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Promoter Sequences Required for Transcription of *Xenopus laevis* Histone Genes in Injected Frog Oocyte Nuclei

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Amphibian oogenesis is accompanied by the accumulation of histone mRNA and proteins in the absence of ongoing DNA replication. To begin an analysis of the mechanisms by which histone gene expression is regulated during frog oogenesis and embryogenesis, we used oocyte injection to examine the upstream sequences required for transcription of genes encoding each of the five histone classes. We found that sequences necessary for maximal levels of transcription are located 100 to 200 base pairs upstream of the corresponding start sites. In this region, each promoter examined contains conserved sequence elements, several of which seem to be histone gene class specific, in addition to other, more common sequence elements believed to be used by general transcription factors.

Histone gene expression in most somatic cells is regulated so that histone synthesis is restricted to cells in the process of replicating their chromosomes. The coupling of histone expression to DNA replication is achieved by transcriptional and posttranscriptional mechanisms (for reviews, see references 21 and 27). Organisms such as *Xenopus*, *Drosophila*, and sea urchins, which undergo rapid cleavages during the initial stages of embryonic development, use maternal stores of histone mRNA or protein, or both, to provide sufficient material for chromosome assembly (21, 27, 42). The histone genes of these organisms may be grouped into a rather large class of maternally active genes that are also expressed during embryogenesis. In *X. laevis*, for example, about 80% of the abundant and moderately abundant gastrula-stage mRNAs are also present in unfertilized eggs (10). Much of the stored maternal mRNA has turned over by this stage of embryonic development, and the mRNA present derives mainly from zygotically active genes (36, 37). An issue of interest is whether and how mechanisms of gene expression during oogenesis differ from the types of regulation imposed subsequently during embryogenesis or differentiation.

Most histone genes in *X. laevis* are organized into at least two distinct types of clusters (9, 31). An examination of histone mRNA accumulation has demonstrated that transcripts from genes in both types of clusters are present throughout oogenesis and embryogenesis (32). Analysis of the expression of cloned *X. laevis* histone genes in cultured *X. laevis* cells, transfected mouse cells, and frog oocytes has demonstrated that their transcription is replication dependent in somatic cells and replication independent in oocytes (28). The conclusions that have emerged from these and related studies (40) are that no major developmental changes occur in the types of histone genes active during embryogenesis, but that their regulation undergoes a transition from constitutive expression during oogenesis to cell cycle-regulated expression at some point in embryogenesis.

We are interested in elucidating the mechanisms by which active histone gene sets in *X. laevis* are regulated during ontogeny. Our approach is to define the DNA sequences and the regulatory factors required for the transcription of these genes in frog oocytes and in developing embryos. The existence of regulatory sequences or factors that are differentially utilized during development could provide evidence that the regulation of these genes undergoes a developmental transition. Evidence for this type of transcriptional regulation, involving different factors in different tissues that regulate the same gene, has been described for several genes, including *yp1*, *yp2* (13, 14), and *hsp27* (23) in *D. melanogaster*. Alternatively, the same sequences and factors may be responsible for transcription in both oocytes and embryos. A mechanism of this sort has been proposed for the regulation of the dual 5S gene system in *X. laevis* (3, 35). Finally, it is of interest to determine whether transcriptional regulation is exerted at the level of the gene cluster or on individual genes and, in the latter case, whether shared regulatory signals or histone class-specific signals are involved.

In this report we present our initial observations regarding the transcription of injected *X. laevis* histone genes. We show that sequences necessary for the transcription of frog histone genes in injected oocytes reside within 200 base pairs (bp) of the transcription initiation site. These promoter regions appear to be complex and to contain multiple regulatory sequences.

