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Multiple Sequence Elements Are Required for Maximal In Vitro Transcription of a Human Histone H2B Gene

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As part of our studies on the cell cycle regulation of human histone gene expression, we examined the elements governing transcription of a human histone H2B gene in nuclear extracts derived from human HeLa cells. Circular templates were transcribed at 5- to 10-fold higher levels than were linear templates. A series of deletion, linker-substitution, and point mutants defined *cis*-acting promoter sequences that were recognized in nuclear extracts. These sequences extended from 118 to 21 base pairs 5′ to the transcription initiation site. Elements recognized included (from 5′ to 3′) a series of direct repeats, a CCAAT homology, a human histone-specific hexamer, an H2B consensus element, and a TATA box. Sequence elements 5′ to the hexamer were required for its function. In contrast, the H2B consensus element could function independently of more-5′ promoter elements and in turn was essential for the function of upstream elements. An interesting feature of this consensus is that its core octanucleotide (ATTTGCAT) is found in several nonhistone genes. By comparison with functional elements in an H4 promoter, we infer that a combinatorial interaction of general and gene-specific factors may contribute to the S-phase elevation of H2B transcription.

It is now well documented that in cultured mammalian cells the expression of most histone genes is elevated during the S phase of the cell cycle (reviewed in references 12 and 18). This control operates at both the transcriptional and posttranscriptional levels (11, 25, 29) to coordinately regulate expression of all histone gene types. It is apparent from sequence comparisons that histone genes contain type-specific promoter elements (see reference 24), suggesting that their transcription may be partly governed by type-specific factors. This possibility is supported by the demonstration that human H4 genes transfected into L cells competitively decrease transcription of their mouse H4 counterparts, with no effect on transcription of other histone types (2), and by the isolation of putative type-specific histone-stimulatory fractions from sea urchin embryos (21).

Our aim is to define the *cis*- and *trans*-acting elements that govern human histone gene expression. One approach that we are taking is to analyze histone gene transcription in cell-free systems. Such analyses have proven powerful for dissecting the mechanisms of action of various transcription factors and enzymes, especially in procaryotes (32). In eucaryotes, fractionation of crude cellular extracts has led to the identification of several general (active in transcription of many genes) and gene-specific RNA polymerase II transcription factors (reviewed in references 4, 9, and 27) that interact with conserved promoter sequence elements. Specific factors may control transcription of nonconstitutively expressed genes, including those that are cell cycle regulated (7, 10, 22). In particular, the use of cell-free systems has proven useful for dissecting the sequence elements controlling transcription of a human histone H4 gene (7). Furthermore, there is evidence that extracts made from cells in S phase are transcriptionally more active for histone genes than are extracts prepared from nonreplicating cells (10), an observation that may be useful in complementation assays for purification of cell cycle-regulated transcription factors.

In this study, we analyzed the elements required for maximal expression of a cell cycle-regulated human histone H2B gene in HeLa cell nuclear extracts. Potentially interesting sequences 5′ to the cap site (as mapped in vivo) are shown in Fig. 9 and comprise putative "general" transcriptional signals (4, 9) that include a TATA box and CCAAT homology, a hexamer (GACTTC) found only in (many) human histone genes (30), and a 15-base-pair (bp) H2B-specific sequence conserved between sea urchin, frog, chicken, and human H2B genes (8, 24, 30). This latter element contains an octamer, ATTTGCAT, that is found (in the same or inverted orientation) in immunoglobulin heavy- and light-chain promoters (6, 23), in the immunoglobulin heavy-chain enhancer (5), in U2 (1, 17) and (some) U1 (3, 15) small nuclear RNA (snRNA) promoters, and (with one mismatch) in the simian virus 40 enhancer (see reference 6).

We report here the promoter boundaries of this H2B gene as assayed in cell-free extracts and present a dissection, by in vitro mutagenesis, of the elements constituting the promoter. In conjunction with a comparison between H2B and H4 promoter elements recognized in vitro, we suggest that multiple transcription factors may interact to control histone gene transcription.

MATERIALS AND METHODS

Suppliers. Radioactive precursors were supplied by New England Nuclear Corp., Restriction and DNA modification enzymes were purchased from Bethesda Research laboratories. Oligonucleotides were synthesized by P. Model and B. Goldstein in the Laboratory of Genetics, Rockefeller University.

Plasmids. Human histone genomic clones pHh4C and pHh4A contain *Eco*RI inserts of the H2B gene used in these studies (30). The gene is split by a natural *Eco*RI site at +230 bp (relative to the cap site). pHh4C contains the 5′ portion and pHh4A the 3′ portion of the gene. A clone containing the human H4 gene inserted into pUC13, p2606, was used as an internal control and was kindly given to us by S. Hanly.

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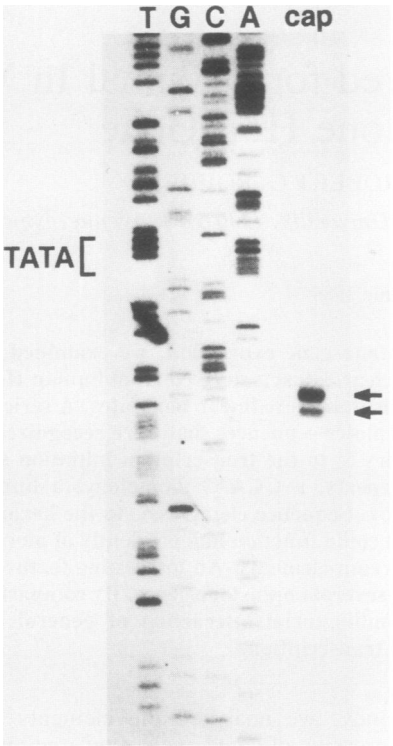

FIG. 1. In vitro H2B cap site mapping. Primer extension analysis of H2B RNA transcribed in a HeLa cell nuclear extract. The template was an uncut 3′ deletion clone (+100) containing the H2B promoter and M13 sequencing primer site (see Materials and Methods). Extension was performed from a 5′-end-labeled universal primer. Autoradiograph of extended products (cap) electrophoresed through a polyacrylamide gel is shown (arrows). Markers (T, G, C, A) are dideoxy sequencing products from the same clone, using the universal primer.

Mutant construction. The 5′ restriction deletion mutants MP11.PE and MP11.XE contained 162 and 2,500 bp of H2B sequence 5′ to the cap site, respectively. Both mutants extended 230 bp 3′ to the cap site. These mutants were constructed by subcloning *Pst*I-*Eco*RI and *Xba*I-*Eco*RI fragments from pHh4C into appropriately cut MP11 by standard cloning procedures (16). The 162-bp PE insert was also cloned into MP10 as MP10.PE.

The strategy used to construct deletion mutants is diagrammed in Fig. 4. 5′ deletion mutants were constructed from a parental plasmid in which a 782-bp *Pst*I-*Hin*dIII pBR322 fragment was inserted into the appropriate polylinker sites adjacent to the MP10.PE insert. The resulting plasmid was linearized with *Pst*I and treated with Bal31 exonuclease under the conditions recommended by the supplier. Portions were removed from the reaction mixture after various digestion times and subsequently processed separately. Digestion was stopped by the addition of 20 mM EDTA on ice, and the resulting fragments were purified by phenol extraction and ethanol precipitation. After filling in the ends with Klenow DNA polymerase, the fragment was again subjected to phenol extraction and ethanol precipitation. *Hin*dIII linkers were ligated to DNA ends, and following phenol extraction and ethanol precipitation, excess linkers were removed by digestion with *Hin*dIII and gel filtration on Sepharose CL4B (16). Since *Hin*dIII cleavage released the pBR322 DNA, the extent of exonuclease digestion at each time point was monitored by following the size of this fragment. Deleted plasmids were recircularized under dilute conditions (1 ng of DNA per μl) to prevent pBR322 DNA reinsertion. Strain JM101 cells were transformed with part of each ligation mix, and plaques were screened with the nick-translated pBR322 fragment as described previously (16). Plaques that did not contain reinserted pBR322 DNA were picked and grown, and phage DNA was prepared from culture supernatants as described previously (20). These DNAs were sequenced by the dideoxy method with the universal M13 primer as previously described (26). Appropriate deletion mutants were grown as the replicative form (16, 20), which was used for in vitro transcription.

