted in a five- to sixfold reduction in transcriptional activity of the H2B promoter (Fig. 1). Additionally, the LS(−85/−80) mutant, which altered sequences immediately upstream of the CCAAT box and reduced transcription in oocytes, also impaired H2B transcription in embryos, presumably by reducing the interactions of proteins that interact with the CCAAT motif. Several distinct proteins are known to bind to the CCAAT motif, and sequences flanking this site are important for determining binding specificity (5, 10, 43). Mutations in the −100 region also reduced H2B transcription in embryos. Previous DNA-binding studies using DNase I protection and gel mobility shift assays showed that a factor

**FIG. 1.** Analysis of promoter elements required for H2B transcription in injected frog embryos. (A) Two-cell embryos were injected with supercoiled plasmids containing mutant H2B promoters, as indicated above each lane. A plasmid containing a histone H2A gene was cojected as an internal control. Embryos were collected at the gastrula stage (stage 10.5), and transcription of the injected genes was analyzed by S1 nuclease protection. The protected fragments expected for accurately initiated transcripts are indicated at the left. The transcript from the LS(+3/+/8) template protected a slightly shorter fragment than did the wild-type H2B promoter because the site of mutagenesis for this template was downstream of the transcription initiation site, resulting in a discontinuity between the probe and the transcript. (B) A separate set of embryos were injected as described above except that a different histone gene (H1) was used as an internal control. (C) The transcription of each H2B promoter mutant was normalized to the activity of the internal control gene and then expressed relative to the transcription of the wild-type H2B promoter. A schematic representation of the Xenopus H2B promoter is shown at the bottom, with wild-type activity arbitrarily set to 1.0.
The absence of neurula expression H2B control. The internal transcription. Templates to correspond to at least five adjacent ATF motif varied between batches of developing embryos. The reduced transcription of the wild-type H2B gene is indicated by plus signs, each of which is expressed at approximately one-third of the level of the wild-type H2B promoter; a minus sign signifies no detectable H2B transcription. Templates marked n.t. were not tested.

These results also showed that an octamerlike element adjacent to the TATA box was required for full activity of the H2B promoter (Fig. 1 and 2). Site-directed mutagenesis of the H2B octamer motif resulted in a template, pLS(−50/−43), that was transcribed at one-third to one-fourth the efficiency of the wild-type H2B promoter in late-blastula-through neurula-stage embryos (Fig. 1; Fig. 2A, lanes 2 and 8). The reduced transcription of an internal deletion mutant that removed the octamer site (Fig. 2A, lane 3) was restored by the insertion of a synthetic oligonucleotide containing this site (Fig. 2A, lane 4). The effect of mutagenesis of the octamer motif on transcription in embryos is remarkably similar to that seen in previous studies that demonstrated octamer-dependent transcription of a human histone H2B promoter in an in vitro transcription system (44) and in synchronized, transfected cells (32). Because full activity of the H2B promoter was dependent on the octamer motif in late-blastula-stage embryos, it appears that the H2B octamer element is active in developing embryos soon after transcriptional activation of the embryonic genome. The results suggest that the octamer motif may participate in the initial activation of H2B transcription during the midblastula transition and that, on the basis of the precedents with synchronized cell cultures, Xenopus histone gene transcription may be coupled to the cell cycle by stage 9, if not earlier.

Finally, the H2B octamer motif (H2B-OCTA) alone was not sufficient to activate transcription of a minimal promoter consisting of the H2B TATA box. Multiple copies of the H2B-OCTA site did not activate transcription of the H2B TATA box in the absence of upstream regulatory elements despite the close proximity of these elements (Fig. 2A; compare lanes 5 and 9 with lanes 6 and 10). This result suggests that proteins bound to one or more nearby sites (i.e., the CCAAT and ATF motifs) are necessary for octamer-dependent H2B transcription.

