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Interaction of the CCAAT Displacement Protein with Shared Regulatory Elements Required for Transcription of Paired Histone Genes

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The H2A and H2B genes of the *Xenopus xlh3* histone gene cluster are transcribed in opposite directions from initiation points located approximately 235 bp apart. The close proximity of these genes to one another suggests that their expression may be controlled by either a single bidirectional promoter or by separate promoters. Our analysis of the transcription of histone gene pairs containing deletions and site-specific mutations of intergenic DNA revealed that both promoters are distinct but that they overlap physically and share multiple regulatory elements, providing a possible basis for the coordinate regulation of their in vivo activities. Using the intergenic DNA fragment as a probe and extracts from mammalian and amphibian cells, we observed the formation of a specific complex containing the CCAAT displacement protein (CDP). The formation of the CDP-containing complex was not strictly dependent on any single element in the intergenic region but instead required the presence of at least two of the three CCAAT motifs. Interestingly, similar CDP-containing complexes were formed on the promoters from the three other histone genes. The binding of CDP to histone gene promoters may contribute to the coordination of their activities during the cell cycle and early development.

Histone genes are ubiquitously expressed in eukaryotic cells, usually in a cell cycle-specific manner, resulting in the accumulation of equimolar amounts of the nucleosome core histones H2A, H2B, H3, and H4. In most cell types, histone protein and RNA synthesis are primarily restricted to the S phase of the cell cycle and require ongoing DNA synthesis (for reviews, see references 17, 18, 26, and 30). Histone gene expression is regulated by both transcriptional and posttranscriptional mechanisms. In cycling cells, transcription of members of each of the five histone gene classes is coordinately activated upon entry into S phase and is then extinguished at the end of the DNA replication period. Histone gene promoters have no obvious transcription factor-binding motifs in common, aside from TATA and CCAAT motifs, leading to the idea that coordinate histone gene transcription is not regulated through the action of a single, common *cis*-acting element (17, 30). Regulatory elements and *trans*-acting factors responsible for basal transcription and S-phase-specific transcription of human H1, H2B, and H4 genes have been identified. An H1 subtype-specific element and a CCAAT motif required for S phase-specific transcription of the H1 promoter interact with distinct factors (13). Activation of H2B and H4 gene transcription during S phase is thought to require the interaction of positive-acting factors with H2B and H4 subtype-specific *cis*-acting elements (reviewed in reference 17).

Histone gene transcription is usually restricted to S phase of the cell cycle, although examples of non-cell cycle-regulated expression of histone gene subclasses are known. Many organisms have distinct sets of histone genes that are transcribed independently of DNA replication (for example, mouse testis-specific histone genes [21]). In *Xenopus laevis*, the same histone genes are transcribed in both DNA replication-dependent and replication-independent modes (29, 34). Histone genes are transcribed in the absence of DNA replication in oocytes which are arrested in the G_2 phase of the first meiotic division. Massive stores of histone mRNA and protein accumulate during oogenesis in preparation for the rapid rounds of cell division that occur during early development. Transcription ceases upon oocyte maturation and resumes after the midblastula transition, when transcription of zygotic genes begins. Histone gene transcription is DNA replication dependent in most cells of postmidblastula transition embryos and adult frogs (29, 35, 40). The mechanisms underlying this change in histone gene transcriptional regulation, from constitutive or basal expression in oocytes to S-phase-specific expression in embryos, are not understood. The dual modes of *Xenopus* histone gene transcription provide an opportunity to investigate mechanisms that regulate transcription during the cell cycle.

Histone genes in *X. laevis* are arranged in several clusters of tandemly repeating units, with each unit consisting of single copies of each of the histone H1, H2A, H2B, H3, and H4 genes (33). Genes in the *xlh3* cluster, a cloned member of the major histone gene repeat in *X. laevis*, are expressed in nondividing oocytes during oogenesis and in cycling cells during early development, as described above (34). The histone H2A and H2B genes in this cluster are transcribed in opposite directions from initiation sites located approximately 235 bp apart. This arrangement of H2A and H2B genes is not unique; it is also found in the genomes of unicellular organisms and other vertebrates (18, 21, 26, 29, 38). Histone H2A and H2B genes are often paired and transcribed in opposite directions, even in the genomes of organisms whose histone genes are not generally clustered (26, 30). The general consequences of H2A-H2B gene pairing for H2A and H2B gene expression are not clear; however, in several such gene pairs, cell cycle-regulated transcription of H2A genes depends on shared S-phase-specific *cis*-acting elements, such as octamer motifs, that are located between the H2A and H2B genes (21, 38). This conserved gene arrangement may play a role in the regulation of H2A and

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H2B gene expression and raises the question of whether these genes are controlled by a single bidirectional promoter, as described for the type IV α1 and α2 collagen genes (15), or by separate promoters.

In previous studies with an isolated *Xenopus* H2B gene, the octamer motif was found to be required for H2B promoter activity in embryos but not in oocytes (19, 20). Functional cooperation between the proteins bound to the octamer motif and a nearby CCAAT motif was necessary for maximal activity in embryos. These studies also demonstrated the activity of an ATF-binding site and showed that the DNA-binding activities of factors that interact with the octamer, CCAAT, and ATF motifs are not detectably different in oocytes and early embryos. From these results, it was concluded that the transition from basal H2B transcription in oocytes to the cell cycle-regulated mode in embryos is due to changes in interactions between transcription factors rather than changes in their abundance or ability to bind DNA. Because the previous studies analyzed transcription of an individual H2B promoter in the absence of the divergently transcribed H2A gene, they did not address possible functional interactions between both promoters and their potential role in coordinating histone gene transcription. In the studies presented here, we investigate the transcriptional regulation of paired H2A and H2B genes in *Xenopus* oocytes and embryos. Our studies show that expression of the H2A and H2B histone genes is controlled by separate promoters that share multiple regulatory elements. We also show that the CCAAT displacement protein (CDP) binds to positively acting regulatory sequences in both promoters as well as to promoters from the remaining major histone gene classes. The binding of CDP to promoters from all five histone gene classes may contribute to the coordination of their activities.