The parental plasmid for 3′ deletion consisted of a *Pst*I-*Bam*HI fragment from pHh4C containing the H2B *Pst*I-*Eco*RI insert and a further 1 kilobase (kb) of pBR325 extending to the vector *Hin*dIII site. This was cloned into the *Pst*I and *Hin*dIII sites of MP10. The plasmid was linearized with *Nco*I, which cuts 14 bp 5′ to the insert *Eco*RI site, and deletion was performed exactly as for the 5′ series.

The H2B plasmid extending from −162 to +1200 was constructed from pHh4C and pHh4A. A 1-kb H2B *Eco*RI-*Hin*dIII fragment from pHh4A, extending from +231 to +1200, was converted into an *Eco*RI fragment by the attachment of *Eco*RI linkers. The fragment was inserted into the *Eco*RI site of MP10.PE. Insert orientation was determined by restriction analysis.

For linker-substitution mutant construction, an H2B *Eco*RI-*Hin*dIII insert from a 3′ deletion mutant was 3′-end filled. The resulting blunt-ended fragment was ligated into an *Hin*dIII-cut, blunt-ended, and calf intestinal phosphatase-treated 5′ deletion plasmid (16). Plasmids were screened for the appropriate orientation of insertion by dideoxy sequencing and for single inserts by restriction analysis.

For point mutant construction, oligonucleotides were lyophilized to dryness and "unblocked" by treatment with NH_4OH at 55°C for 8 h. Residual NH_4OH was removed by lyophilization from H_2O. Mutagenesis was done essentially by the method of Zoller and Smith (31). The parental plasmids for mutant construction were the clones extending from −162 to +1200 and the 5′ deletion mutant extending from −77 to +230. Phage DNA was hybridized to a kinase-treated mutant oligonucleotide and to non-kinase-treated M13 universal primer. Extension was carried out at 15°C for 12 h. After transformation and plating, nitrocellulose plaque lifts were prehybridized for 8 h at 25°C and hybridized with 5′-end-labeled oligonucleotide for 12 h. Filters were washed sequentially in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) at 25, 45, and 60°C. Plaques remaining hybridized at the highest temperature were picked, eluted in L broth (16), and replated to attain plaque homogeneity. Secondary plaques were randomly picked and grown. Phage DNA was sequenced by the dideoxy method to verify mutant genotype.

Nuclear extract preparation and in vitro transcription. Nuclear extracts were prepared from HeLa cells synchronized to the S phase as described previously (10). For technical reasons we did not analyze extracts from nonreplicating cells. Transcriptions were carried out in 10-μl reaction volumes containing 5 μl of nuclear extract, 0.4 μg of specific template DNA, 120 μM each ATP, CTP, and UTP, 12 μM GTP, 5.0 μCi of [^{32}P]GTP (600 Ci/mmol), 5 mM creatine phosphate, and 60 mM KCl. After incubation for 45 min at 30°C, transcription reactions were terminated by the addition of 50 μg of carrier RNA and 0.5% sodium dodecyl sulfate. After phenol-chloroform and chloroform extractions, nucleic acids were ethanol precipitated and analyzed

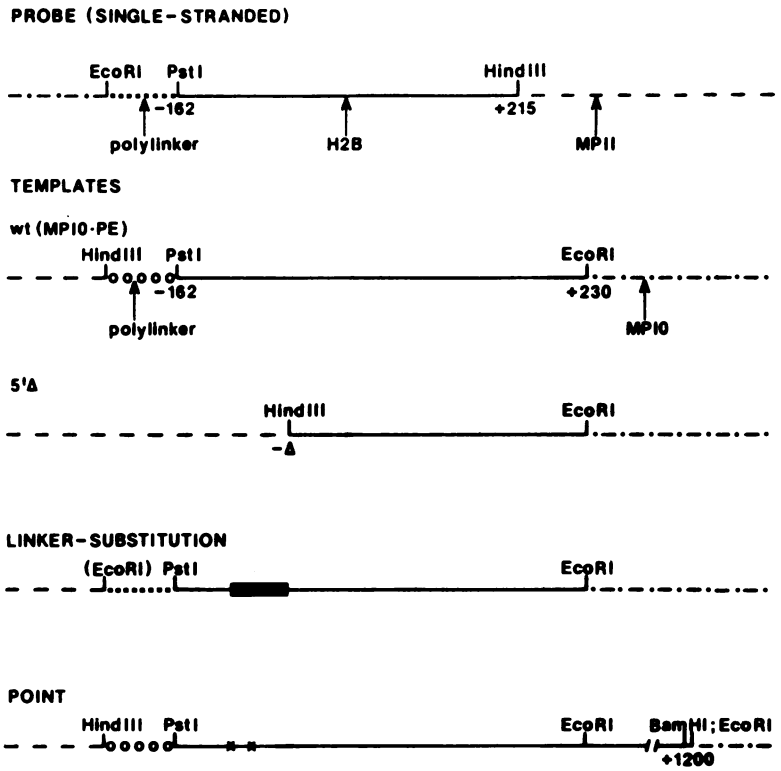

FIG. 2. Templates, probe, and S1 mapping strategy. All templates consisted of an H2B insert cloned into the polylinker of MP10 (except MP11.PE and MP11.XE; see Materials and Methods) and extended at least to an *Eco*RI site at +230 bp (relative to the cap site). The wild-type template (MP10.PE) extended from −162 and contained polylinker from the *Pst*I to the *Hin*dIII site of MP10. 5′ deletion inserts comprised an *Hin*dIII linker and variable lengths of 5′ H2B sequence. Linker-substitutions were formed from the fusion of 3′ and 5′ deletions (see Materials and Methods) and therefore had polylinker from the (destroyed) *Eco*RI to *Pst*I sites. Point mutants were constructed in a clone similar to MP10.PE but extending to +1200. Probe consisted of a 3′ deletion extending from −162 to +215 (see Materials and Methods) and containing polylinker from the *Eco*RI to *Pst*I sites. This insert was cloned into MP11 so that single-stranded phage DNA would contain the H2B coding strand and therefore be capable of hybridizing to H2B RNA. A 3′ discontinuity between RNAs transcribed from the templates shown and this probe was therefore created at +215, so that a discrete H2B RNA 215 nucleotides long would be protected from S1 nuclease digestion. Since all templates also had a 5′ discontinuity with respect to the probe, other discrete RNAs would be protected. These would include products initiating in the vector or, for circular H2B templates, readthrough RNAs from the H2B promoter traversing the entire vector. Resultant S1-protected bands would include those the entire length of the probe insert or, when a further 5′ discontinuity existed, less than complete probe insert lengths. wt, Wild type.

on 6% polyacrylamide–urea gels. Transcription products were visualized autoradiographically.

S1 nuclease analysis. The S1 mapping strategy is diagrammed in Fig. 2. A single-stranded phage DNA probe was constructed by cloning a 3′ deletion mutant *Hin*dIII-*Eco*RI insert into MP11 so that the phage DNA contained sequences complementary to H2B RNA. The mutant extended from −162 to +215. The S1 mapping procedure was a modification of that described in reference 7. Total nucleic acid, including radioactively labeled RNA, was purified from each in vitro transcription reaction and suspended in 70% formamide–0.4 M NaCl–10 mM PIPES (piperazine-*N*,*N*′-bis-2-ethanesulfonic acid, pH 6.4)–1 mM EDTA–0.5 μg of probe DNA in a total volume of 20 μl. Samples were hybridized at 45°C for 4 h. Then, 200 μl of a solution containing 0.25 M NaCl, 50 mM sodium acetate (pH 4.5), 4.5 mM zinc sulfate, and 25 U of S1 nuclease was added to each reaction mixture. After incubation at 30°C for 30 min, digestion products were ethanol precipitated and analyzed by electrophoresis through 6% polyacrylamide–urea gels. S1-protected RNAs were visualized autoradiographically.

Primer extension. Primer extension analysis of unlabeled in vitro-transcribed RNA was done as described previously (14), using the universal M13 sequencing primer 5′-end labeled. Products were run on an 8% sequencing gel and visualized autoradiographically.