Requirement of the CCAAT element for octamer-dependent H2B gene transcription. To test the hypothesis that the activity of the H2B octamer motif requires the presence of an additional regulatory element or elements and to identify which sequences were required for octamer-dependent H2B transcription, additional mutations were introduced into promoters that contained altered ATF, CCAAT, and octamer elements. The resulting promoters contained site-specific mutations in pairwise combinations of these three elements and were analyzed in injected embryos as described above. The results of one experiment are shown in Fig. 3A, and the data from three separate experiments are...
FIG. 4. Formation of a specific complex between the ATF promoter element and extracts from oocytes and embryos. (A) Extracts from oocytes (lane 1) and staged embryos (lanes 2 to 12) were used in an electrophoretic mobility shift assay with an oligonucleotide probe containing the Xenopus H2B ATF/TEF-1-binding site. The specific complex formed with this probe is indicated by the arrow on the right. (B) A gastrula-stage (stage 10.5) embryo extract was used in an electrophoretic mobility shift assay with an oligonucleotide probe containing either an ATF-, TEF-1-, or ATF/TEF-1-binding site, as indicated. The specificity of each complex formed was tested by adding a 150-fold molar excess of unlabeled homologous oligonucleotide competitor (lanes 2, 5, and 8) or unlabeled nonspecific oligonucleotide competitor (lanes 3, 6, and 9).

The activity of the wild-type H2B gene (A+C+O+) was arbitrarily set to 100 U. Individual regulatory elements were assigned activity values based on the amount of accurately initiated mRNA produced and assuming that each element functioned independently. The −33 5′-deletion mutant, which contained only the H2B TATA box, was transcribed 10-fold less efficiently than the wild-type H2B promoter (Fig. 3A, lanes 1 and 2); therefore, 10 U was taken as the activity of the H2B TATA element. An activity of 6 U was assigned to the ATF element, since the double mutant that disrupted both the CCAAT and octamer elements (A+C−O−) produced 16 U of mRNA, of which 10 U was due to the TATA element. Activities of 20 U for the CCAAT element and 1 U for the octamer element were similarly assigned. These results show that in the presence of the TATA box, a single ATF or CCAAT element was able to activate H2B transcription, whereas the H2B octamer site alone was insufficient in this regard.

The value determined for the activity of each promoter element was used to predict the amount of correctly initiated mRNA that would be produced by combinations of independently acting promoter elements; the predicted values were then compared with those actually observed (Fig. 3B). Thus, the observed value of 36 U for the promoter containing intact ATF and CCAAT motifs (A+C+O+) is very close to the predicted value of 39 U (Fig. 3B). This result suggests that in the context of this promoter, the ATF and CCAAT elements function independently in activating H2B transcription. The ATF element also appeared to act independently of the octamer motif, since the observed and predicted amounts of transcription of the promoter containing these elements (A+C−O+) were identical. In contrast, the activity observed when the CCAAT and octamer motifs were present together (A−C+O+) was more than twice the sum of the individual activities. This result shows that the activity of the H2B octamer site is dependent on the CCAAT motif. This conclusion is consistent with results showing that single and multiple copies of the octamer site did not activate H2B transcription and that the activity of the ATF site was independent of the CCAAT and octamer motifs. The sum of the activities of each individual promoter element (37 U) did not account for the activity of the intact promoter. Even considering the cooperativity of the octamer motif and the CCAAT box, the observed activity of the promoter in embryos was still about 50% more than predicted (71 U). These results suggest that functional interactions that take place in the intact promoter do not occur when individual regulatory elements are examined in isolation or in simple combinations.

Proteins that bind to the H2B ATF and CCAAT elements increase slightly in abundance during early development. The H2B ATF element is located adjacent to a potential TEF-1 (AP-5)-binding site (8, 51), but only the ATF element formed a specific complex with proteins from oocyte extracts (21). To determine whether embryos express novel factors that interact with this region, we performed electrophoretic mobility shift assays using an oligonucleotide that contains both binding sites. Incubation of the ATF/TEF-1 probe with extracts from oocytes and staged embryos resulted in the formation of two electrophoretically distinct complexes (Fig. 4A). The more rapidly migrating complex was present at a constant low level in embryos from all developmental stages tested. In contrast, the more abundant, slowly migrating complex (indicated with an arrow) was present at a relatively
constant level in oocytes and embryos until late blastula stage (stage 9.5) and then steadily increased in abundance by approximately threefold through the early neurula stage (stage 14).