MATERIALS AND METHODS

Plasmids and mutagenesis. The histone genes used in this study are derived from the *xlh3* cluster (33). It was originally reported that the H2A and H2B gene promoters in this cluster were separated by about 2 kb and transcribed in the same direction. Subsequent restriction mapping and DNA sequencing studies showed that the initial orientation of the *Hin*dIII restriction fragment containing the H2B gene was inverted relative to the rest of the cluster. The correct orientation places the H2A and H2B transcription initiation sites approximately 235 bp apart. Exonuclease deletions and site-specific mutations were prepared as described elsewhere (19). The histone H1, H2A, and H2B constructs used in this study contain a synthetic, in-frame linker (*Eco*RI for H2A and H2B and *Bam*HI for H1) within their coding regions to allow transcripts from injected templates to be distinguished from endogenous mRNAs (16). To prepare 5′ deletions, linker-scanning mutants were digested at the *Asp* 718 site that was generated by linker scanning mutagenesis and an upstream site (*Bam*HI for H2A and *Sal*I for H2B). After the 3′ ends were filled with DNA polymerase I, the resulting plasmid DNA was recircularized with T4 DNA ligase. Plasmids containing mutations in CCAAT1 and CCAAT3 were prepared by joining separate fragments containing single CCAAT1 and CCAAT3 mutations at a unique *Hin*dIII site (nucleotide [nt] 707; see Fig. 1). The CCAAT2 mutant constructs were prepared by PCR amplification of the appropriate template with mutagenic oligonucleotides designed to replace CCAAT2 with a *Bgl*II site.

Preparation of oocytes and embryos. For oocyte injections, ovaries were removed from female frogs, washed several times in modified Barth's solution (MBS [31]), and then swirled in MBS containing 2 mg of collagenase (type 1A; Sigma) per ml at room temperature for approximately 4 h to remove follicle cells. Oocyte germinal vesicles were then injected with 20 nl of a solution containing 100 pg of supercoiled H2A-H2B DNA and 100 pg of control histone H1 DNA. After incubation for 12 to 24 h at 20°C, oocytes of normal appearance were harvested and used immediately or quick-frozen on dry ice and stored at −70°C until use.

For microinjection of embryos, egg laying was induced by injection of 100 U of pregnant mare serum gonadotropin into the dorsal lymph sacs of adult female frogs. On the following day, the frogs were injected with 500 U of human chorionic gonadotropin. On the following day, eggs were stripped from frogs into petri dishes. Testes were then surgically removed from adult male frogs, and a sperm suspension was prepared by teasing a portion of testis tissue apart in MBS. Eggs were fertilized by incubation in sperm suspension diluted in 0.1× MBS until cortical rotation was observed in at least 90% of the eggs (approximately 30 min). Fertilized eggs were then dejellied for 5 min in 2.5% (wt/vol) L-cysteine–0.6% (wt/vol) Tris (pH 7.9) and were then washed extensively with 0.1× MBS. Dejellied embryos were transferred to MBS containing 4% (wt/vol) Ficoll and were microinjected with a DNA solution at the two-cell stage. At 3 to 4 h after fertilization, embryos were washed with 0.1× MBS and were maintained in this buffer until approximately stage 10 (28), a point well past the onset of transcription of zygotic genes at the midblastula transition (stage 8.5). Harvested embryos were used immediately or quick-frozen on dry ice and stored at −70°C until further use.

RNA purification and analysis. RNA was extracted from injected oocytes or embryos by homogenization in 400 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 1 mg of proteinase K per ml and 1% (wt/vol) sodium dodecyl sulfate. The homogenate was then incubated for 30 min at 37°C with occasional mixing. Forty microliters of 3 M sodium acetate was then added, and the mixture was deproteinized by extraction with an equal volume of water-saturated phenol and then by several extractions of the resulting aqueous phase with an equal volume of phenol-chloroform (1:1) until a white interface no longer formed. Nucleic acids were then precipitated by the addition of 3 volumes of absolute ethanol, incubated for at least 30 min at 40°C, and recovered by centrifugation for 15 min at 10,000 × *g*. Nucleic acids were then resuspended in 10 μl of water per oocyte or embryo. Specific transcription of injected H1, H2A, and H2B genes was analyzed by the S1 nuclease protection assay. Radiolabeled probes were prepared by using dephosphorylated *Eco*RI-digested (H2A or H2B) or *Bam*HI-digested (H1) DNA and T4 polynucleotide kinase with [γ-^{32}P]ATP. For S1 nuclease analysis, a mixture containing 50,000 cpm of each probe, 80% (vol/vol) formamide, 0.4 M NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 1 mM EDTA, and one or two oocyte or embryo equivalents of RNA in a total volume of 10 μl was incubated for a minimum of 4 h at 50°C. After hybridization, single-stranded (unhybridized) nucleic acids were digested by incubation with 10 U of S1 nuclease for 30 min at 37°C in 100 μl of a solution containing 0.25 M NaCl, 0.03 M sodium acetate (pH 4.5), and 1 mM $ZnCl_2$. After digestion, the solution was extracted with an equal volume of phenol-chloroform, and nucleic acids were precipitated with 3 volumes of absolute ethanol. Samples were then resuspended in 50 μl of electrophoresis sample dye (80% [vol/vol] formamide and 1 mM EDTA containing xylene cyanol and bromophenol blue), and the suspension was heated for 10 min at 68°C and subjected to electrophoresis on 4% acrylamide (acrylamide-bisacrylamide, 20:1)–50% (wt/vol) urea gels containing 0.5× TBE (44.5 mM Tris [pH 8.3], 44.5 mM boric acid, 1 mM EDTA). Urea was removed from the gels by soaking them in water, and the gels were then dried. The dried gels were subjected to autoradiography with X-ray film or were scanned with a PhosphorImager (Molecular Dynamics), and the levels of gene-specific transcripts were quantitated. H2A and H2B transcription levels were normalized to the transcription levels of a coinjected H1 gene.

Electrophoretic mobility shift assays. HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C and 6% CO_2. The *Xenopus* tissue culture (XTC) cell line was grown in 67% L-15 medium supplemented with 10% fetal bovine serum at room temperature in sealed T flasks. Nuclear extracts were prepared from HeLa cells as described by Abmayr and Workman (1). For preparation of nuclear extracts from XTC cells, the cells were washed with 67% phosphate-buffered saline (PBS) (100% PBS is 50 mM sodium phosphate [pH 7.6] and 150 mM NaCl), and KCl was omitted from the hypotonic buffer. H1, H2A-H2B, H3, and H4 promoter probes were prepared by digesting appropriate linker-scanning mutant constructs. The resulting DNA fragments were purified by elution from agarose gels, precipitated, and labeled by phosphorylation with [γ-^{32}P]ATP by T4 polynucleotide kinase.

Each electrophoretic mobility shift reaction mixture contained 5 to 10 μl of nuclear extract (approximately 25 μg of protein), 3 μg of poly(dI-dC), 10 mM dithiothreitol, 10 mM $MgCl_2$, 0.5% (wt/vol) Ficoll, and 10,000 to 50,000 cpm of DNA probe brought to a 25-μl volume with buffer C (25% glycerol, 20 mM Tris-HCl [pH 7.3], 50 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol). In some experiments, a 50- to 100-fold molar excess of a DNA fragment was used as a competitor. Reaction mixtures were incubated for 30 min on ice and then subjected to electrophoresis on 4% (wt/vol) polyacrylamide gels containing 0.05% (wt/vol) bisacrylamide and 0.5× TBE. Electrophoresis was performed at 150 V until the bromophenol blue tracking dye neared the bottoms of the gels. The gels were then dried and subjected to autoradiography.