MATERIALS AND METHODS

Construction of plasmids. Restriction fragments containing each of the five histone genes from pXlh3, a subcloned member of the major tandemly repeated histone gene cluster from *X. laevis* (31), were cloned into pUC vectors as follows: H1A, the 1,540-bp *Eco*RI-*Hind*III fragment; H2A, the 1,700-bp *Pst*I-*Bam*HI fragment; H2B, the 950-bp *Bal*I-*Xba*I fragment; H3, the 1,090-bp *Bgl*II-*Bst*EII fragment; H4, the 1,430-bp *Hind*III-*Xmn*I fragment. The top panel of Fig. 1 shows the orientation of all of the subcloned fragments with respect to the gene cluster from which they were derived. Preliminary injections with plasmids bearing the H1A gene indicated that upon electrophoresis the corresponding mRNA comigrated with an endogenous radiolabeled RNA species of unknown origin. To allow transcripts of the exogenous H1A gene to be distinguished from the endogenous transcript, a 224-bp *Ava*II-*Pvu*II fragment was excised from within the central coding region of the gene, and the flanking fragments were joined without shifting the original

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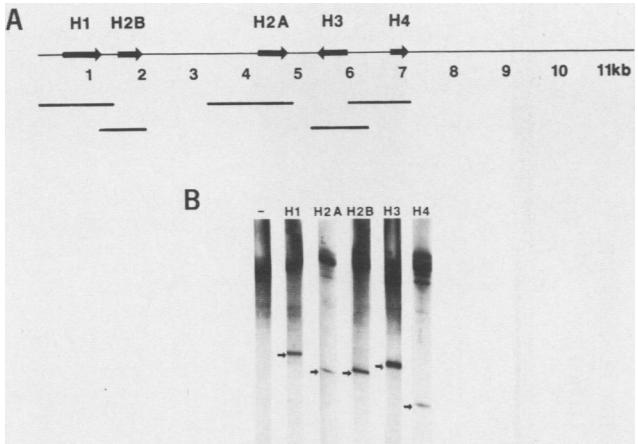

FIG. 1. Genomic organization of genes in the major *X. laevis* histone gene repeat and their transcription in injected oocyte nuclei. (A) The organization and transcriptional polarity of genes in the major histone gene repeat are indicated by the solid arrows. The lines below each histone gene represent the DNA fragments used for the initial oocyte injections and for subsequent deletion mutagenesis. (B) A 200-pg portion of each supercoiled template, as indicated above the lanes, was coinjected with [α-^{32}P]GTP into frog oocyte nuclei. Radiolabeled total oocyte RNA was extracted and analyzed by polyacrylamide gel electrophoresis. The first lane represents RNA from oocytes injected with [α-^{32}P]GTP alone (no DNA). Arrows indicate template-specific transcripts. Abbreviation: kb, kilobase pairs.

translation frame. Injection of this truncated minigene resulted in the formation of a shortened transcript that could be easily resolved from the endogenous species. Each of the histone gene templates was modified further by the insertion of synthetic linker sequences at unique sites within histone-coding regions, with care taken to avoid shifting the translation reading frame. These modifications potentially permit transcripts from the exogenous marked genes to be distinguished from those of the endogenous genes in a primer extension assay (although this approach was not used for the studies described here). We have not investigated in detail potential effects of these internal nucleotide changes on the stability of the corresponding transcripts.

Deletion mutagenesis of the 5'-flanking regions was performed with *Bal*31 nuclease as previously described (31) with *Kpn*I linkers. Deletion endpoints were confirmed by dideoxynucleotide sequence analysis of supercoiled plasmid DNA (4). As a consequence of multiple sequence determinations, we found it necessary to revise portions of the published sequence for the H1 and H2B gene promoter regions. For the revised sequences, see Fig. 4.

Oocyte injections and RNA analysis. Adult female *X. laevis* oocytes were obtained from Nasco, Fort Atkinson, Wis. The oocytes were swirled gently on a rotary shaker overnight at 18°C in modified Barth solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES (*N*-2-hydroxyethylpiperatine-*N'*-2-etharesulfonic acid; pH 7.5), 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$] containing 100 μg of penicillin per ml, 50 μg of streptomycin per ml, and 2 mg of collagenase (type IA; Sigma Chemical Co., St. Louis, Mo.) per ml.