To determine which element was responsible for complex formation, oligonucleotides containing either an ATF- or TEF-1-binding site or the combined ATF/TEF-1-binding sites were tested with early-gastrula-stage (stage 10.5) extracts. Both the ATF and ATF/TEF-1 oligonucleotides formed two complexes similar to those previously observed (Fig. 4B, lanes 1 and 7). Only the less abundant, more rapidly migrating complex was formed with the TEF-1 oligonucleotide (lane 4). The sequence specificity of the complexes was determined by using homologous and heterologous oligonucleotide competitors. The rapidly migrating complexes formed with ATF and ATF/TEF-1 probes and the complex formed with the TEF-1 oligonucleotide appeared to be nonspecific, since their formation was inhibited by an excess of the homologous oligonucleotides and by an oligonucleotide containing a consensus octamer-binding site. In contrast, the more slowly migrating complex formed with ATF and ATF/TEF-1 oligonucleotides was competed for only by an excess of the homologous unlabeled oligonucleotide. The ATF oligonucleotide also disrupted formation of the specific ATF/TEF-1 complex (data not shown), demonstrating that proteins present in gastrula-stage embryo extracts interact specifically with the ATF element and suggesting that the activity of the H2B ATF motif during early embryogenesis is mediated by a protein or proteins similar to those present in oocytes.

The ability of the CCAAT motif to promote the activity of the H2B octamer site in embryos but not in oocytes suggests that the CCAAT-binding proteins in embryos may be distinct from those in oocytes. To investigate whether distinct CCAAT-binding proteins were differentially expressed during embryogenesis, we analyzed the complexes formed with an oligonucleotide probe containing the H2B CCAAT motif. Two complexes were formed when this probe was incubated with oocyte and embryo extracts (Fig. 5A). The more rapidly migrating complex was present at fairly constant levels throughout early development and was not specific, since its formation was inhibited by an excess of either the homologous oligonucleotide or a heterologous competitor (Fig. 5B). The more slowly migrating complex formed with the CCAAT motif appeared to increase in abundance during development in a biphasic manner. The amount of this complex formed in oocyte extracts increased approximately fivefold by stage 6, remained constant until early gastrulation, and then increased gradually during subsequent development (Fig. 5A). The magnitude of the initial increase of this complex during early development was variable and appeared to be due to the fact that the oocytes used for these extracts were not obtained from the same female used to prepare the embryos. Similar results were obtained with extracts from two independent sets of staged embryos (data not shown). The slowly migrating complex formed with stage 10.5 embryo extract was judged specific, since excess CCAAT oligonucleotide (Fig. 5B, lane 2) but not OCTA oligonucleotide (lane 3) was able to disrupt its formation. Identical results were obtained with competition studies using an oocyte extract (data not shown). The simplest interpretation of these results is that the same protein or proteins interact with the H2B CCAAT element in oocytes and early embryos. However, the formal possibility exists that oocytes and embryos contain different CCAAT-binding proteins with similar electrophoretic mobilities.

**Formation of multiple octamer-specific complexes with embryo extracts.** Octamer-dependent H2B transcription in cycling cells is thought to be mediated by Oct-1. Oct-1 in oocytes binds specifically to a consensus octamer site, but its binding to the octamer motif in the *Xenopus* H2B promoter is much weaker (21). It is therefore possible that the increased transcriptional activity of the H2B octamer motif in embryos was due to an increased binding to this site by Oct-1 or other octamer-binding proteins.