RESULTS

Overlapping H2A and H2B promoters. H2A and H2B histone genes are present in the genomes of many species as divergently transcribed gene pairs. A cloned representative of the major histone gene repeat in *X. laevis* contains an H2A-H2B gene pair whose transcription initiation sites are approximately 235 bp apart (Fig. 1). Several transcription factor-binding motifs can be recognized in the intergenic region, including two TATA boxes, three CCAAT boxes, an octamer-like motif, and an ATF-binding site. Additionally, this region

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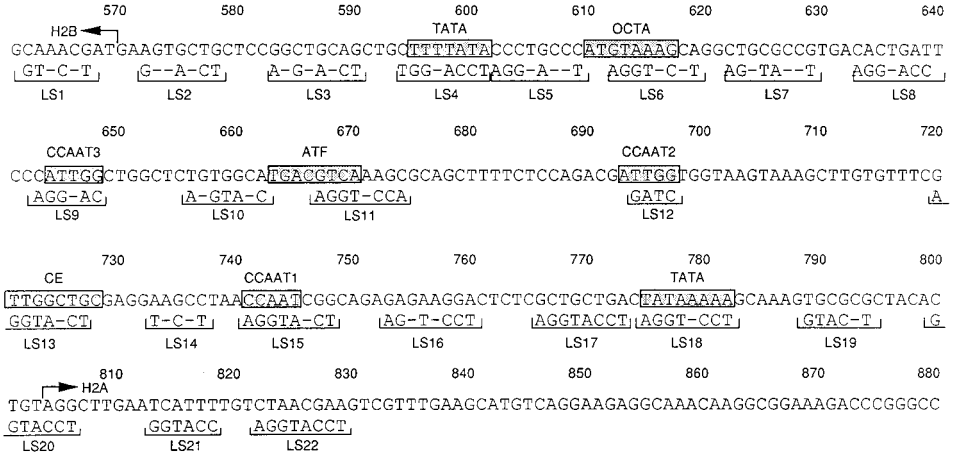

FIG. 1. Nucleotide sequence of the H2A-H2B intergenic region. The bases changed in each mutation are indicated below the sequence. Arrows indicate transcription initiation points for H2A and H2B as labeled. Boxed and shaded regions contain functional promoter elements. TATA, TATA box; OCTA, octamer-like motif; CCAAT, CCAAT box; ATF, ATF site; CE, H2A conserved element. Nucleotide numbering starts with the *Xba*I site approximately 803 bp 5′ of the H2A transcription initiation site.

contains a conserved element (CE) that is present in the H2A promoters of many species and resembles a consensus CCAAT motif (33).

To determine which portions of the intergenic region are required for transcription of each gene, two sets of deletions were prepared and tested for their ability to support H2A and H2B transcription in injected oocytes and embryos (Fig. 2). The transcriptional activity of the H2A gene promoter in oocytes was decreased when sequences containing the H2B octamer motif were deleted from the 5′ end of the H2A promoter (Fig. 2A). Deletion of the 28-bp fragment containing the octamer motif had a more substantial effect on H2A transcription in embryos, suggesting that the level of activity of this element is increased in embryos. H2A transcription in oocytes and embryos was further diminished by deletion of CCAAT3 and was then extinguished by deletion of the ATF-binding site.

The activity of the H2B promoter in oocytes was decreased by deletion of a 68-bp fragment containing the H2A TATA box (Fig. 2B). This result suggested that H2B transcription depends on one or more elements located upstream of the H2A TATA box or on the H2A TATA box itself. The reduced level of H2B transcription persisted through subsequent deletions until the ATF site was deleted. As observed for transcription of the H2A promoter, deletion of the ATF site inactivated the H2B promoter. Similar results were observed when these deletion mutants were analyzed with injected embryos (Fig. 2B). These results show that sequences necessary for the activity of the H2A and H2B gene promoters in oocytes and embryos are present in overlapping portions of the intergenic region.

Shared regulatory elements required for H2A and H2B transcription. To examine the function of specific sequences in H2A and H2B transcription, a series of site-specific mutations were prepared (Fig. 1). The effects of these mutations on the activities of the H2A and H2B promoters in oocytes and embryos were assayed (Fig. 3). As predicted from the deletion studies described above, mutation of several motifs had significant effects on H2A transcription (Fig. 3A). More specifically, mutation of OCTA, CCAAT3, ATF, CCAAT2, CE, CCAAT1, and the H2A TATA box decreased H2A transcription. Several mutations that did not disrupt obvious transcription factor-binding motifs also diminished the levels of H2A transcription. In one case (LS8), the mutation may have interfered with the binding of one or more factors to the adjacent CCAAT motif, while another (LS21) may have perturbed the interaction of factors with the H2A transcription initiation site. Disruption of the CCAAT3, CCAAT1 and H2A TATA motifs reduced H2A transcription in both embryos and oocytes, whereas disruption of the OCTA element decreased H2A transcription preferentially in embryos, and mutagenesis of the ATF, CCAAT2, and CE motifs decreased H2A transcription levels in oocytes only. Therefore, these last elements appear to contribute to H2A transcription more in oocytes than in developing embryos. Since the relative rates of transcription of the injected templates in oocytes and embryos are unknown, it is not clear whether the absolute activities of these elements (ATF, CCAAT2, and CE) were reduced in embryos compared with oocytes or whether the activities of the other elements (including CCAAT1 and H2A TATA) were increased. Interestingly, mutation of the H2B TATA box increased H2A transcription (by approximately twofold) in embryos, suggesting that H2A transcription in embryos may be dependent on a common, limiting factor that functions through TATA motifs.

Mutations disrupting the H2A TATA box and the ATF-binding site had significant effects on the level of H2B transcription (Fig. 3B), as expected from deletion studies discussed above. In addition, mutagenesis of the H2B TATA box, OCTA, and CCAAT3 substantially decreased H2B transcription. As observed previously, mutations in regions lacking recognizable transcription-binding motifs affected H2B transcription. In this case, the LS5 and LS8 mutations disrupted regions adjacent to the octamer motif and CCAAT3, respectively, and may have interfered with the interaction of regulatory factors with these sites.

These results suggest that physically overlapping regions of the H2A-H2B intergenic region are required for H2A and H2B transcription. That is, the intergenic region cannot be physically divided into distinct H2A and H2B promoters each of which is capable of supporting maximal levels of H2A and H2B transcription. Additionally, some regulatory elements are required for transcription of both genes (e.g., CCAAT1, CCAAT3, ATF, octamer, and H2A TATA motifs), demonstrating that the two promoters share functional regulatory elements.