RESULTS

H2B gene is faithfully transcribed in nuclear extracts. Our analyses involved the human histone H2B gene, which is part of the plasmid pHh4C (30). Expression of this gene is cell cycle regulated, as shown by S1 mapping of RNA from synchronized HeLa cells (30). We first wished to establish that this gene was transcribed in vitro and that the transcription start site was similar to the in vivo site. Runoff transcripts generated from transcription of variably truncated templates indicated that this promoter was active in HeLa cell nuclear extracts (data not shown). The in vitro cap site was more accurately mapped by primer extension (see Materials and Methods). The data in Fig. 1 show the presence of two transcription start sites, expressed at a ratio of about 3:1. The major and minor sites mapped 24 and 27 nucleotides 3′ to the 3′ border of the TATA box, respectively. The major site was virtually coincident with the in vivo cap site mapped by S1 analysis (R. Zhong, unpublished

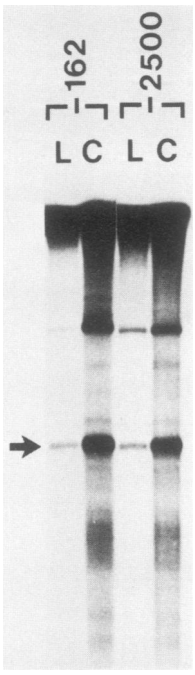

FIG. 3. Comparison of −162 and −2500-bp H2B promoter mutants. Autoradiograph of in vitro transcription products from clones containing 162 or 2,500 bp 5′ to the H2B cap site (see Materials and Methods). RNAs were trimmed by the S1 nuclease assay diagrammed in Fig. 2. Templates were either linearized (L) at the *Eco*RI site 230 bp 3′ to the cap site or assayed as circular molecules (C), at equal concentrations in parallel transcription reactions (see Materials and Methods). Arrow, specifically initiated H2B transcripts. The prominent higher-molecular-weight RNA bands resulted from protection of the entire H2B insert in the S1 probe by RNAs initiating in vector sequences, and for circular templates include readthrough products from the H2B promoter that transcribed the entire clone.

data). Therefore, elements of this H2B gene that specify transcription initiation in vivo are faithfully recognized in nuclear extracts.

Promoter elements recognized in vitro lie between −118 and −21. To delineate the 5′ and 3′ borders of the H2B promoter, we constructed an extensive set of deletion mutants. Initially, we determined the approximate 5′ boundary of this promoter by generating two deletion mutants (containing 2,500 and 162 bp 5′, to the cap site) by cleavage with appropriate restriction enzymes (see Materials and Methods). These were transcribed as linear (cleaved at a unique restriction site) or circular (uncleaved, initially supercoiled) templates in a HeLa cell nuclear extract. Since transcription from circular templates terminated heterogeneously in our extracts, specifically initiated RNAs were monitored by S1 hybridization analysis. The strategy, using uniformly labeled RNA and single-stranded M13 probes, is detailed in Fig. 2 and in Materials and Methods. Figure 3 shows the results of a representative analysis. First, 162 bp 5′ to the H2B cap site was sufficient for maximal expression in vitro, since equivalent transcription was seen from templates with 2.5 kb or 162 bp of upstream sequence. Second, the levels of transcripts from the circular templates were about 10-fold higher than those from linearized templates.

On the basis of this result, a series of 5′ deletion mutants was generated by Bal31 nuclease digestion of the −162 parental H2B clone as diagrammed in Fig. 4 and detailed in Materials and Methods. Figure 5A shows the levels of runoff transcripts produced from linearized 5′ deletion mutant templates in a representative assay. (Quantitation of assays from several experiments is presented in Fig. 9.) Specific H2B transcription decreased approximately fivefold as sequences between −162 and −39 were deleted. Transcription efficiency was altered incrementally, suggesting that discrete elements contributed to a fully functional promoter. Wild-type (−162 mutant) transcription levels were maintained in a −118 mutant. However, when sequences between this point and −100 were removed, transcription decreased on average to about 80% of the wild-type level. This region encompassed a series of 5-bp direct repeats. Deletion from −100 to −77, a region including the CCAAT homology, further depressed transcription to about 50% of the wild-type level. Curiously, as shown by comparison of the −69 and −60 mutants, deletion of sequences that included the histone-specific hexamer increased transcript levels to about 60% of wild-type. However, further deletion to −39 lowered transcription to 20% of its wild-type value. This latter deletion includes removal of an H2B-conserved element. Further deletion to −32 did not alter this "basal" transcript level. Finally, deletion of the TATA homology (−24) completely abolished specific transcription. This result indicated that upstream H2B promoter elements were recognized in HeLa cell nuclear extracts and that those sequences contributing to maximal transcription included several regions of "general" or histone gene-specific homology. Figure 5B shows that the effects of deletion were not due to a *trans*-acting inhibitor present specifically in mutant template preparations. Runoff analysis of 5′ H2B deletion mutants was performed in the presence of an H4 gene, serving as an internal control to which H2B transcription levels were normalized. To avoid potential competition for shared (non-TATA) transcription factors between H2B mutants and the H4 gene, we used a 5′ H4 deletion mutant that lacks sequences distal to −49 and is transcribed at basal levels in vitro (7). The results of this analysis were identical to those obtained when the H2B mutants were transcribed alone.

The effect of template topology on the activity of the 5′ deletion mutants was analayzed by transcribing these as circular plasmids and visualizing accurately initiated RNAs with the S1 mapping strategy shown in Fig. 2. The results of one analysis are shown in Fig. 5C, and several similar assays are quantitated in Fig. 9. While the promoter elements recognized were the same as those recognized in the linear templates (above), the effects of deletion were greater with the circular templates. For example, deletion to −100 decreased transcription to 50% of the wild-type level, removal of the CCAAT box (by a −77 deletion) depressed transcription to 25% of its maximal level, and H2B consensus deletion (to −39) reduced transcription to about 10% of wild-type levels. The increase in transcription observed in the linearized −60 mutant was also reflected in the analysis of the circular template, in which transcription increased (from 25% in the −69 mutant) to about 40% of the wild-type level. The greater magnitude of deletion effects from circular templates was not a result of the S1 assay, since S1 analysis of transcripts from linear templates yielded relative H2B RNA levels very similar to those seen by runoff analysis (see Fig. 9). The absolute difference in expression between linear and circular templates (shown earlier in Fig. 3) was reproduced in the 5′ deletion analyses, in which the levels of H2B RNA produced from circular templates were 5- to 10-fold greater than those from the corresponding linearized template (Fig. 5C). When circular templates were assayed, the