At least three distinct complexes were formed when an oligonucleotide containing a consensus octamer-binding site (OCTA) was incubated with extracts from oocytes and embryos (Fig. 6A, lanes 1 to 12). The most slowly migrating complex (Cl) was present at a low level in oocytes, increased two- to threefold by the 16-cell stage (lane 3), and then remained relatively constant in abundance until late gastrulation. The most rapidly migrating complex was present at a very low level in oocytes but increased significantly in early-cleavage-stage embryos. The abundance of this complex remained constant until early gastrulation, after which it quickly disappeared. A third complex with an intermediate electrophoretic mobility was first detected at late blastula stage (stage 9), was present at a constant level until late gastrula stage (stage 11.25), and then declined to a low level during neurulation.

A surprisingly different binding pattern was observed when an oligonucleotide containing the *Xenopus* H2B octamer-binding site (H2B-OCTA) was used as a probe (Fig. 6A, lanes 13 to 24). The major complex formed with H2B-OCTA had an electrophoretic mobility indistinguishable from that of the Cl complex formed with OCTA, although significantly less Cl was formed with H2B-OCTA. Interestingly, a large increase in the amount of the Cl complex formed with H2B-OCTA occurred in rapidly cleaving em-
bryos, after which there was a sharp decline to the low maternal level by the early gastrula stage (stage 9), which was subsequently maintained throughout early development. Identical results were obtained for several extract preparations from independent sets of staged embryos. The basis for the increase in $C_1$ during cleavage is not known, but since the increase was transient, the amount of $C_1$ in embryos after the midblastula transition was nearly identical
to the maternal level observed in oocytes. Similar changes were not observed when other sites, including the ATF, CCAAT, and consensus octamer sites, were used as probes.

The sequence specificity of the complexes formed with the consensus octamer site and the H2B octamer motif was tested by using oligonucleotide competitors. All three complexes formed with the OCTA oligonucleotide and early-gastrula-stage (stage 9.5) extracts (Fig. 6B, lane 1) were disrupted by an excess of unlabeled OCTA oligonucleotide (lane 2), whereas a similar amount of an unrelated ATF oligonucleotide had no effect (lane 3). Oligonucleotide competitions were also performed with the H2B-OCTA oligonucleotide probe and an early-cleavage-stage (stage 6) extract (lanes 8 to 21). At least three complexes were formed with the H2B-OCTA probe. The most abundant complex (C1) had the most reduced electrophoretic mobility and was specifically competed for with an excess of the OCTA, but not the ATF, oligonucleotide. The least abundant and most rapidly migrating complex also appeared to be specific, since its formation was inhibited by the OCTA oligonucleotide but not by the ATF competitor (lanes 15 to 17). In contrast, an intermediate complex was not sequence specific. An excess of oligonucleotide containing the ATF site actually increased the level of this complex (lane 10). However, this increase was seen only with the ATF oligonucleotide; other non-specific competitors prevented formation of this complex (data not shown). No additional sequence-specific complexes were observed when extracts from embryos were incubated with the H2B octamer site.

A polyclonal antiserum directed against bacterially expressed *Xenopus* Oct-1 was used to determine which, if any, of the complexes contained Oct-1. The addition of immune serum to reactions containing the consensus octamer site significantly inhibited C1 formation in extracts from gastrula-stage embryos (Fig. 6B, lane 5), whereas the other two complexes were not affected. Moreover, normal rabbit serum did not affect complexes formed with the OCTA probe (lane 4). An extract from early-cleavage-stage (stage 6) embryos was used to determine whether Oct-1 was present in C1 formed with the H2B-OCTA oligonucleotide. This developmental stage was chosen for investigating whether the elevated level of the C1 complex observed during cleavage was due to Oct-1 or to other octamer-binding proteins with mobilities similar to that of Oct-1. The results show that the addition of the Oct-1 antiserum specifically and quantitatively recognized C1 formed with the H2B-OCTA probe (lanes 12 and 19). Similar results were obtained when a gastrula-stage extract was used with the H2B-OCTA probe (data not shown). Therefore, *Xenopus* Oct-1 binds to both the consensus octamer site and the H2B octamer motif and is responsible for the formation of C1.