Functional cooperativity between the octamer motif and elements in the intergenic region. Previous studies showed that transcription of the *Xenopus xlh3* H2B gene in embryos re-

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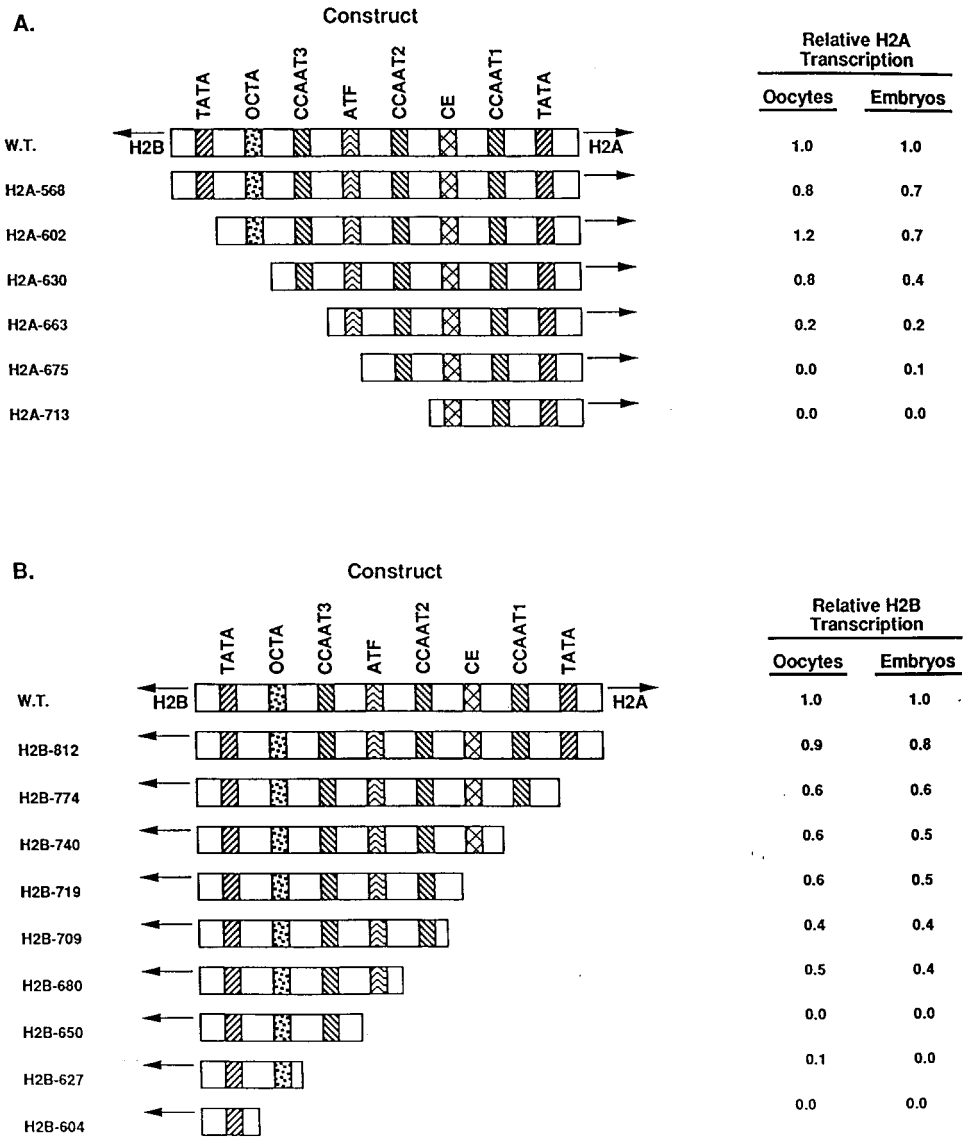

FIG. 2. Effects of deletion of 5′-flanking regions on H2A and H2B transcription in oocytes and embryos. H2A and H2B transcription levels were determined by PhosphorImager analysis of S1 nuclease protection assays, were normalized to H1 transcription levels, and were expressed relative to the transcription level of the wild-type plasmid (W.T.). (A) Transcriptional activity of H2A 5′ deletion constructs in injected oocytes and embryos; (B) transcriptional activity of H2B 5′ deletion constructs in injected oocytes and embryos. The results are average values obtained from three individual experiments. The standard deviations were approximately 10%.

quired functional cooperation between CCAAT3 and the octamer motif (20). These conclusions were based on studies with an H2B gene promoter that included only the H2B-proximal portion of the H2A-H2B intergenic region (up to nt 712) and which showed that the activity of the octamer motif required an intact CCAAT3 site. Functional cooperativity between Oct-1 and CCAAT box-binding proteins is not unique to the H2B promoter; similar interactions have been reported for transcription of a U3 promoter (2). Since the complete H2A-H2B intergenic region contains several regulatory elements (including a CCAAT motif) that were not present in the template used in the previous study, it is possible that one or more of these elements substitutes for the CCAAT3 site in its functional interaction with the octamer motif.

To examine the activity of the octamer motif in the context of the intact H2A-H2B gene pair, transcription of promoters containing single and double mutations was analyzed as previously described (16), except that the H2A gene and the H2A-proximal portion of the intergenic region were included (Fig. 4). The results show that mutagenesis of the CCAAT3 and octamer motifs together reduced H2A and H2B transcription more than mutagenesis of either element alone. In addition, H2B transcription in oocytes was nearly eliminated upon mutagenesis of both regulatory elements. We conclude from these results that the octamer motif can function independently of the CCAAT3 element in the context of the intact H2A-H2B gene pair, presumably because of alternative interactions with other elements in the intergenic region.

Multiple CCAAT elements required for binding of CDP. DNA-binding assays were used to explore interactions between nuclear factors and regulatory elements of the H2A-H2B promoter. An electrophoretic mobility shift assay was performed