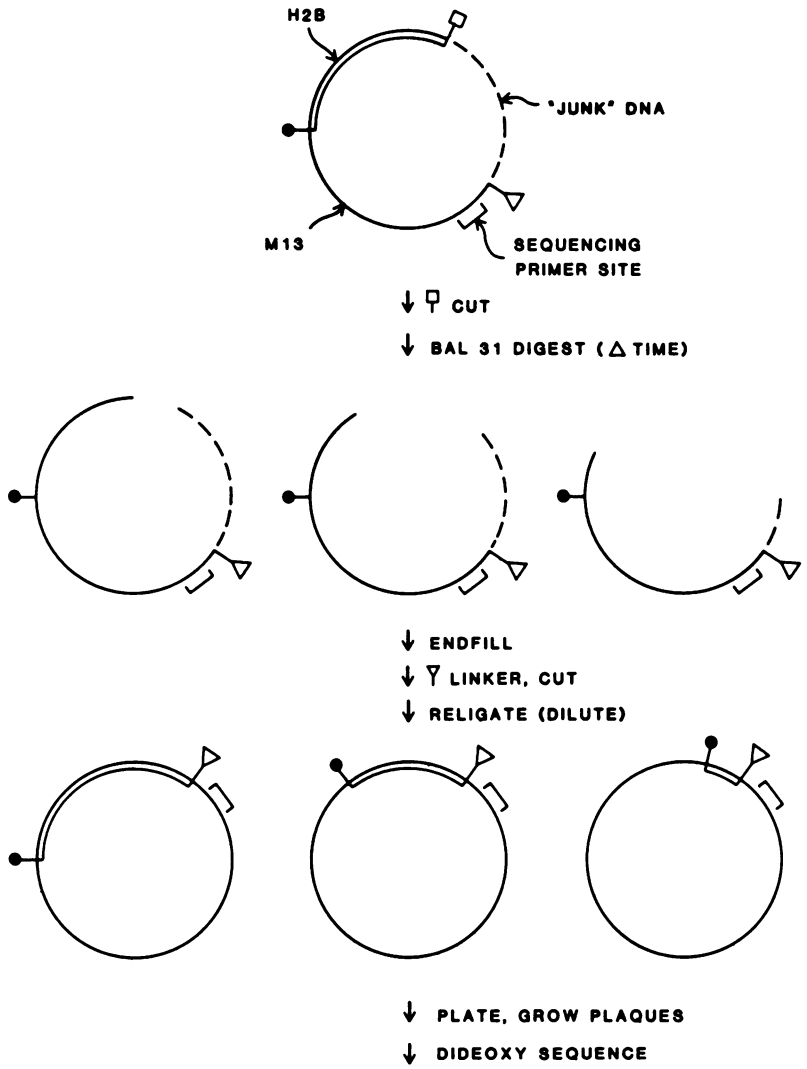

FIG. 4. Intramolecular strategy for H2B deletion mutant construction. An H2B insert extending from −162 to +230 bp (relative to the cap site) was cloned in either orientation in MP10 (see Materials and Methods). "Junk" DNA consisting of plasmid sequence was inserted between the M13 universal sequencing primer site and the H2B insert. After digestion with an enzyme cleaving near the junk-H2B junction, Bal31 nuclease digestion was carried out for various lengths of time. The junk DNA prevented removal, by nuclease action, of the primer-binding site. DNA ends were blunted, and *Hin*dIII linkers were attached and removed after secondary digestion with *Hin*dIII by chromatography on Sepharose CL4B. This cleaved the junk DNA from the vector. Recircularization was performed under dilute conditions to prevent reinsertion of the junk DNA. After plating and transformation, clones were sequenced by the dideoxy method to determine deletion endpoints. For both 5′ and 3′ deletion mutant constructions: ▽, *Hin*dIII site, for 5′ deletions: ●, *Eco*RI site; □, *Pst*I site, for 3′ deletions: ●, *Pst*I site; □, *Nco*I site.

increased concentrations of RNAs that ran near the top of the gel reflected protection by the S1 probe (see Fig. 2) of readthrough products from the H2B promoter into vector sequences, while the array of RNAs whose size decreased with increasing 5′ deletion reflected initiation in vector sequences.

To delineate the 3′ border of the H2B promoter, a series of 3′ deletion mutants were generated (see Fig. 4 and Materials and Methods) and transcribed. The 3′-most mutant contained about 1.2 kb of sequence 3′ to the cap site or about 700 bp 3′ to the end of the mature H2B mRNA. A runoff analysis of templates linearized in the vector is shown in Fig. 6. When normalized for transcript length, no change in transcription efficiency was observed until deletion had removed H2B sequences 3′ to +8 bp (relative to the cap site). The +8 mutant was transcribed at approximately 70% of the wild-type level. Further deletion to +2 depressed transcription to 30% of wild type; however, a −21 mutant was transcribed at nearly 70% of the maximal level. Deletion to −33, which removes the TATA box but retains all 5′ conserved elements, abolished specific H2B transcription. These results indicated that H2B sequences 3′ to the TATA box were not essential for efficient in vitro expression of this gene and could largely be replaced by vector sequences. However, since transcription was depressed to some extent by removal of H2B sequences near the cap site, either these sequences played some specific role in effecting maximal transcription of this gene or the introduced vector sequences were not compatible with efficient initiation.

Discrete sequences interact to potentiate H2B transcription. To define the contribution made by discrete promoter elements, we used two approaches to construct mutants in

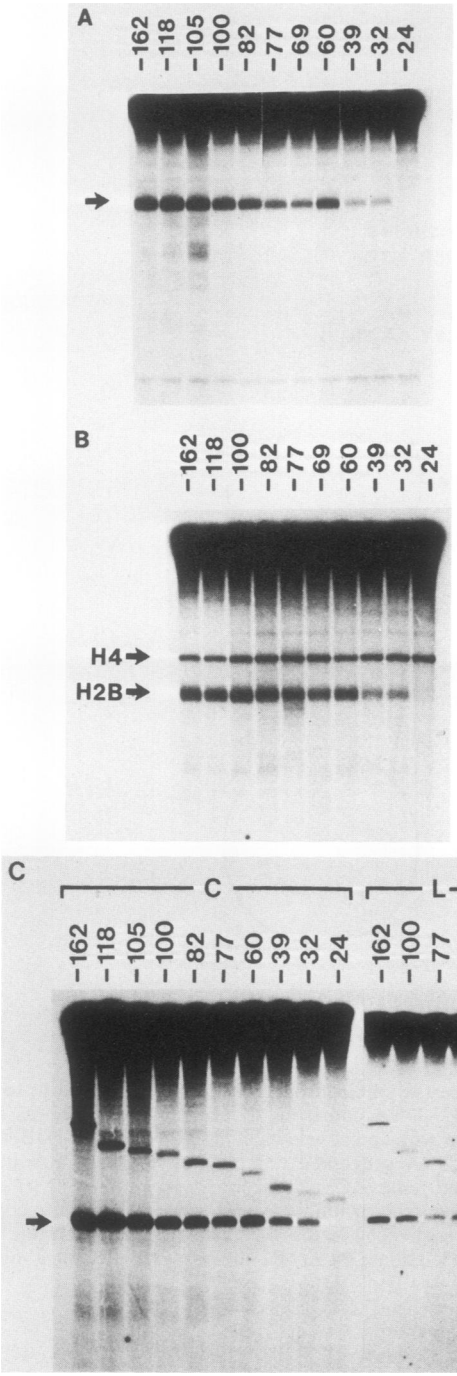

FIG. 5. Transcriptional analysis of 5′ H2B deletion mutants. (A) H2B 5′ deletion mutants were constructed as described in the legend to Fig. 4 and linearized at the *Eco*RI site 230 bp 3′ to the cap site. Equal concentrations of each mutant (40 μg/ml) were transcribed in HeLa cell nuclear extracts as detailed in Materials and Methods. Runoff transcripts were visualized autoradiographically after electrophoresis through a denaturing polyacrylamide gel. Positions of the 5′ deletion endpoints are indicated above the appropriate lanes. The arrow indicates specific H2B transcripts. (B) Similar transcription analysis as in panel A, with the inclusion in each reaction of 50 ng (5 μg/ml final concentration) of an H4 −49 deletion mutant cloned into pUC13 (see Materials and Methods and reference 7) as an internal control to which H2B transcription levels were normalized. The H4 template was linearized at a unique *Acc*I site 285 bp 3′ to the H4 cap site. Arrows, H4 and H2B runoff transcripts. (C) Transcription of H2B 5′ deletion mutants assayed by S1 analysis as detailed in the legend to Fig. 2 and in Materials and Methods. Equal concentrations of circular (C) or *Eco*RI-linearized (L) templates were transcribed as in panel A. Specific H2B transcripts are shown with an arrow; the transcripts whose length decreased with increasing H2B 5′ deletion initiated in M13.

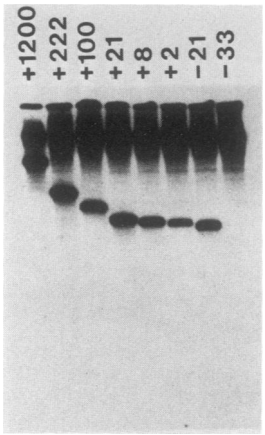

FIG. 6. Runoff transcriptional analysis of H2B 3′ deletion mutants. H2B 3′ deletion mutants were constructed as diagrammed in Fig. 4 and linearized at the unique M13 *Bgl*II site, located 3′ to the deletion endpoint of each mutant. This site is 660 bp 3′ to the +230 mutant insert-vector junction and therefore gave a runoff transcript of approximately 900 nucleotides from this mutant and relatively shorter transcripts from more-3′-deleted templates. The +1200 mutant was constructed as described in Materials and Methods and linearized at a unique *Bam*HI site near the junction of insert and vector sequences. Equal concentrations of DNA were transcribed, as detailed in Materials and Methods, and transcripts were analyzed autoradiographically after electrophoresis through a denaturing polyacrylamide gel. The 3′ endpoint of each mutant is shown above the appropriate lane.

which single elements were removed. First, appropriate 5′ and 3′ deletion mutants were ligated to form linker-substitution (19) mutants over the direct repeats and over regions including either the hexamer or the H2B consensus. In these mutants a 14-bp "filled-in" *Hin*dIII linker (see Materials and Methods and Fig. 7) replaced various H2B sequences with little or no change in the spacing of the 5′ and 3′ parts of the promoter. Second, one-, two-, or three-nucleotide changes in the octamer core of the H2B consensus element or in the hexamer were constructed by oligonucleotide-mediated mutagenesis (see Materials and Methods).