At least three other octamer-binding proteins are expressed during early development (22). To determine whether these proteins were present in complexes formed with the consensus and H2B octamer motifs, we tested the ability of a polyclonal antiserum directed against bacterially expressed proteins to recognize the complexes. An antiserum that recognizes the maternally expressed POU domain protein Oct-60 specifically disrupted the formation of the most rapidly migrating complex formed with both probes (Fig. 6B, lanes 7 and 21). An antiserum directed against the POU domain protein Oct-91 specifically recognized the intermediate complex formed with the OCTA probe (lane 6) but not any of the complexes formed with the H2B-OCTA site. These results show that at least one POU domain protein (Oct-60) in addition to Oct-1 specifically recognizes the H2B octamer motif. Oct-91, which formed a specific complex with the consensus octamer motif, did not bind stably to the H2B-OCTA site, demonstrating that octamer-binding proteins in early embryos vary in the ability to interact with the H2B promoter element. The much greater abundance of the complex containing Oct-1 compared with that of the other complexes formed with the H2B-OCTA site strongly suggests that the activity of the H2B octamer motif in embryos is mediated by Oct-1.

**Relative binding efficiency of Oct-1 to the H2B octamer site and a consensus octamer site.** Two observations suggest that Oct-1 in embryos may be functionally heterogeneous. First, Oct-1 appeared to bind less efficiently to the H2B octamer motif than to a consensus octamer site. This finding might simply reflect an overall reduced affinity of Oct-1 for the H2B octamer site, or it might indicate the binding of a distinct subpopulation of Oct-1. Second, Oct-1 from rapidly cleaving embryos showed a dramatic transient increase in binding to H2B-OCTA but not to the consensus octamer motif, again suggesting that different populations of Oct-1 were bound to each site. To investigate these possibilities, oligonucleotide competitions were performed with both binding-site probes and an extract from gastrula-stage embryos. Increasing amounts of an oligonucleotide containing the consensus octamer site specifically and efficiently inhibited the binding of embryo Oct-1 to the OCTA site probe (Fig. 7, lanes 1 to 4). The binding of embryo Oct-1 to the OCTA oligonucleotide was significantly reduced by the addition of a 0.5- to 1.5-fold molar excess of unlabeled homologous competitor. More than a 50- to 150-fold molar excess of H2B-OCTA was required to achieve a similar degree of competition (lanes 5 to 10). A vast excess of an oligonucleotide containing an unrelated binding site (ATF/TEF-1) did not inhibit Oct-1 binding to the consensus site (lane 11). Similar results were obtained when the H2B octamer site was used as a probe (lanes 12 to 26). As was seen when the OCTA oligonucleotide was used as probe, a 0.5- to 1.5-fold molar excess of OCTA oligonucleotide significantly reduced Oct-1 complex formation with the H2B-OCTA probe, whereas a 50- to 150-fold molar excess of H2B-OCTA was required to achieve a similar degree of competition. These results show that Oct-1 from embryos binds to the H2B octamer site with a 100-fold-lower affinity than to the consensus octamer-binding site and suggest that these sites bind the same population of Oct-1 molecules.

**DISCUSSION**

Regulatory sequences and factors required for transcription of a histone H2B promoter in developing frog embryos were analyzed to investigate a transition in histone gene expression that was predicted to occur during amphibian early development. Full activity of the H2B promoter in embryos required multiple, positive-acting promoter elements. Three of these regulatory sequences (the ATF [CREB] motif, the CCAAT box, and the TATA box) are structurally homologous to regulatory elements required for expression of a human H2B histone gene in S-phase and non-S-phase cells, suggesting that these elements participate in induced as well as basal expression of the H2B gene throughout the cell cycle. This view is supported by our previous report that these H2B promoter elements are required for expression of the *Xenopus* H2B gene in oocytes (21). A fourth regulatory element required for H2B gene expression in embryos consists of a conserved subtype-
From the study of our early development, transcription of the H2B gene octamer during development is conserved and requires cooperative interactions with additional factors bound to the CCAAT box. The activity of Oct-1, which interacts synergistically with other cellular and viral promoters, also strengthens by hormone-responsive transcription in vitro but not for uninduced expression. Thus, the octamer motifs in the MMTV and H2B promoters may have similar functions, since they each mediate induced transcription but not basal expression. The U1, U2, and U3 small nuclear RNA genes are transcribed by RNA polymerase II, but they lack TATA boxes and appear to use a transcriptional mechanism that is qualitatively different from that which activates mRNA synthesis 