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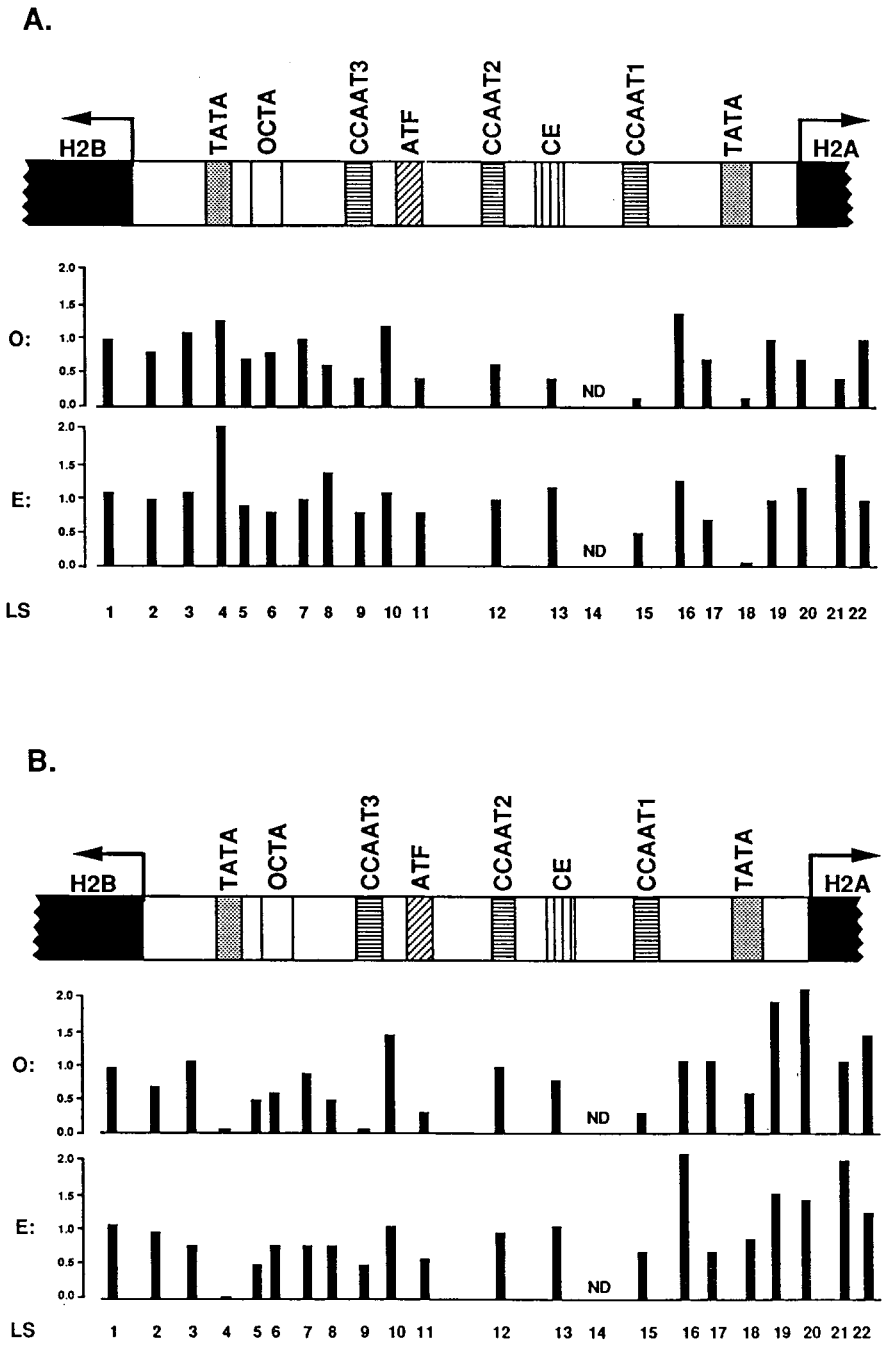

FIG. 3. Effects of site-specific mutations on the activities of H2A and H2B promoters. The transcription levels of individual templates are expressed relative to the H2A (A) and H2B (B) transcription levels of the wild-type plasmid (W.T.). The results from three separate experiments are depicted graphically as described in the legend to Fig. 2. O, oocytes; E, embryos; LS, linker-scanning mutant; ND, not determined.

with probes spanning the entire H2A-H2B intergenic region (Fig. 5A). A DNA-protein complex was observed whose formation was inhibited by an excess of unlabeled H2A-H2B promoter DNA (lane 2) but not by an unrelated DNA fragment of similar length (lane 3). A specific DNA-protein complex was also formed with this probe and nuclear extracts from a *Xenopus* cell line (XTC [Fig. 5B]). These results suggest that the protein or proteins in this complex are ubiquitous and evolutionarily conserved, as expected for a DNA-protein complex presumed to be involved in regulating histone gene transcription. The complex was not disrupted by addition of up to 2 M urea, up to 10% Triton X-100, or a mixture of 0.9% Nonidet P-40 and 1.5% sodium deoxycholate (12). Treatment with nonionic detergents has been reported to dissociate multiprotein complexes that bind DNA (9, 10), suggesting that the complex may contain a single protein.

To identify sequences involved in formation of the complex, DNA probes spanning different portions of the H2A-H2B promoter region were used as probes and competitors in mobility shift assays. The results of several such assays show that se-

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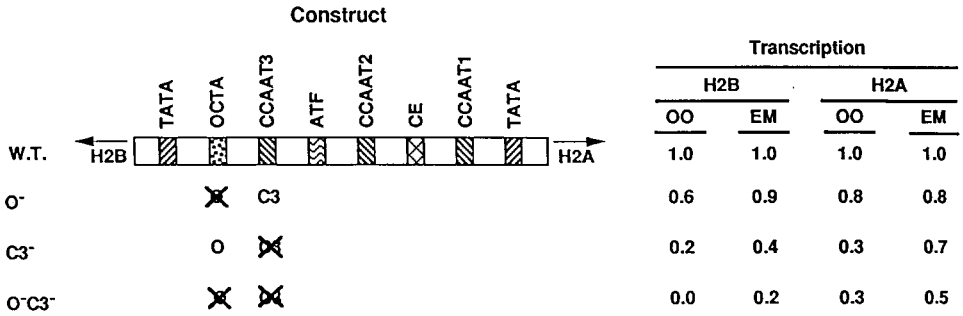

FIG. 4. Effect of octamer and CCAAT3 motif disruptions on H2A and H2B transcription in oocytes (OO) and embryos (EM). The transcription levels of each mutant promoter are expressed relative to the H2A and H2B transcription levels of the wild-type construct (W.T.) as described in the legend to Fig. 2. O^-, octamer mutant; $C3^-$, CCAAT3 mutant; O^-C3^-, double mutant.

quences spanning the three CCAAT boxes were necessary for efficient complex formation (Fig. 6). Furthermore, the complex was formed with probes that span CCAAT1 and CCAAT2 or CCAAT2 and CCAAT3, suggesting that at least two CCAAT motifs are required for complex formation.

The protein or proteins involved in complex formation did not appear to be related to the known CCAAT box-binding factors H1TF2 (13), NF1/CBP (36), and CBF (25), since addition of antibodies specific for these proteins did not affect the formation of the complex or its electrophoretic mobility (12). Addition of antiserum raised against purified human CDP (27) specifically disrupted the sequence-specific complex but not the more rapidly migrating, nonspecific complexes, whereas the addition of nonimmune serum to DNA-binding reaction mixtures did not affect the formation of any of the complexes (Fig. 5A). These results demonstrate that CDP or an immunologically related protein is present in a specific complex formed with the H2A-H2B intergenic region. We were unable to detect CDP binding activity in extracts from *Xenopus* oocytes or gastrula-stage embryos, suggesting that this protein is either absent or expressed at a low level during early amphibian development.