A representative transcription assay of linear and circular linker-substitution mutants is shown in Fig. 7, and the results of several such assays are quantitated in Fig. 9. A mutant that replaced the series of direct repeats between −115 and −100 was transcribed at about 60% of the wild-type level as either a circular or linear template. A mutant that replaced sequences between −74 and −60, a region that encompasses the hexamer element, was transcribed at 80% of the wild-type level as a circular template and at 100% of the wild-type level as a linear template. Replacement of the H2B consensus element between −53 and −39 depressed transcription to approximately 40% of the maximal level with either template topology. These data confirmed the role of sequences between −115 and −100 and the H2B consensus element in efficient transcription of this gene, but a positive or negative role of the hexamer remained ambiguous.

In the experiment shown in Fig. 7, the transcripts whose

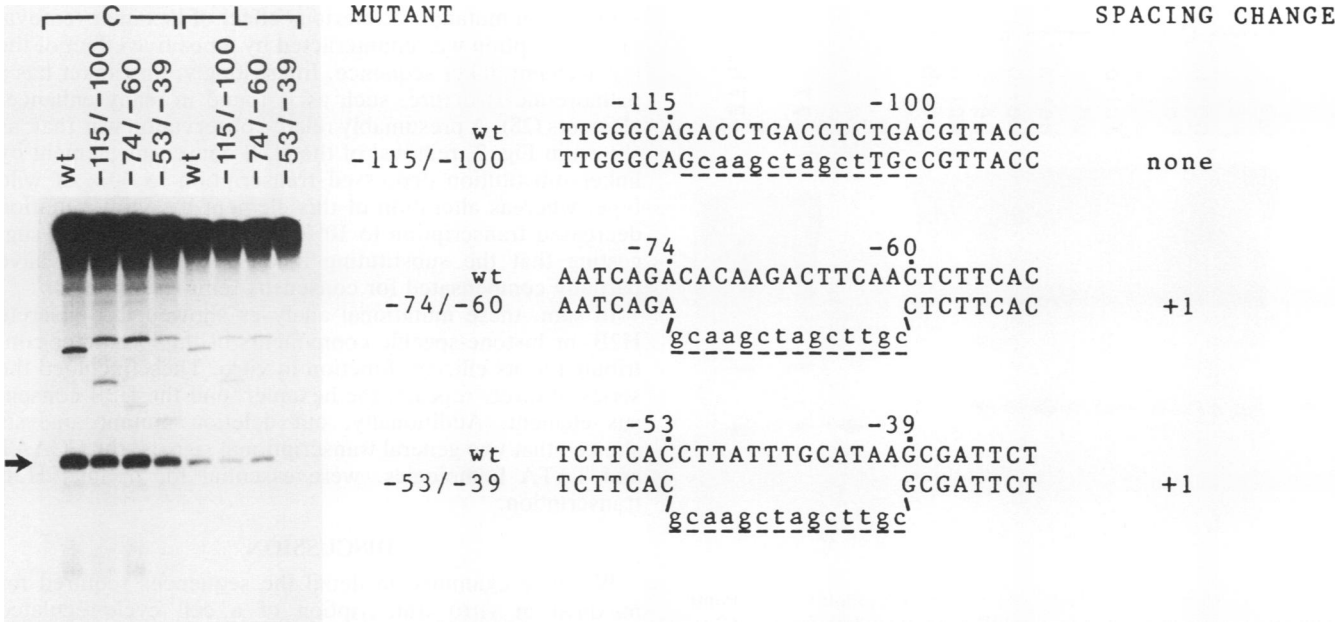

FIG. 7. Transcriptional analysis of H2B linker-substitution mutants. H2B linker-substitution mutants were constructed by religating 3′ and 5′ deletion mutants so that the promoter extended from −162 to +230 (relative to the H2B cap site), with replacement of 14 to 15 bp of H2B sequence by a filled-in *Hin*dIII linker, as described in Materials and Methods. The exact sequences replaced are shown beside the autoradiograph. The inserted linker sequence is underlined. For the −115/−100 mutant, the linker replaced H2B sequence with no spacing change in the promoter. Nucleotides unaltered from the wild-type (wt) sequence are shown as capital letters, while altered nucleotides are lowercase letters. The −74/−60 and −53/−39 mutants each contained one additional nucleotide in the inserted sequence. There are therefore 14 ways in which the wild-type and mutant sequences can be aligned. Since we do not know which alignment most accurately represents how the promoter is recognized in vitro, the inserted sequence is shown in lower case. Templates were either linearized (L) at the *Eco*RI site 230 bp 3′ to the H2B cap site or transcribed as circular (C) molecules at equal DNA concentrations, as described in Materials and Methods. Transcripts were mapped by the S1 strategy described in the legend to Fig. 2, electrophoresed through a denaturing polyacrylamide gel, and visualized autoradiographically. The sequences replaced in each mutant are indicated above the appropriate lanes as 5′ endpoint/3′ endpoint of replaced sequences. The wild-type (wt) template extended from −162 to +230 bp with no H2B sequence replaced. Arrow, specific H2B transcripts. Transcripts whose length decreased with more-3′ H2B sequence substitution initiated in vector sequences, while discrete higher-molecular-weight bands represent protection of the complete probe insert. See text for discussion.

sizes decreased as sequences nearer the cap site were replaced resulted from initiation in the vector. The discrete higher-molecular-weight RNAs whose changing intensities mirrored those of the H2B band (arrows) resulted from protection of the entire probe insert (see Fig. 2). Although such readthrough RNAs should have been cleaved at the site of linker insertion, it is likely that the palindromic structure of the transcribed linker (Fig. 7) prevented S1 digestion. We do not understand why the intensity of this band paralleled H2B promoter activity. While transcripts initiated at the H2B promoter and extending through the entire vector could have effected this result from circular templates, this was not possible from linear templates. One explanation could be that the activity of the H2B promoter influenced the activity of a fortuitous vector promoter(s).

The role of the H2B consensus and hexamer sequences was further examined by point mutagenesis of these elements. A representative transcription assay of these mutants is depicted in Fig. 8A, and quantitation of several similar assays is presented in Fig. 9. The mutants oct.a and oct.b each contain two nucleotide changes, altering the octamer "core" of the H2B consensus element from ATTTGCAT to AGTTGAAT and ATTGGCAG, respectively. Each mutation resulted in a dramatic transcriptional decrease with either linear or circular templates, oct.a to about 10% and oct.b to about 25% of wild-type levels. This result confirmed that the octamer sequence was essential for efficient H2B transcription.

The hex.a mutant contains a single transversion, converting the wild-type hexamer GACTTC to GAATTC, and was transcribed at wild-type levels. This was not surprising, since this genotype is found in at least one functional cell cycle-regulated histone gene (30). The hex.b and hex.c mutants contain two and three nucleotide changes which convert the hexamer to TACGTC and CACGGC, respectively. Both these mutations decreased H2B transcription to 50% of wild-type levels when assayed as linear or circular templates. This result, in contrast to those of previous assays (Fig. 5B, 5C, and 7), indicated a positive role for this element. The prominent higher-molecular-weight RNAs represent protection of the entire probe, since the S1 digestion conditions were not stringent enough to cleave single-base mismatches. As in the linker-substitution assay, however, we do not understand why the intensity of these bands paralleled H2B promoter activity.