Oocytes remaining viable were rinsed several times with fresh modified Barth solution containing antibiotics before being injected with 20 nl of 10 mM Tris (pH 7.4), 0.5 mM EDTA containing 100 pg of test template, 100 pg of control template, and 0.1 μCi of [α-^{32}P]GTP (<3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). Plasmids for injection studies were purified by CsCl density gradient centrifugation in the presence of ethidium bromide. The amount of template DNA injected (100 to 200 pg per oocyte) was determined to be within the linear range (0.05 to 1.0 ng) for expression of these genes in injected oocytes. Injected oocytes were incubated for 12 to 18 h at 18°C.

Total RNA was extracted from injected oocytes by homogenization in 10 mM Tris (pH 7.4)–1 mM EDTA–1% sodium dodecyl sulfate–0.3 M sodium acetate (40 μl per oocyte), extraction twice with phenol-chloroform (1:1) and once with chloroform, and precipitation with 3 volumes of 95% ethanol. Samples were suspended in 95% formamide before electrophoresis on 6% polyacrylamide gels containing 50% urea and 0.5× TBE (1× TBE is 89 mM Tris, 89 mM boric acid, and 8 mM EDTA). About 20 μg of RNA from each sample (corresponding to 5 oocyte equivalents) was loaded per lane.

Dried gels were subjected to autoradiography, with exposure times adjusted so that signals were in the linear range of the film. Transcript sizes were calculated by comparison with radiolabeled DNA fragments of known sizes. The expected sizes of accurately processed transcripts derived from the exogenous genes are as follows: H1A, 550 nucleotides; H2A, 500 nucleotides; H2B, 490 nucleotides; H3, 520 nucleotides; and H4, 410 nucleotides. The accumulation of gene-specific transcripts was quantitated by densitometric scanning of the autoradiographs with a DU-8 densitometer (Beckman Instruments, Inc., Fullerton, Calif.). Results obtained for the transcription of each test template were normalized to those for the coinjected internal-control gene.

RESULTS

Transcription of individual *X. laevis* histone genes in injected frog oocytes. Previous work from Birnstiel and coworkers (22, 33) has shown that the promoter and mRNA-processing signals of sea urchin histone genes are recognized by the frog oocyte transcriptional machinery with greatly differing efficiencies. In particular, posttranscriptional formation of the proper 3' terminus of H3 mRNAs was shown to occur by an RNA-processing event that requires specific structural features at the 3' end of the mRNA (16) and nuclear factors, containing U7 RNA, extracted from sea urchin embryos (39).

To ascertain whether transcripts from exogenous *X. laevis* histone genes are processed efficiently in injected oocytes, we analyzed the accumulation of mature histone transcripts. Accurate transcription initiation and 3' terminus formation after injection of H4 genes derived from other *X. laevis* histone gene clusters were previously reported (5, 29), and the formation of mature mRNAs of the predicted sizes was shown to be contingent on initiation of transcription at the proper site and efficient processing of the 3' end of each mRNA. That these events occur on injection of *X. laevis* histone genes from the major histone gene cluster is shown in the lower panel of Fig. 1. The coinjection of [α-^{32}P]GTP with each template resulted in radiolabeling of discrete template-specific transcripts of the appropriate sizes, as well as endogenous transcripts of presumed nucleolar origin. The template specificity of the indicated transcripts was demonstrated by analysis of RNA produced in oocytes injected with [α-^{32}P]GTP alone (Fig. 1B, lane −). In the absence of injected template DNA, transcripts of the expected size did not accumulate. Background labeling of endogenous RNA species was independent of injected template and exhibited variation with each batch of injected oocytes.

We considered the possibility that incomplete processing of de novo histone transcripts results in a pool of pre-mRNAs of perhaps heterogeneous sizes. We tested this by assaying for specific S1 nuclease-resistant products larger than mature mRNA that result from hybridization of radiolabeled RNA from injected oocytes to excess quantities of the appropriate single-stranded M13 histone gene DNA. These results (not shown) indicated that most, if not all, transcripts from marked exogenous *X. laevis* histone genes are efficiently processed to yield the mature mRNA product. We conclude that in this system, transcriptional events rather than posttranscriptional processes constitute the rate-limiting steps in histone mRNA synthesis. This conclusion is supported by the results presented below.

