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Interaction of a common factor with conserved promoter and enhancer sequences in histone H2B, immunoglobulin, and U2 small nuclear RNA (snRNA) genes

(octanucleotide/electrophoretic mobility shift/"footprinting"/HeLa cell nuclear extract)

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ABSTRACT **We have examined the interaction of factors in HeLa cell nuclear extracts with a human histone H2B gene (*H2B*) promoter. Protein–DNA mobility-shift and DNase I protection assays detected a factor(s) binding to a 15-base-pair consensus element that is essential for efficient *H2B* transcription *in vitro*. Part of this consensus sequence is the octanucleotide ATTTGCAT, which is apparently a functional component of several non-histone genes. A subset of these genes, including a human U2 small nuclear RNA (snRNA) gene promoter, a mouse immunoglobulin heavy chain enhancer, and a mouse light chain promoter, were shown to interact with the H2B consensus sequence-binding factor(s). These results suggest that a common factor or closely related factors may contribute to the regulation of these and other genes that share the octanucleotide sequence.**

Previous studies of a human histone H2B gene (*H2B*) promoter have identified a number of discrete functional elements, each of which is required for maximal levels of accurate transcription in nuclear extracts derived from HeLa cells (1). These elements are localized between base pairs (bp) −118 and −21 (relative to a cap site) and include (in the 5′ → 3′ direction) a region with two pairs of repetitive elements, a "CAAT" sequence, a hexamer sequence conserved among all human histone genes, an *H2B*-specific consensus sequence, and a "TATA" element (see Fig. 1). The *H2B* consensus sequence (CCTTATTTGCATAAG) extends from −53 to −39 and is conserved between *H2B* promoters in sea urchin, frog, chicken, and human (2). This element is essential for efficient transcription of this gene; its removal by 5′ deletion, linker-substitution, or point mutagenesis decreases transcription as much as 90% *in vitro*.

An essential part of this consensus sequence, the octamer ATTTGCAT, is found in a variety of non-histone genes. It is found in the same orientation in immunoglobulin κ chain promoters (3–5), in the putative promoter regions of several *Drosophila melanogaster* genes (6, 7), and within the immunoglobulin heavy chain enhancer (8, 9). It is found in inverted orientation in immunoglobulin heavy chain promoters (4, 5), in human U2 (10, 11) and amphibian U2 (12) and U1 (13, 14) small nuclear RNA (snRNA) gene promoters, and (with one mismatch) in the simian virus 40 (SV40) enhancer (4). The wide distribution of this sequence among a diverse set of genes and the lack of a fixed orientation or position relative to the transcription initiation sites suggested that the octamer might bind one or more transcription factor(s) (presumably protein) that can act in a variable manner.

To search for factors interacting with the *H2B* promoter, we have used protein–DNA mobility-shift and DNase I protection ("footprint") assays with nuclear extracts and chromatographic fractions derived from human HeLa cells. Our results show that the *H2B* consensus sequence binds a factor present in nuclear extracts and that an immunoglobulin light chain promoter, a heavy chain enhancer, and a U2 snRNA promoter specifically compete for this factor. These data suggest that a common factor, or a related group of factors, may recognize a consensus octamer in many genes.

MATERIALS AND METHODS

Enzymes. Restriction and DNA-modification enzymes were purchased from Bethesda Research Laboratories.

Plasmids. The *H2B* mutant plasmids used have been described (1). All constructs were cloned in bacteriophage M13 mp10. The human U2 snRNA mutant plasmids have been described (10) and consisted of inserts extending from −198 or −256 to +442 cloned in pBR322. The mouse T1 κ chain immunoglobulin 5′-deletion mutants, p90-gpt and p160-gpt (4), contained respectively 60 and 130 bp 5′ to the cap site. *Eco*RI–*Xba* I fragments used in the competitions extended from the deletion endpoint to approximately +900. Immunoglobulin heavy chain enhancer plasmids comprised the adenovirus major late promoter extending from −51 to +192 cloned in pUC12, either with or without juxtaposition at −51 of a 1-kbp *Xba* I fragment containing the enhancer from a mouse γ_{2b} (IgG2b heavy chain) gene (9).