We next asked whether the hexamer required upstream sequences for its function. A −77 5′ deletion mutant that lacked a CCAAT box and other more distal sequences, but retained an intact hexamer, was mutated to the hex.c sequence. The resulting mutant, which contains three transversions in the hexamer, was transcribed as either a linear or circular template. A representative analysis is shown in Fig. 8B, and several assays are quantitated relative to the wild type in Fig. 9. The parental −77 and −77 hex.c mutants were transcribed at similar levels as either circular or linear templates. The fact that the hex.c mutation reduced

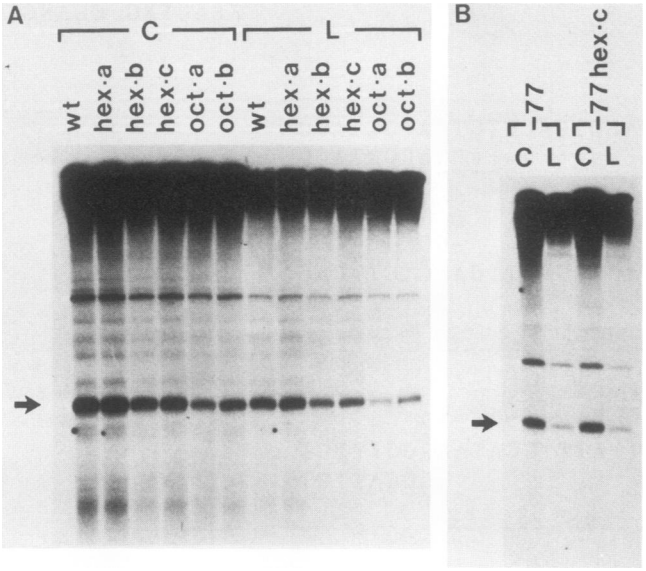

FIG. 8. Transcriptional analysis of H2B point mutants. (A) Point mutants were constructed in an H2B gene extending from −162 to +1200 relative to the cap site; construction of this clone and of the mutants is detailed in Materials and Methods. The wild-type (wt) template was this parental gene. hex.a, hex.b, and hex.c are 1-, 2-, and 3-nucleotide changes in the histone-specific hexamer centered at −65, respectively; oct.a and oct.b are each different 2-nucleotide changes in the H2B consensus element centered at −45. Exact nucleotide changes are detailed in the text. Mutants were either transcribed as circular templates (C) or linearized (L) at a unique *Bam*HI site approximately 1,200 bp 3′ to the H2B cap site. Equal concentrations of each DNA were transcribed, as described in Materials and Methods. RNAs were subjected to the S1 mapping procedure shown in Fig. 2, electrophoresed through a denaturing polyacrylamide gel, and visualized autoradiographically. Arrow, specific H2B transcripts. The prominent higher-molecular-weight band represents protection of the entire H2B insert in the S1 probe by readthrough transcription from the H2B promoter across the entire vector from circular templates and from transcripts initiating in the vector from both linearized and circular templates (see text). (B) An H2B −77 5′ deletion mutant (extending to +230 bp) was altered by the same three nucleotides in the hexamer element as the hex.c mutant (−77 hex.c). Mutants were transcribed as circular templates (C) or were linearized (L) at the *Eco*RI site at +230 and assayed as described for panel A. Arrow, specific H2B transcripts.

transcription when the promoter was otherwise intact but did not appreciably reduce transcription when sequences 5′ to −77 had been deleted indicated that the hexamer required upstream H2B sequences for its function.

We were initially unsure whether the hexamer acted positively, negatively, or indeed at all. As the hexamer cannot act in the absence of upstream sequences, we would predict no change in transcription levels upon its deletion; in fact, as shown earlier in Fig. 5, transcription increased 15% between a −69 and −60 mutant. The transcriptional increase was not caused by adjacent vector sequences, since it was still observed when several 5′ deletions were recloned so that the abutting vector sequence was completely different (data not shown). While we would further predict that a linker-substitution mutant over the hexamer should have shown decreased transcription, such a mutant was transcribed at approximately wild-type levels (as shown in Fig. 7). On the basis of these results we believe that the *Hin*dIII linkers juxtaposed to deletion endpoints could act to increase transcription in the −60 mutant, while in the linker-substitution mutant a deleterious effect of hexamer removal on transcription was counteracted by a positive effect of the replacement linker sequence. Interestingly, this linker has a palindromic structure, such as is found in many enhancer elements (28). A presumably related observation was that, as shown in Fig. 7, removal of the H2B consensus element by linker-substitution depressed transcription to 40% of wild type, whereas alteration of this element by point mutation decreased transcription to 10% of the wild-type level, suggesting that the substituting linker sequences may have partially compensated for consensus removal.

In sum, these mutational analyses showed that discrete H2B- or histone-specific components of this promoter contributed to its efficient function in vitro. These included the series of direct repeats, the hexamer, and the H2B consensus element. Additionally, our deletion mutant analysis showed that two general transcriptional signals, the CCAAT and TATA homologies, were essential for optimal H2B transcription.

DISCUSSION

We have examined in detail the sequences required for maximal in vitro transcription of a cell cycle-regulated human histone H2B gene. Transcripts of this gene were correctly initiated in HeLa cell nuclear extracts. By deletion mutagenesis, the 5′ and 3′ borders of the promoter were mapped between 118 and 21 bp upstream of the cap site. Progressive deletion and removal of discrete promoter elements by linker-substitution and point mutagenesis demonstrated that the H2B promoter is complex, consisting of several discrete regions. As will be discussed below, these comprise both general transcriptional elements found in many histone and nonhistone promoters and a histone-specific and an H2B conserved element. Maximal promoter function requires the interaction of all these elements. It seems likely that the cell cycle transcriptional regulation of this promoter is effected by factors that interact with histone or H2B-specific promoter elements or both.

In particular, sequences recognized were those between −118 and −100 bp (relative to the cap site), a CCAAT homology centered at −80, a human histone-specific hexamer between −69 and −63, a 15-bp H2B conserved consensus element centered at −45, and a TATA box whose 5′ end is at −30. Of these elements, those most commonly found in other promoters were the TATA and CCAAT homologies. The TATA box was apparently indispensable for H2B promoter function, as 5′ or 3′ (see below) deletions impinging on this sequence completely abolished specific transcription. 5′ deletion of sequences between −100 and −77, a region which included the CCAAT box, depressed transcription to 50% of the −100 level (Fig. 9). The complete consensus for this element is PuCCAAT (4), and indeed a −82 mutant that replaced this 5′ purine with a cytosine from the linker attached to the deletion endpoint (Fig. 7) was transcribed 15% less efficiently than the −100 mutant. It is of course also possible that non-CCAAT sequence elements in the −100 to −82 region contributed to this decrease.

The region between −115 and −100 contains a series of direct repeats. When uncut templates were assayed, transcription decreased maximally to 50% of wild type when this region was deleted. Furthermore, when this sequence was replaced by an *Hin*dIII linker, transcription decreased to 60% of wild type from both linear and circular templates. We do not know whether the direct repeats per se are important or whether a subset of this region not connected with the repeat structure enhances transcription.