Functional analysis of histone gene regulatory elements by DNA injection into frog oocyte nuclei. A comparative analysis of the DNA sequences that flank corresponding genes in two distinct *X. laevis* histone gene clusters has revealed the presence of multiple conserved upstream sequence elements whose locations upstream of each gene are remarkably similar among the histone gene classes (31). Several of these conserved regions were known to be transcriptionally functional for histone genes from other species. These observations prompted the speculation that functionally related transcriptional regulatory elements might occupy similar upstream positions (31). To begin to examine the role of these sequences in transcription and to test more directly for other important regulatory signals whose structures and locations might not be as conserved, we constructed a series of 5′ deletion mutants with mutations in each of the five histone genes shown in Fig. 1. Attention was given to obtaining deletions that flanked both sides of the conserved sequence elements in each promoter. Purified supercoiled plasmid templates containing deletion mutants to be tested were mixed with an equal mass of a separate histone gene template functioning as an internal control. Transcripts were identified by the incorporation of coinjected radiolabeled precursor into RNA of the appropriate size, as described above. Owing to occasional variability in the level of transcription of injected templates, each deletion mutant series was injected into at least two different batches of oocytes.

Since the H4 mRNA transcripts were electrophoretically well resolved from the other histone mRNAs (Fig. 1), the H4 gene was generally used a convenient internal control. The H3 gene was used as an internal control for injections of the H4 5′ deletions. Although the amount of injected DNA (200 pg) was not in excess of the transcriptional capacity of the oocyte (about 1 ng [6, 15, 19]), it is possible that competition for a limiting factor occurred between the internal control gene and several test templates. Such competition might alter relative levels of transcription, but should not qualitatively affect the conclusions.

Results of the injection of the H1A 5′ deletion mutants are shown in Fig. 2. The upper portion of Fig. 2 shows the data obtained from one set of injections. The normalized results, and those from three other sets of injections of H1A 5′ deletion mutants, are quantitatively displayed in the lower part of the figure. In this figure, as for the studies below with the other histone genes, each datum point plotted represents the average result obtained from multiple (two to four) sets of injections. It was apparent that DNA from about position −600 to about position −100 may be removed without significant effect on H1A gene transcription. Particularly relevant was our finding that the removal of two distal conserved sequence elements (approximately 170 and 300 bp upstream of the transcription initiation site) had no obvious

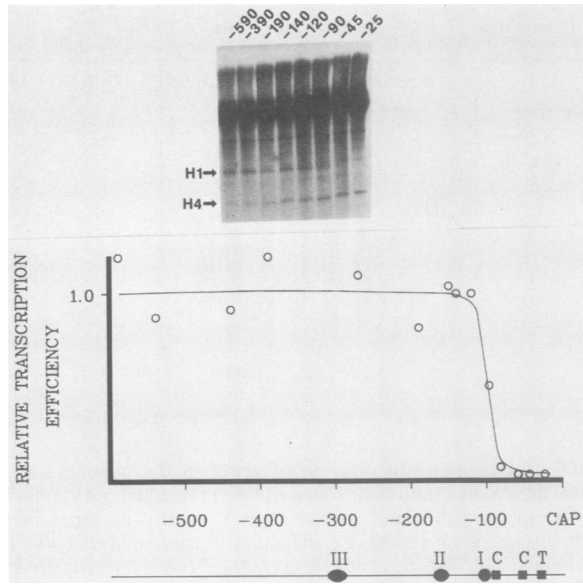

FIG. 2. Transcriptional analysis of H1 5′ deletion mutants by injection into frog oocyte nuclei. (Top) Results obtained by coinjection of a set of H1 5′ deletion mutants with an internal control gene (H4) and [α-^{32}P]GTP. (Bottom) Results obtained above, as well as results from injections of other H1 deletion mutants. Labeled symbols on the bottom line represent conserved sequences within the H1 promoter region, including the TATA and CCAAT boxes and upstream sequence elements I, II, and III (see also Fig. 4).

consequences for H1A transcription. These results are consistent with a previous study in which deletion of the AAACACA motif (present at about position −160 for the frog gene) was shown to have no effect on transcription of a chicken H1 gene in injected frog oocytes (43). Subsequent studies have shown, however, that this motif is required for cell cycle-regulated transcription of the same chicken H1 gene in stably transfected HeLa cells (8) and for expression of a late H1 gene in sea urchin embryos (25). These results suggested that H1 gene transcription in oocytes may be regulated by a subset of those factors used in cycling cells.