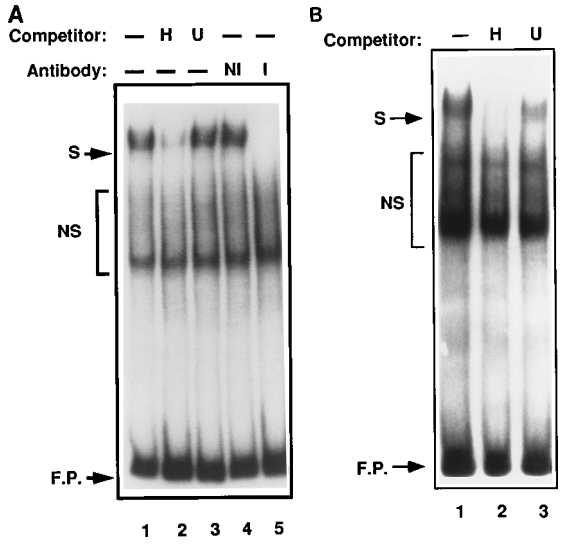

FIG. 5. Binding of nuclear proteins to the H2A-H2B intergenic region probe. (A) Electrophoretic mobility shift assay with the H2A-H2B probe (nt 590 to 817) and a HeLa nuclear extract. Competition experiments were performed by using a 100-fold molar excess of the homologous, unlabeled fragment (H) and an unrelated competitor (U). The presence of CDP was tested by using nonimmune guinea pig serum (NI) and guinea pig serum containing antibodies raised against human CDP (I). (B) DNA-binding assays with the H2A-H2B promoter probe and an XTC nuclear extract with a 100-fold molar excess of competitors. S, specific complex; NS, nonspecific complexes; F.P., free probe.

The involvement of the CCAAT boxes in the formation of the CDP-containing complex was further analyzed in a competition assay with the wild-type H2A-H2B promoter probe and competitors containing mutations in one or more CCAAT boxes (Fig. 7A). Formation of the complex was abolished by a 100-fold excess of the homologous wild-type competitor (lane 2). Mutagenesis of CCAAT1, but not CCAAT2 or CCAAT3, slightly reduced the ability of the competitor to inhibit complex formation. The presence of mutations in CCAAT1 and either CCAAT2 or CCAAT3 further decreased the ability to inhibit formation of the CDP-containing complex (compare lanes 6 and 7 with lanes 3 to 5), whereas the presence of mutations in both CCAAT2 and CCAAT3 did not significantly reduce competition with the probe. Mutagenesis of all three CCAAT motifs resulted in a fragment that failed to efficiently compete with the wild-type probe for complex formation. These results suggest that CCAAT1 is the most important of the elements analyzed for efficient complex formation and that the combination of two or more CCAAT motifs is sufficient for complex formation. These conclusions were substantiated in assays with DNA fragments containing single or multiple CCAAT motif mutations (12). Synthetic oligonucleotides containing individual CCAAT boxes did not compete for complex formation and failed to form the complex (12, 20).

Since multiple CCAAT boxes were required for complex formation, we asked whether a correlation existed between complex formation and promoter activity by testing the effects of mutations at single and multiple CCAAT elements on H2A and H2B gene transcription. As shown above, mutagenesis of either CCAAT1 or CCAAT2 specifically diminished H2A transcription (Fig. 7B). The activity of the H2A promoter was reduced more by mutagenesis of CCAAT1 than by that of CCAAT2. Moreover, the introduction of base changes in both sites together nearly eliminated H2A transcription without affecting H2B promoter activity. In contrast, mutagenesis of CCAAT3 reduced the levels of transcription of both histone genes. DNA templates containing second-site mutations at either CCAAT1 or CCAAT2, in addition to the mutation at CCAAT3, showed severely reduced levels of transcription of both histone genes. Mutagenesis of all three CCAAT boxes completely abolished H2A and H2B transcription in oocytes and embryos. In conjunction with the studies discussed above, these data demonstrate the importance of the CCAAT boxes for H2A and H2B transcription and indicate that CCAAT3 participates in the transcription of both the H2A and H2B genes, whereas CCAAT1 and CCAAT2 are principally re-

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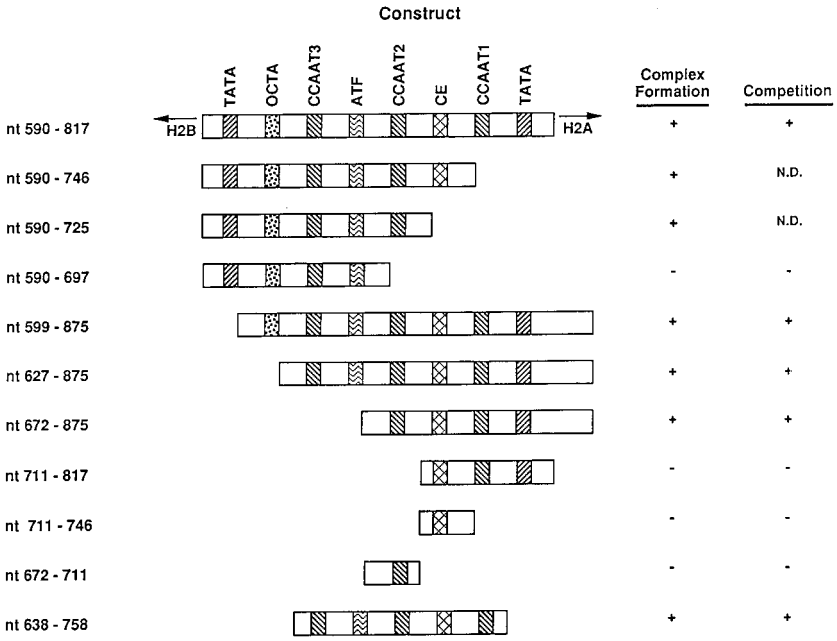

FIG. 6. Summary of results of DNA-binding assays performed with various intergenic region fragments. The region spanned by each DNA fragment is indicated in the left-hand column. Nucleotides are numbered as described in the legend to Fig. 1. Assays involving intergenic region fragment competitors were performed with two different probes that spanned nt 590 to 817 and nt 638 to 758, with identical results for each probe. N.D., not determined.

quired for H2A gene transcription. Sequences important for complex formation correlate closely with those required for the activity of the H2A histone gene promoter.

CDP binding to other *Xenopus* histone gene promoters. CCAAT motifs are among the few regulatory elements commonly found in histone gene promoters. Therefore, we asked whether CDP-containing complexes could be formed with sequences from other *Xenopus* histone gene promoters. When fragments containing H1, H3, and H4 histone gene promoter sequences were used as probes, a specific DNA-protein complex was formed with each probe and with similar electrophoretic mobilities (Fig. 8A). Formation of these complexes was abolished by the addition of anti-CDP antibodies (12). Competition experiments with an excess of DNA fragments spanning the H1, H3, and H4 promoter regions abolished the binding of CDP to the H2A-H2B intergenic region probe (Fig. 8B). Together with the previous data, these results show that CDP binds to CCAAT boxes in the H2A and H2B histone gene promoters as well as to sites, presumably CCAAT boxes, in promoters for the other three *Xenopus* histone gene classes.