Recent studies have shown that the octamer motif is required for expression of the early H2B histone gene in developing sea urchin embryos (3). These results suggest that the function of the H2B octamer site has been highly conserved during metazoan evolution. The activity of the H2B octamer motif in developing embryos suggests that histone gene transcription is coupled to the cell cycle during early development, probably when zygotic histone gene transcription is initiated, and provided experimental support for a transition in the regulation of histone gene transcription during amphibian early development. This idea is further strengthened by the recent observation that histone mRNA accumulation in developing frog embryos is dependent on ongoing DNA synthesis (42). The observation that H2B transcription was dependent on the octamer motif during amphibian embryogenesis suggests that a significant fraction of histone expression is S-phase specific after the initiation of zygotic transcription. This could result from a particularly low degree of basal histone gene transcription due to the short G1 and G2 periods that occur during early Xenopus development (14, 35).

Analysis of the activity of individual promoter elements in our study shows that H2B transcription in embryos resulted from multiple synergistic interactions among factors bound to the promoter. By systematically altering single and multiple regulatory elements in the H2B promoter, we found that activity of the H2B octamer motif in embryos was dependent on the CCAAT box. The activity of octamer motifs in certain other cellular and viral promoters also requires cooperative interactions with additional promoter elements. Oct-1 interacts synergistically with factors bound to a hormone-responsive element in the mouse mammary tumor virus (MMTV) promoter (4), the SPH motif in the chicken U1 distal sequence element (DSE) (41), the Sp1 motif in the U2 DSE (27), and a CCAAT motif in the U3 DSE (1). The octamer site in the MMTV promoter is required for hormone-induced transcription in vitro but not for uninduced expression. Thus, the octamer motifs in the MMTV and H2B promoters may have similar functions, since they each mediate induced transcription but not basal expression. The U1, U2, and U3 small nuclear RNA genes are transcribed by RNA polymerase II, but they lack TATA boxes and appear to use a transcriptional mechanism that is qualitatively different from that which activates mRNA synthesis (46, 48). Nevertheless, the cooperativity between the octamer site and a variety of regulatory elements in different promoters suggests a common mechanism of action for Oct-1, which appears to mediate the activity of octamer motifs in the promoters of ubiquitously expressed genes. Cooperation between the octamer motif and other transcriptional regulatory elements is presumably based on interactions, perhaps indirect, between Oct-1 and proteins bound to the other sites. Our results showed that the activity contributed by each element to the TATA motif was always less than the decrease in activity observed upon mutagenesis of that element. This finding indicates that the H2B promoter elements are more active when present in an intact promoter than when present in isolation and strengthens the conclusion that the activity of the H2B promoter in embryos is due to multiple synergistic interactions. A similar analysis of the function of these elements in oocytes indicates that functional cooperativity among factors bound to the H2B promoter is involved in basal H2B transcription, which is independent of the octamer motif (21). It is possible that Oct-1 increases the basal activity of the protein or proteins bound to the CCAAT motif rather than independently activating transcription in embryos. In this regard, octamer-dependent transcription could result from protein interactions that stabilize the binding of proteins to nearby regulatory elements (e.g., the H2B CCAAT motif) without necessarily introducing additional activation domains.