We found that no significant reduction in H1A transcription was obtained unless all but about 100 bp of upstream sequence was removed. The 5′ boundary of this region occurred within 20 bp of an element at position −110 that is conserved among H1 genes and that resembles a GC (SP1) box. Further deletion of more-proximal DNA sequences resulted in undetectable levels of H1 transcription. (For the revised nucleotide sequences of the promoter region for H1 and of the other histone genes promoters studied here, see Fig. 4.)

Results obtained for the analysis of sequences required for H2A transcription are shown in the upper left portion of Fig. 3. The first two lanes in the top portion of Fig. 3A depict the levels and size of RNA produced after injection of separate H2A and H4 templates, thus providing independent identification of both histone gene transcripts. Although the deletion of nearly 700 bp of 5′-flanking DNA (from positions −815 to −130 and including the conserved element at position −200) did not change H2A transcription significantly, removal of 30 bp between positions −130 and −100 resulted in an approximately 10-fold decrease in H2A transcription. This decrease correlated with removal of a series of 18 consecutive C residues immediately preceding an

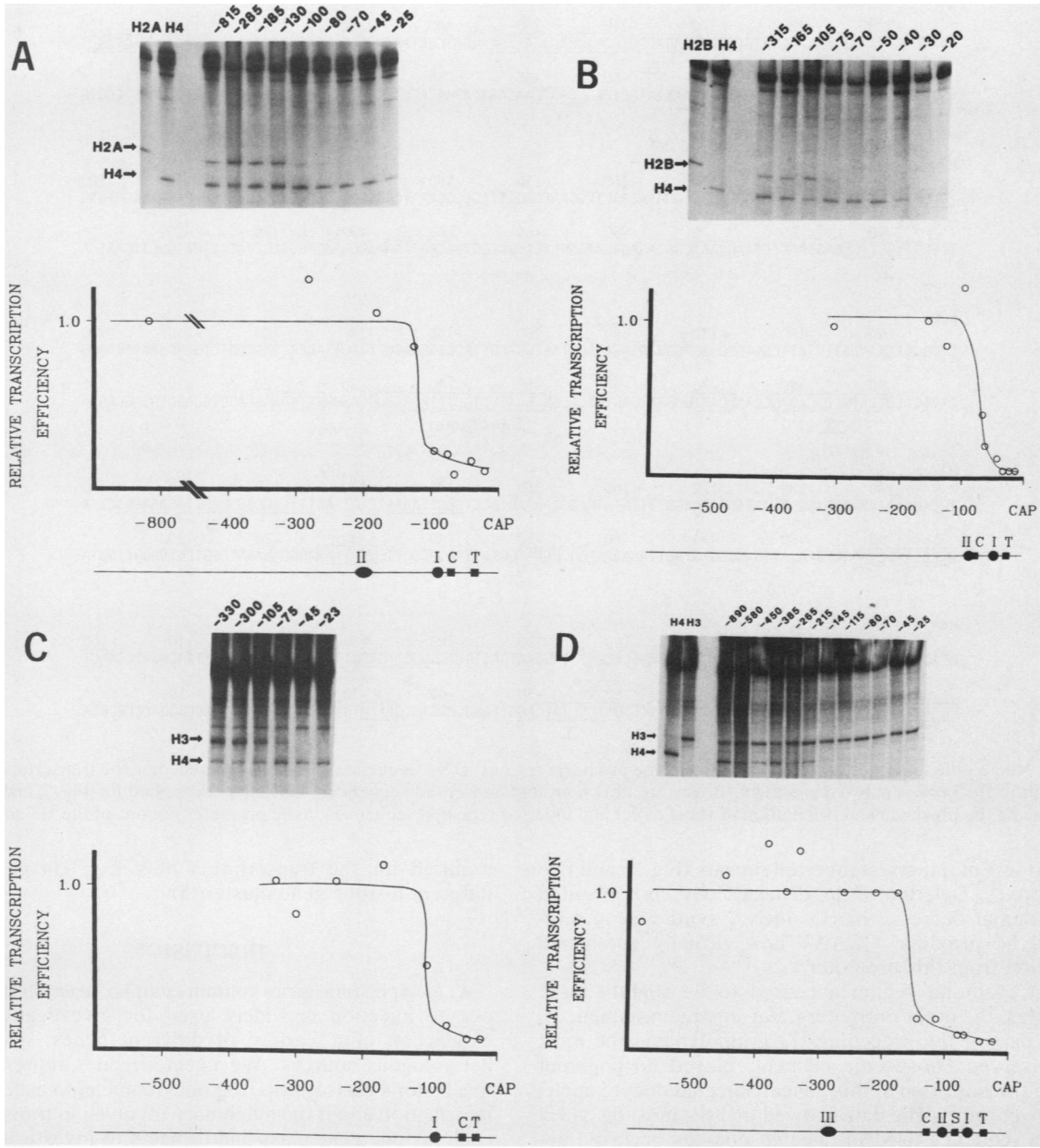

FIG. 3. Transcription analysis of H2A, H2B, H3, and H4 5′ deletion mutants. Sets of 5′ deletion mutants were injected into frog oocytes. The top portion of each panel depicts the results of a typical set of injections, and the bottom portion presents a summary of these results and those from other injections: 5′ deletion mutant injections of H2A (A), H2B (B), H3 (C), and H4 (D), respectively. Labeled symbols on the bottom line in each panel represent conserved sequences, as described for Fig. 2.