The H1 and H3 histone gene promoters contain two consensus CCAAT boxes, whereas the H4 promoter contains one consensus CCAAT box and two other sites that vary from the consensus by a single base mismatch. Each CCAAT-like motif contains sequences that have been previously shown to bind CDP (3, 5, 14). To determine whether the CCAAT motifs in

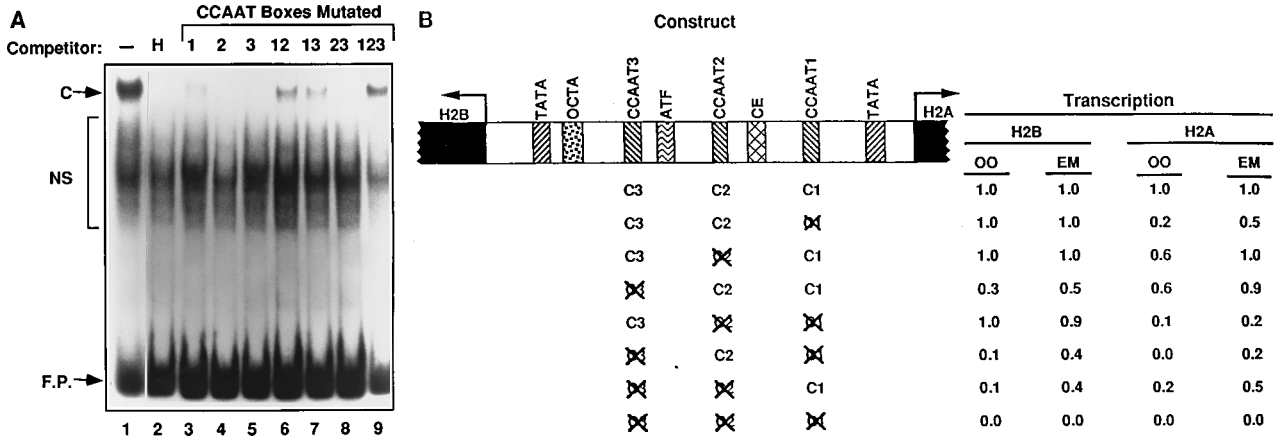

FIG. 7. Involvement of CCAAT boxes in H2A and H2B gene transcription. (A) DNA fragments containing disruptions in one, two, or three CCAAT boxes used as competitors in mobility shift assays with HeLa nuclear extracts and labeled wild-type H2A-H2B promoter probes (nt 590 to 817). H, homologous competitor (numbers indicate disrupted CCAAT boxes [e.g., 13 indicates that CCAAT1 and CCAAT3 are both mutated]); C, specific complex; NS, nonspecific complexes; F.P., free probe. (B) Transcription of H2A and H2B promoters containing mutations in one, two, and three CCAAT boxes assayed in oocytes (OO) and embryos (EM). Average values of three independent experiments are depicted as described in the legend to Fig. 2.

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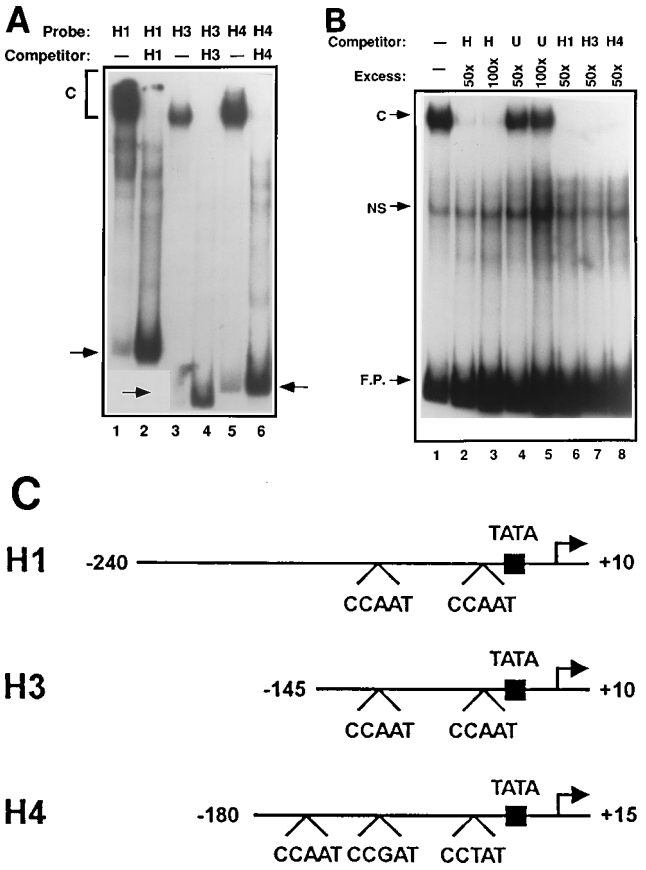

FIG. 8. Complex formation with histone H1, H3, and H4 promoter fragments. (A) DNA-binding assays performed with H1, H3, and H4 promoter probes and a HeLa nuclear extract in the absence and presence of a 100-fold molar excess of the homologous unlabeled competitor; (B) assays performed with the H2A-H2B intergenic region probe and various competitors. The molar excess of each competitor relative to the probe is indicated for each lane. C, specific complex; NS, nonspecific complexes; F.P. or unlabeled arrow, free probe; H, homologous competitor; U, unrelated competitor. (C) Depiction of regulatory elements in the H1, H3, and H4 promoter fragments used in panels A and B.

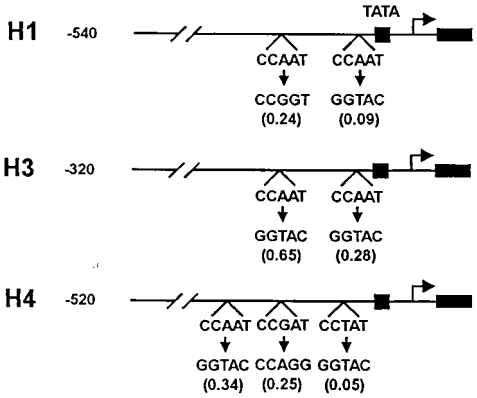

FIG. 9. Function of potential CDP-binding sites in histone gene transcription in frog oocytes. Mutations were introduced into individual CCAAT motifs in the *xlh3* H1, H3, and H4 promoters, and the activity of each promoter mutant was assayed by S1 nuclease protection after microinjection into frog oocytes. The activity of each mutant (shown in parentheses) was normalized to that of the corresponding wild-type promoter (1.0).

the H1, H3, and H4 histone gene promoters were important for transcription, we analyzed the activities of promoters containing mutations at these sites. When templates containing the altered promoter sequences were tested in injected frog oocytes, the mutant promoters were transcribed less efficiently than the corresponding wild-type promoter, suggesting that each CCAAT motif was a functional promoter element (Fig. 9). As observed with the H2A and H2B promoters, mutagenesis of TATA box-proximal CCAAT motifs decreased promoter activity to a greater extent that did mutagenesis of more-distal sites. Although the ability of CDP to bind to specific sites in the H1, H3, and H4 histone gene promoters was not tested directly, the results from the DNA-binding studies, competition assays, and oocyte injection studies support the conclusion that CDP binds to important positive-acting regulatory sites in each of the five *Xenopus* histone gene promoters.