The binding of Oct-1 to the H2B octamer site was inefficient relative to its binding to a consensus octamer motif and
was increased transiently during early cleavage. It is unlikely that the increased Oct-1 binding is directly responsible for octamer-dependent H2B transcription, because binding to the H2B octamer site returned to the basal maternal level before the activation of transcription at the midblastula stage. Binding of proteins to each of the H2B promoter elements in the developmental stage used for these studies (stage 10.5) was increased two- to threefold, in contrast with the 10\(^6\) - to 10\(^7\)-fold increase in the number of cells and the amount of DNA during this time. We conclude that the transition from basal H2B transcription in oocytes to octamer-dependent transcription in embryos is mediated by changes in properties other than the binding of Oct-1 to the H2B octamer site. It is possible that increased binding of Oct-1 to the H2B promoter during early cleavage prevents inactivation of the H2B promoter before transcriptional activation by precluding the assembly of nucleosomes over this region. However, assuming that Oct-1 could maintain the H2B promoter in an active chromatin conformation, this mechanism of action appears insufficient to account for the functional cooperativity between the CCAAT box and the octamer motif.

Our interpretations of the data are summarized in Fig. 8. Our current working model for H2B transcription in oocytes is shown in Fig. 8A. Maternal factors that bind to the ATF and CCAAT sites appear to interact with a transcription initiation complex formed over the H2B TATA motif and transcription initiation site. We presume that these factors interact indirectly with TFIID, although this has not been shown for the H2B promoter. The binding of Oct-1 to the H2B promoter does not activate transcription in oocytes, presumably because it is unable to sustain an interaction with the CCAAT-binding protein. Moreover, the weak affinity of Oct-1 for the H2B octamer motif may be insufficient to permit stable binding to the H2B promoter in lieu of additional interactions. Three different, although not necessarily mutually exclusive, models that might account for octamer-dependent H2B transcription in embryos are shown in Fig. 8B to D. The first model (Fig. 8B) suggests that Oct-1 and the CCAAT-binding protein interact via an adapter molecule that stabilizes the binding of Oct-1 to the H2B promoter. The proposed adapter molecule would be functionally analogous to cellular factors that mediate the interaction of Oct-1 with the herpes simplex virus transactivator VP16 (12, 30, 45) except that it would instead promote interactions of Oct-1 with the protein bound to the H2B CCAAT box and might introduce an additional activation domain into the transcription complex. At present, there is no direct evidence suggesting that Oct-1 in embryos is present in a complex with other proteins, although it is possible that the conditions used in our binding studies were not favorable. The two other models are similar to each other and propose that posttranscriptional modifications of Oct-1 (Fig. 8C) or the CCAAT-binding protein (Fig. 8D) result in interactions between these proteins. A feature common to all three models is that octamer-dependent H2B transcription is controlled by alterations in the interactions of Oct-1 with the CCAAT-binding protein. As a consequence of this interaction, we predict that the binding of Oct-1 and the CCAAT-binding protein to the promoter would be increased and activation domains associated with Oct-1 or the adapter molecule would become available to facilitate H2B transcription. In principle, strengthened interactions of the CCAAT-binding protein with the H2B promoter could be sufficient to activate H2B transcription in an octamer-dependent manner.

The modification of preexisting factors, for example by phosphorylation, would be a convenient pleiotropic mecha-
nism for rapid and reversible control of their activity. Indeed, Oct-1 is phosphorylated on multiple sites in a cell cycle-dependent manner (40). Phosphorylation of Oct-1 during the G2 and M phases of the cell cycle decreases the ability of Oct-1 to bind DNA and provides a potential mechanism for inactivating octamer-dependent transcription at the end of S phase (40). The transient increase in binding of Oct-1 to the H2B octamer motif in extracts from early embryos might reflect an increase in Oct-1 binding activity due to the absence of G1 and G2 phases during rapid cleavage. The results described here show that the activity of the H2B octamer motif and, we presume, the ability of Oct-1 to activate H2B transcription are dependent on the presence of other functional promoter elements. Although attention has been focused on modifications of Oct-1, the regulation of the interactions of this protein with other factors could also be accomplished by modifications of the other components of these interactions.

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