H2A-specific element at position −95 (Fig. 4). The H2A-specific element has been shown to be required for maximal transcription of a sea urchin H2A gene injected into frog oocyte nuclei (17) and apparently functions as a transcriptional enhancer in vitro (18). In our study, deletion of additional DNA, including the H2A element, was accompanied by a gradual diminution in the accumulation of H2A transcripts.

Analysis of the sequences required for transcription of the H2B gene (Fig. 3, upper right) demonstrated that most of the spacer DNA between the H1 and H2B genes (Fig. 1) can be eliminated without effect on H2B transcription. The first quantitative decrease in transcription occurred after removal of a region containing an element at position −90 that was initially identified as a potentially species-specific conserved sequence present upstream of *X. laevis* H2B histone genes (31). Further deletion of the adjacent CCAAT box reduced transcription to background levels. The remaining promoter sequences, including the TATA box and the proximal H2B-specific element containing the octamer motif (shown to be required for cell cycle regulation of a human H2B gene [24]), were insufficient to drive high levels of transcription of this gene.

Transcriptional analysis of mutants with H3 gene 5′ deletion mutations (Fig. 3, lower left) indicated that sequences important for transcription of this gene lie in a region about 150 bp from the transcription initiation site, although the precise upstream boundary is uncertain. This region con-