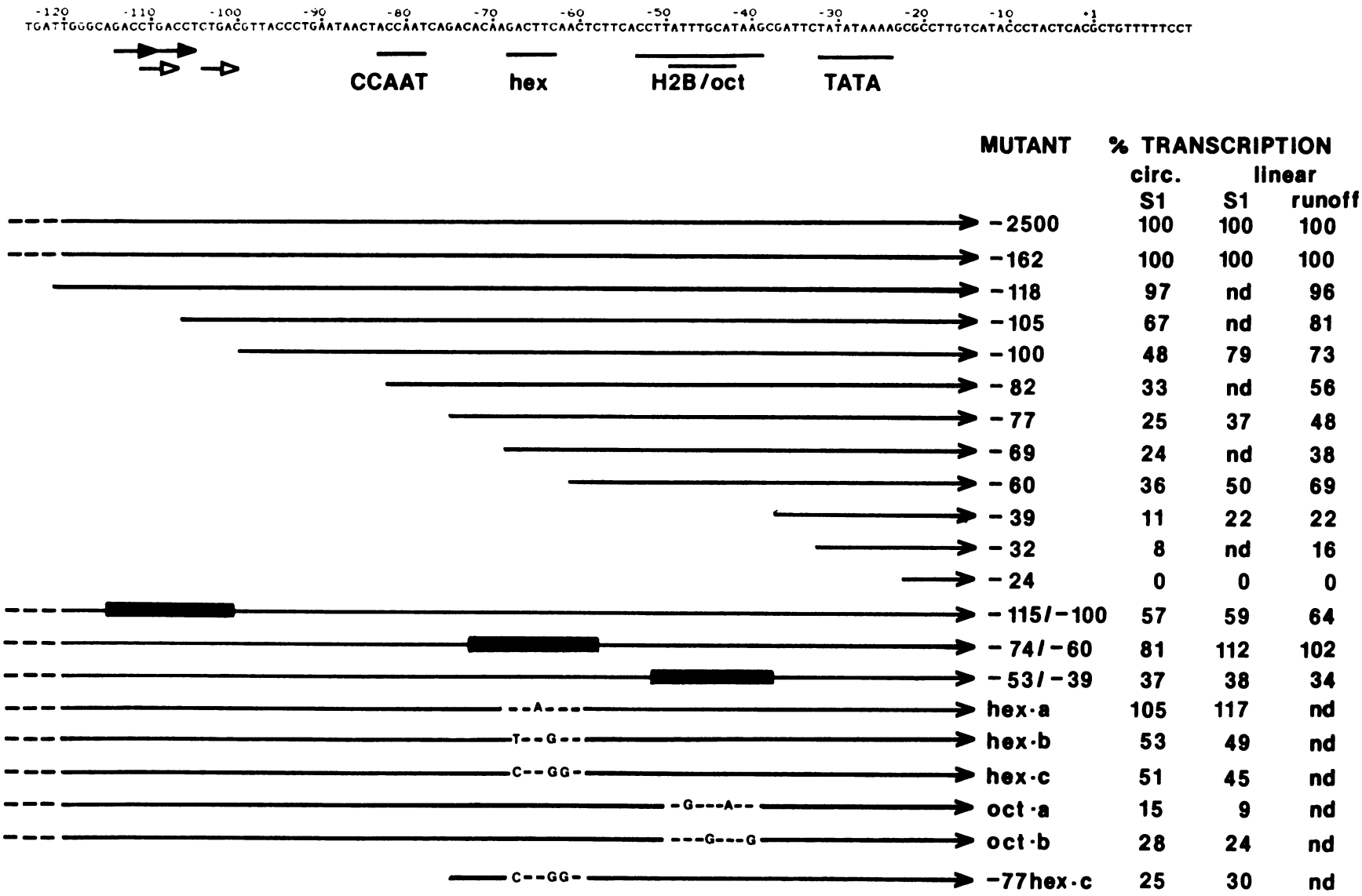

FIG. 9. Summary of H2B promoter analysis. Mutant designations are as described in the text and appropriate figure legends. The numbers shown are the means of at least three independent transcription assays, presented as the percentage of wild-type transcription. In all cases the standard deviation of each point was less than 15% of the mean. Quantitation was performed densitometrically, and at least two different autoradiographic exposures of each assay were quantitated to ensure that scans were performed in the linear range of the film and densitometer. nd, Not determined. Runoff data for −115/−100, −74/−60, and −53/−39 are not shown.

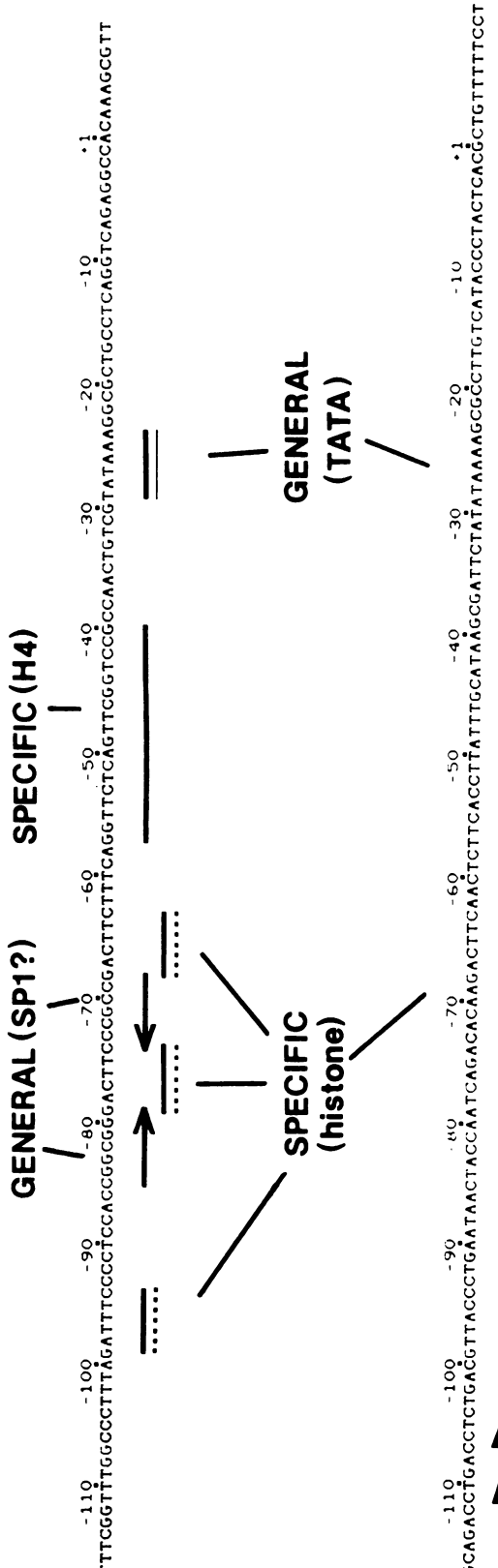

FIG. 10. Comparison of H2B and H4 promoter structure. DNA sequences are aligned with respect to the H2B and H4 cap sites. Conserved or unusual elements and the nature of transcription factors with which they may interact are indicated.

Point mutagenesis of a human histone-specific hexamer sequence located between −69 and −63 indicated that this element played a positive role in effecting efficient H2B transcription, since its removal decreased transcription by 50% from both linear and circular templates. Furthermore, we were able to show that the hexamer could act only in conjunction with more-5′ H2B sequence, since when a −77 5′ deletion clone was altered by mutation in the hexamer, no further decrease in transcription was observed. The functional upstream element closest to the hexamer is the CCAAT box, and it is possible that this is the element with which the hexamer interacts (presumably through the action of CCAAT- and hexamer-specific DNA-binding proteins).

The role of the H2B consensus element was confirmed by deletion, linker-substitution, and point mutagenesis. Its removal by 5′ deletion maximally (from circular templates) depressed transcription to about 10% of wild type. Point mutagenesis of an otherwise intact promoter indicated that the core octamer of the H2B consensus was essential for its function. Furthermore, since transcription of one point mutant was reduced to the same level seen in a deletion that removed all H2B sequences 5′ to the TATA box (Fig. 9), we conclude that the H2B consensus is essential for the function of upstream promoter elements.

It will be exceptionally interesting to determine whether the factor with which the H2B consensus (presumably) interacts can also interact with the snRNA promoters (1, 3, 15, 17) and immunoglobulin promoters (6, 23) and enhancers (5) that contain (at least) the octamer homology. The fact that functional sequence elements surrounding this consensus are quite different among these genes raises the possibility that the function of the octamer may be modulated by flanking elements. In particular, the proximity of the H2B consensus element to the TATA box suggests that factors recognizing these two elements may interact in a way analogous to that of the adenovirus major late promoter, for which it has recently been shown (27) that the TATA-binding factor stabilizes the association of a factor that binds to a contiguous upstream element.

3′ deletion analysis indicated that no elements essential for transcription lay 3′ to the TATA box. While deletion to +2 decreased transcription to 30% of wild type, further deletion resulted in 70% of maximal expression. Our results appear to differ from those obtained from analysis of a sea urchin H2B gene, assayed in *Xenopus laevis* oocytes (21). This gene apparently contains, within the transcribed region, sequences responsive to factors extracted from sea urchin chromatin. However, the authors did not demonstrate that this response was transcriptional rather than posttranscriptional.

The effects of template topology on levels of H2B RNA were dramatic. We consistently observed that circular templates were expressed at 5- to 10-fold-higher levels than their linear counterparts. We cannot rule out that this effect is partly posttranscriptional, since transcripts from circular templates are on average much longer than runoffs and might therefore be more resistant to 3′ exonuclease activity. However, as the nonequivalent expression of linear and circular templates persisted even when runoff lengths varied 20-fold (from 230 to 4,500 nucleotides; data not shown), it appears that much of the difference may be transcriptional. Although template DNAs were supercoiled at the start of the transcription reaction, we do not know the state of the template after incubation and cannot conclude whether (initial) superhelicity or circularity per se was required for the enhancement. Similar differences, dependent on template topology, in the in vitro expression of other histone and nonhistone genes have been documented (7, 13). No H2B sequence element(s) appeared to be specifically responsive to circularity, although the deleterious effects of 5′ deletions were about twofold greater in circular than linear templates. In either point or linker-substitution mutants, however, relative transcription differences were similar when linear and circular templates were compared. We cannot explain this discrepancy.