DISCUSSION

In this study, we examined regulatory elements that control expression of a divergently transcribed histone gene pair. Our results show that the H2A and H2B genes in a conserved *Xenopus* histone gene cluster have separate, yet physically and functionally overlapping, promoters. Transcription of both genes required the combined activities of shared and promoter-specific regulatory elements. The presence of shared regulatory elements in the H2A and H2B histone gene promoters was not surprising (21, 38). The particular arrangement of the H2A and H2B promoters, separated by less than 250 bp, suggested a priori that their activities might be mutually dependent. In view of the complex and often cooperative interactions between diverse transcriptional regulatory proteins that have been described, it might be expected that the presence of a transcription complex on one promoter would increase the activity of an adjacent promoter. However, our results suggest that the transcriptional activity per se of one histone gene promoter did not contribute significantly to the activity of the other, since several promoter elements appeared to function in a promoter-specific manner. For example, mutations at CCAAT1 and CCAAT2 abolished H2A transcription but did not alter the activity of the H2B promoter. Likewise, H2A transcription was unaffected by deletion of the H2B promoter or base changes at a site (H2B TATA box) that was strictly required for H2B transcription. Thus, transcription of neither histone gene was highly dependent on the activity of the other promoter, indicating that the assembly of transcription complexes on these promoters occurred independently rather than cooperatively. Differences in the activities of promoter elements presumably reflect the binding of distinct proteins to these sites. The involvement of particular regulatory sequences in the transcription of both histone genes may be due to the ability of the corresponding factors to bidirectionally promote transcription. In addition, the ability of factors to bidirectionally stimulate transcription may be altered by factors bound to nearby sites.

Previous studies suggested that H2B transcription in embryos requires functional cooperation between Oct-1 bound to the octamer motif and a protein binding to the nearby CCAAT box (19, 20). The experiments presented here demonstrate that elements further upstream can substitute for the H2B-proximal CCAAT box in mediating Oct-1-dependent transcription. Two additional CCAAT boxes, CCAAT1 and CCAAT2, are plausible substitutes for CCAAT3 since these sites were required for H2B transcription with promoters that lacked the CCAAT3 site but not with the wild-type promoter. This result suggests that the upstream CCAAT boxes can perform one or

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more functions that are normally provided by CCAAT3. Together with the previous results, these data suggest that H2B transcription requires functional interactions between Oct-1 and one or more factors bound to elements further upstream. The interaction of Oct-1 with factors bound to elements required for activity of both promoters might account for the involvement of the octamer motif in H2A transcription.

The binding of CDP to the overlapping H2A and H2B promoters, as well as to the three other histone gene promoters, is particularly interesting and raises the possibility that CDP participates in regulating the transcription of *Xenopus* histone genes. CDP and related mammalian proteins (4, 27) are members of a homeodomain protein family that includes the *Drosophila* Cut protein, a regulator of cell specification in multiple lineages (7–9, 22, 24). Members of the Cut protein family share two unique structural characteristics. First, the Cut homeodomain is distinct in containing a histidine at the ninth position of the recognition helix. The amino acid in this position determines the DNA-binding specificities of some homeodomain proteins. Second, members of the Cut protein family contain three other motifs, called the Cut repeats, which share about 60% amino acid identity with each other and bind DNA specifically. Thus, CDP and other members of the Cut protein family contain four distinct DNA-binding domains. The presence of multiple DNA-binding domains in CDP could explain the requirement for multiple CCAAT boxes in the H2A-H2B intergenic region to form the CDP-containing complex. This suggestion is supported by the observations that cloned CDP products bind to multimerized sites more efficiently that to monomeric sites (3, 4), and the CCAAT consensus sequence is among the variety of those selected from a pool of random oligonucleotides by glutathione *S*-transferase fusion proteins containing individual Cut repeats (14). The fact that oligonucleotides containing individual CCAAT boxes from the H2A-H2B intergenic region fail to stably bind CDP and do not compete efficiently with the intact promoter fragment for CDP binding suggests that these sites bind CDP weakly when analyzed individually and that the combination of two or more of these sites is necessary for formation of the CDP-containing complex.

CDP was first described as a possible repressor that interacts with a sea urchin testis-specific H2B gene promoter (6). Subsequently, CDP and related mammalian proteins were implicated in repressing expression of several other genes, including human myelomonocyte-specific *gp91-phox* (37), mouse *NCAM* (39), human c-*myc* (11), and the mouse myosin heavy-chain gene (4). The mechanisms by which CDP regulates transcription are not yet clear. Investigations of CDP function have been complicated by its widespread expression as well as the lack of a well-defined consensus DNA-binding motif (3, 4, 6, 37, 39). Although CDP has been generally viewed as a transcriptional repressor, transcriptional activating functions have not been ruled out. Our results show that CDP binds to positive-acting sequences required for histone gene transcription, but they do not distinguish between an activation and a repression function for CDP. Preliminary studies indicate that CDP is expressed at a low level, if at all, in *Xenopus* oocytes and early embryos (12), suggesting that the CCAAT box requirement for transcription of histone gene promoters at these stages reflects the binding of factors other than CDP. Several factors involved in cell cycle-specific transcription of mammalian H1, H2B, and H4 histone genes have been identified (H1TF1 and H1TF2, Oct-1, and H4TF2, respectively; reviewed in reference 17). Interestingly, at least one of these factors, H1TF2, interacts with a CCAAT motif. Moreover, S-phase-specific transcription of mammalian thymidine kinase genes was found to depend on a CCAAT motif (23, 32), and a CCAAT box located near the octamer motif in the mouse TH2A-TH2B intergenic region appears to be important for TH2B gene transcription (21). CDP might play a role in regulating *Xenopus* histone gene expression by displacing positive-acting regulatory factors from CCAAT boxes and, perhaps, other binding sites in histone gene promoters as originally proposed (6). The binding of CDP to *Xenopus* histone gene promoters might be important to repress histone gene transcription in differentiated and quiescent cells or in cycling cells outside S phase. The present data are consistent with a model in which the activity of histone gene promoters is controlled by the interplay between positively and negatively acting factors that interact in a mutually exclusive manner with shared regulatory elements. It remains to be determined whether CDP is a passive, permissive participant in this regard or whether this protein plays a more complex, regulative role.

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