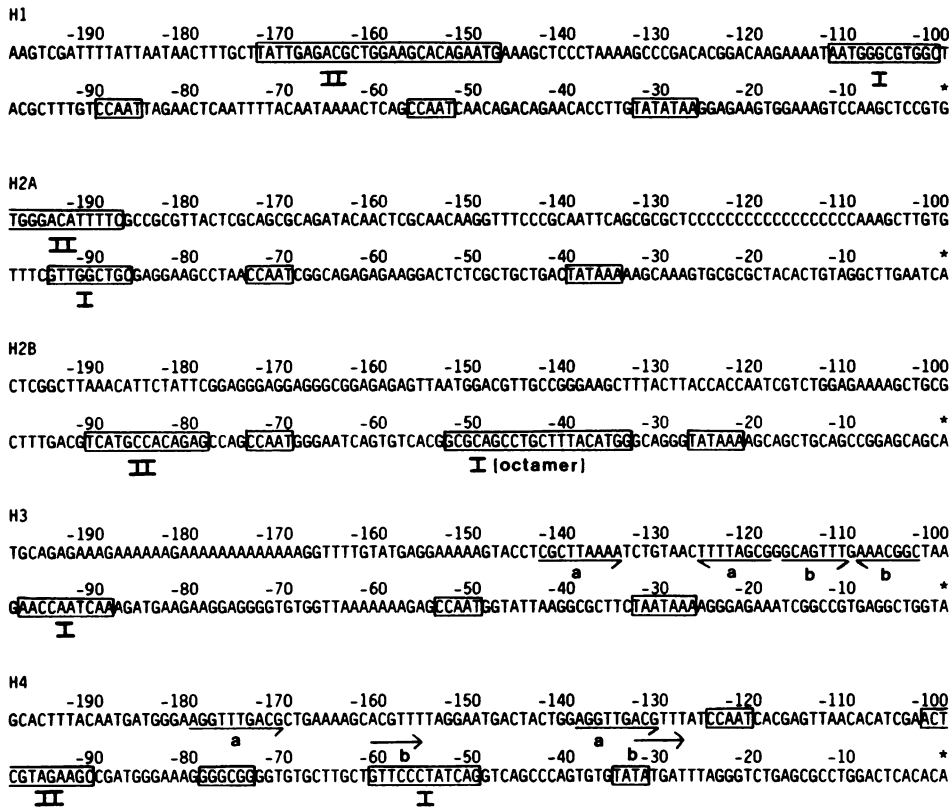

FIG. 4. Nucleotide sequence of *X. laevis* histone gene promoter regions. DNA sequence of the 200 bp preceding the transcription initiation site for each of the genes analyzed is shown. Boxes are drawn around conserved sequences, labeled as described for Fig. 2 and 3. Labeled arrows indicate the presence and orientation of short direct and inverted repeated sequences in the promoter regions of the H3 and H4 genes.

tained two sets of imperfect inverted repeats (Fig. 4) and two CCAAT boxes. Deletion of the distal CCAAT box resulted in a substantial decrease in H3 mRNA synthesis, and removal of the proximal CCAAT box virtually eliminated transcription from this promoter.

The H4 promoter region appeared to be slightly more complex than the other promoters. Far-upstream sequences, including part of the preceding H3 promoter and the most distal conserved H4-specific element, played no apparent role in the transcription of this gene in injected oocyte nuclei (Fig. 3, lower right). The data showed that the most distal H4 regulatory sequence used in injected oocytes occurred approximately 200 bp upstream of the transcription initiation site. Although no conserved sequences specific to H4 were found in this region, two sets of repeated sequences were noted. The first set is a 10-bp sequence at position −180 that is imperfectly repeated (9 of 10 matches) at position −139; the second is a 6-bp sequence at position −161 that was also found at position −133 (Fig. 4). Deletion of a region containing the first sequence of both sets resulted in significantly decreased H4 transcription. Deletion of the remainder of both sets of repeats and of an adjacent CCAAT box was without overt effect. Removal of an element at position −100 that appears to be specific to *X. laevis* H4 genes resulted in essentially background levels of transcription. Other more-proximal sequence elements whose function cannot be tested by this method included a GC (SP1) box at position −80, an additional H4 gene-specific element at position at position −60, and the TATA box at position −35 (Fig. 4). Elements corresponding in sequence and position to the H4 class-specific element at position −60 have been shown to be required for the transcription of a frog H4 gene from a different histone gene cluster (5).

DISCUSSION

***X. laevis* histone genes contain complex promoters.** *X. laevis* oocyte injection is widely used for investigations of the expression of a variety of different genes, usually from heterologous sources. We injected genes representing the five histone classes into frog oocyte nuclei to ascertain basic information about the machinery involved in transcription of each histone gene class and to begin to investigate possible transitions in their regulation during development. In recent years it has become clear that many eucaryotic genes possess complex promoters and enhancers consisting of multiple functional elements (reviewed in reference 26). Our studies indicated that frog histone gene promoters possess such complex structures. We found that each of the genes studied requires 100 to 200 bp of 5′-flanking DNA for maximal levels of transcription. These regions generally contain multiple conserved elements, some of which may be specific for particular histone classes, and short repeated sequences. Each gene contains, in addition, other conserved sequences farther downstream that represent likely signals for general transcription factors (i.e., GC, CCAAT, and TATA box regions). We found no consistent evidence for sequences interacting with negatively acting factors (repressors) as described for the expression of yeast H2A and H2B genes (30).