This study does not address which (if any) of the H2B elements identified are responsible for the elevation in transcription of this gene during the S phase. However, some insight into the mechanism by which histone gene transcription is regulated can be gained by comparing the structure of this H2B promoter with that of a cell cycle-regulated H4 gene. As assayed in vitro, this H4 promoter, like the H2B, comprises both histone-specific and more general elements. As diagrammed in Fig. 10, the H4 gene contains three copies of the human histone-specific hexamer, while the H2B gene contains a single copy of this sequence. Both genes contain a type-specific element of comparable length. This is located in a similar position relative to the TATA box, which in turn is the only general transcriptional signal shared by both genes. Additionally, the H4 promoter contains two GC-rich repeats that resemble transcription factor SP1 binding sites (4) and that are interspersed with the hexamer sequences, while the H2B promoter contains a CCAAT box in close apposition to the hexamer element.

In vitro transcription of deletion mutants in the H4 gene indicates that the hexamer and GC repeats are key elements in H4 promoter function (7). By these assays, the H4 consensus sequence was not independently recognized in vitro in the absence of upstream sequences. This contrasts to the H2B promoter, in which the H2B consensus element can function without more-5′ sequence, even though its effect is augmented by upstream elements. Furthermore, while H2B sequences 3′ to the TATA box could largely be functionally replaced by vector sequence, 3′ deletion of the H4 gene to −15 reduced transcription to less than 5% of wild type (7).

This comparison suggests that if coordinate S-phase transcriptional regulation of the H2B and H4 promoters involves a common DNA-binding factor, it must interact with the hexamer since this is the only histone-specific shared promoter element. It is also possible that histone type-specific factors contribute to or control cell cycle regulation of these genes. The activity of any of these putative regulatory factors could be modulated during the cell cycle by changes in protein concentration, or, in view of the rapid change in histone gene transcription rates associated with DNA synthesis (29), factor activity may be modulated by protein modification(s).

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ADDENDUM IN PROOF

Recent studies (H. Sive and R. Roeder, Proc. Natl. Acad. Sci. USA, in press) have demonstrated a HeLa cell factor which binds both to the H2B consensus element of the H2B

promoter and to the conserved octanucleotide core in promoters and enhancers of immunoglobulin and snRNA genes. A HeLa factor that interacts with the octanucleotide core of immunoglobulin genes was also reported by others [H. Singh, R. Sen, D. Baltimore, and P. A. Sharp, Nature (London) **319**:154–158].

LITERATURE CITED

1. **Ares, M., Jr., M. Mangin, and A. M. Weiner.** 1985. Orientation-dependent transcriptional activator upstream of a human U2 snRNA gene. Mol. Cell. Biol. **5**:1560–1570.
2. **Capasso, O., and N. Heintz.** 1985. Regulated expression of mammalian histone H4 genes in vivo requires a trans-acting transcription factor. Proc. Natl. Acad. Sci. USA **82**:5622–5626.
3. **Ciliberto, G., R. Buckland, R. Cortese, and L. Philipson.** 1985. Transcription signals in embryonic Xenopus laevis U1 RNA genes. EMBO J. **5**:1537–1543.
4. **Dynan, W. S., and R. Tjian.** 1985. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA binding proteins. Nature (London) **316**:774–778.
5. **Ephrussi, A., G. M. Church, S. Tonegawa, and W. Gilbert.** 1985. B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science **227**:134–140.
6. **Falkner, F. G., and H. G. Zachau.** 1984. Correct transcription of an immunoglobulin K gene requires an upstream fragment containing conserved sequence elements. Nature (London) **310**:71–74.
7. **Hanly, S. M., G. C. Bleecker, and N. Heintz.** 1985. Identification of promoter elements necessary for transcriptional regulation of a human histone H4 gene in vitro. Mol. Cell. Biol. **5**:380–389.
8. **Harvey, R. P., A. J. Robins, and J. R. E. Wells.** 1982. Independently evolving chicken histone H2B genes: identification of a ubiquitous H2B-specific 5′ element. Nucleic Acids Res. **10**:7851–7863.
9. **Heintz, N., and R. G. Roeder.** 1982. Transcription of eukaryotic genes in soluble cell-free systems, p. 57–89. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering, vol. 4. Plenum Publishing Corp., New York.
10. **Heintz, N., and R. G. Roeder.** 1984. Transcription of human histone genes in extracts from synchronized HeLa cells. Proc. Natl. Acad. Sci. USA **81**:2713–2717.
11. **Heintz, N., H. L. Sive, and R. Roeder.** 1983. Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. **3**:539–550.
12. **Hentschel, C. C., and M. L. Birnstiel.** 1981. The organization and expression of histone gene families. Cell **25**:301–313.
13. **Hirose, S., M. Tsuda, and Y. Suzuki.** 1985. Enhanced transcription of fibroin gene in vitro on covalently closed circular templates. J. Biol. Chem. **260**:10557–10562.
14. **Jones, K. A., K. R. Yamamoto, and R. Tjian.** 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell **42**:559–572.
15. **Krol, A., E. Lund, and J. E. Dahlberg.** 1985. The two embryonic U1 RNA genes of Xenopus laevis have both common and gene-specific transcription signals. EMBO J. **4**:1529–1535.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Mattaj, I. W., S. Lienhard, J. Jirichy, and E. M. DeRobertis.** 1985. An enhancer-like sequence within the Xenopus U2 gene promoter facilitates the formation of stable transcription complexes. Nature (London) **316**:163–167.
18. **Maxson, R., R. Cohn, and L. Kedes.** 1983. Expression and organization of histone genes. Annu. Rev. Genet. **17**:239–277.
19. **McKnight, S. L., and R. Kingsbury.** 1982. Transcriptional control signals of a eukaryotic protein coding gene. Science **217**:316–324.
20. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–79.
21. **Mous, J., H. Stunnenberg, O. Georgiev, and M. L. Birnstiel.** 1985. Stimulation of sea urchin H2B histone gene transcription by a chromatin-associated protein fraction depends on gene sequences downstream of the transcription start site. Mol. Cell. Biol. **5**:2764–2769.
22. **Nasmyth, K.** 1985. A repetitive DNA sequence that confers cell-cycle START (cdc28)-dependent expression on a set of cell type specific genes in yeast. Cell **42**:225–235.
23. **Parslow, T. G., D. L. Blair, W. J. Murphy, and D. K. Granner.** 1984. Structure of the 5′ ends of immunoglobulin genes: a novel conserved sequence. Proc. Natl. Acad. Sci. USA **81**:2650–2654.
24. **Perry, M., G. H. Thomsen, and R. G. Roeder.** 1985. Genomic organization and nucleotide sequence of two distinct histone gene clusters from Xenopus laevis: identification of novel conserved upstream sequence elements. J. Mol. Biol. **185**:479–499.
25. **Rickles, R., F. Marash, F. Sierra, S. Clark, J. Wells, J. Stein, and G. Stein.** 1982. Analysis of histone gene expression during the cell cycle in HeLa cells by using cloned human histone genes. Proc. Natl. Acad. Sci. USA **79**:749–753.
26. **Sanger, F., N. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
27. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:165–175.
28. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eukaryotic gene transcription. Trends Genet. **1**:224–230.
29. **Sittman, D. B., R. A. Graves, and W. F. Marzluff.** 1983. Histone mRNA concentrations are regulated at the level of transcription and mRNA degradation. Proc. Natl. Acad. Sci. USA **80**:1849–1853.
30. **Zhong, R., R. G. Roeder, and N. Heintz.** 1983. The primary structure and expression of four cloned human histone genes. Nucleic Acids Res. **11**:7409–7425.
31. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3**:479–488.
32. **Zubay, G.** 1980. Regulation of transcription in prokaryotes, their plasmids, and viruses, p. 153–214. *In* D. Prescott and S. Goldstein (ed.), Cell biology. Academic Press, Inc., New York.