Several of the histone class-specific sequences and other less conserved sequences seemed to be required for tran-

scription in frog oocytes. In particular, removal of conserved elements in the −100 region was associated with large transcriptional decreases for the H1 and H2B genes. The other frog histone genes also contained conserved sequences in this region, but their function was not demonstrable because of transcriptional requirements for sequences upstream. A transcriptional requirement for less highly conserved, perhaps gene-specific, sequences was seen with the other frog histone genes. Specifically, this was observed for transcription of the H2A gene, which appeared to require an 18-bp stretch of C residues, and of the H3 and H4 genes, each of which required distinct sets of repeated sequences.

Several of the more distal conserved sequences lacked apparent function in this system. In particular, a conserved motif known to be required for H1 expression in cultured mammalian cells (8) and in sea urchin embryos (25) appeared not to be functional in frog oocytes (43; see above). At present, we cannot rule out the possibility that factors (acting either positively or negatively) that bind to distal promoter sequences are present in oocytes in low abundance and are titrated out by an excess of injected template. Although it is clear that the contributions of individual sequences and factors to the transcriptional regulation of frog histone genes must be analyzed further, the results reported here provide tentative support for a model in which distinct factors and sequences are used by cells in different growth states and developmental stages to regulate histone gene transcription.

Relationship of present work to previous studies. Our results were generally consistent with those obtained for histone genes from other organisms. In cases for which it was most closely examined —namely, the human H2B and H4 genes —a region important for transcription was functionally identified in the −100 region. For both genes, this region contains distinct sets of short repeated sequences (20, 38), as seen slightly farther upstream with the frog H3 and H4 genes examined here. Nuclear proteins that interact specifically with this region have been identified for the human H4 gene (7), although the functional significance of this binding has not been demonstrated.

Farther downstream (at about position −50), both human histone genes have been shown to contain conserved class-specific sequences with which specific nuclear proteins interact (7, 12). The H2B element in this region consists, in part, of the octamer motif —an increasingly ubiquitous consensus sequence that is important for the transcription of several types of genes (2, 11, 38), viral DNA replication in vitro (34, 41), and cell cycle regulation of a human H2B gene in vivo (24). An octamer-binding protein from human cells has been shown to stimulate H2B transcription in vitro (12). As with the H2B octamer motif, a protein in extracts from human cells has been shown to interact with the H4-specific element in the −50 region (7); in this case, however, no function has been directly demonstrated for the interaction. Indirect evidence suggesting a functional significance for this interaction comes from the demonstration that the presumed homologous motif is required for transcription of an *X. laevis* H4 gene in injected frog oocytes (5).

Studies of functional sequences required for histone gene transcription in other species, including sea urchin (17, 18), hamster (1), and chicken (8), support the view that histone gene promoters consist of multiple functional domains. Recent reports with a human H2B gene (24) and a chicken H1 gene (8) have demonstrated that conserved motifs critical for cell cycle-regulated transcription of these genes reside at position −50 for the H2B gene and position −120 for the H1 gene. Taken together, these results do not support the previous suggestion that functionally equivalent promoter elements occupy strictly analogous upstream positions (31). This conclusion stems from the fact that the octamer motif is immediately adjacent to the H2B gene TATAA box, whereas the apparently functionally analogous H1 element is separated from the H1 gene TATAA box by almost 100 bp, including a GC box and a CCAAT box.

In view of the multiplicity of functional and conserved elements present in each of these promoters, it is reasonable to predict that the transcriptional regulation of these genes is dependent on combinatorial interactions between multiple factors binding to distinct regulatory elements, as is generally believed for promoters and transcriptional enhancers. As suggested, the interplay of multiple distinct factors and sequences could provide the framework necessary for regulation of inducible gene transcription. *X. laevis* histone genes seem to represent a system that may be suitable for addressing some of these issues in a developmental context.

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