Mobility-Shift Assays. Nuclear extracts employed in these assays were prepared from HeLa cells synchronized to the S phase, as described in ref. 15. Assays were a modification of the procedure described in ref. 16. Each binding-reaction mixture contained 4% (wt/vol) Ficoll, 1 mM $MgCl_2$, 20 mM Hepes (pH 7.9), 60 mM KCl, 0.25 μg of sonicated salmon sperm DNA, and 1.5 μg of protein consisting of a 1:10 dilution of HeLa cell nuclear extract in BC100 [100 mM KCl/20% (vol/vol) glycerol/20 mM Hepes, pH 7.9/2 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride]. Various amounts of competitor DNAs, as noted in figure legends, were added either 5–10 min before or simultaneously with 0.1–0.5 ng of 3′ end-labeled H2B promoter fragment. Total reaction volume was 20 μl. After incubation at 20°C for 15–30 min, reaction mixtures were loaded onto 4% polyacrylamide/0.25× TBE gels (1× TBE is 89 mM Tris/89 mM boric acid/1 mM EDTA) and electrophoresed at 100 V (≈15 mA). Labeled complexes were visualized autoradiographically.

DNase I Protection Assay. Protein used in these assays consisted of a 0.4 M KCl fraction from nuclear extract (prepared as described in ref. 17) chromatographed on heparin-agarose. DNA–factor complexes were formed in the usual manner; however, the labeled DNA, protein, and salmon sperm DNA concentrations were respectively 2 ng, 6 μg, and 0.75 μg per 20 μl. Additionally, 1 μg of specific

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Abbreviations: snRNA, small nuclear RNA; bp, base pair(s).

competitor DNA for the −147/−162 complex was included in each reaction. After binding, samples were treated with DNase I (2 μg/ml) for 1 min at 20°C. Digestion was stopped by the addition of 20 mM EDTA, and electrophoresis of complexes was performed as usual. Appropriate bands were excised from the gel and the DNA was electroeluted in 0.25× TBE at 50 mA. Recovered fragments were passed through a cotton plug to remove acrylamide, extracted with phenol/chloroform (1:1) and chloroform, and precipitated with ethanol. DNA was electrophoresed in an 8% polyacrylamide/urea sequencing gel and visualized autoradiographically.

RESULTS

***H2B* Sequences Interact with Factors in Nuclear Extracts.** The initial objective was to determine whether *H2B* sequences interacted in a site-specific way with factors (presumably proteins) in HeLa cell-derived nuclear extracts and whether such interactions could be correlated with functional *H2B* promoter elements. These analyses employed an electrophoretic assay that exploits the high-affinity binding properties of site-specific DNA-binding proteins and results in a shift in the mobility of a labeled DNA fragment when the protein is bound. This assay has been used successfully to define site-specific protein–DNA interactions in several other genes (18–21). In the present case, a radioactively labeled *H2B* promoter fragment was incubated with nuclear extract in the presence of an excess of carrier DNA, which binds nonspecific DNA-binding proteins and facilitates resolution of specific DNA–protein complexes. Of the several bands seen in addition to that of the unbound DNA, those resulting from sequence-specific factor–DNA interactions were deduced by competition studies with unlabeled wild-type and mutant *H2B* promoter-containing plasmids.

Three major bands were apparent in the initial assays with a labeled *H2B* fragment extending from −162 to +20 (Fig. 1). The pattern and band intensities seen with carrier DNA alone (data not shown) were exactly the same as those seen in the presence of an unlabeled *H2B* promoter-containing plasmid (5′Δ−39) lacking all promoter elements 5′ to the TATA box (lane 6). In the case of band 1, the intensity was greatly reduced by all *H2B* plasmid competitors (indicated above the lanes) containing an intact *H2B* consensus sequence (−53 to −39); these included the wild-type competitor (lanes 1 and 7), 5′ deletions to −147 (lane 8) and to −60 (lane 9), and a 3′ deletion to −33 (lane 2). In contrast, no specific competition for band 1 was seen with competitors lacking an intact *H2B* consensus sequence; these included plasmids with a 5′ deletion to −39 (lane 6), a 3′ deletion to −53 (lane 10), a linker-substitution mutant replacing sequences between −53 and −39 (lane 3), and a double point mutation (see figure legend) in the *H2B* consensus octamer (lane 4). These results suggested that the complex in band 1 contained a factor that interacted specifically with the *H2B* consensus sequence.

The wild-type *H2B* competitor (Fig. 1, lanes 1 and 7) reduced (by a factor of 6) the level of band 2 relative to the level observed with competitors containing 5′ deletions to −60 and to −39 (lanes 5, 6, and 9) or in the absence of specific competitor (data not shown, see above). The loss of competition with the deletion of 5′ sequences to −147 (lane 8) indicates that the 5′ boundary of the interaction site lies between −162 and −147, while effective competition with a 3′ deletion mutant extending to −115 (data not shown) indicates that the 3′ boundary lies upstream of this region. In agreement with these results, and clearly showing that the *H2B* consensus sequence is not involved in the interaction associated with band 2, competition was observed with the linker substitution (lane 3), double point (lane 4), and 3′Δ−53 (lane 10) mutants.

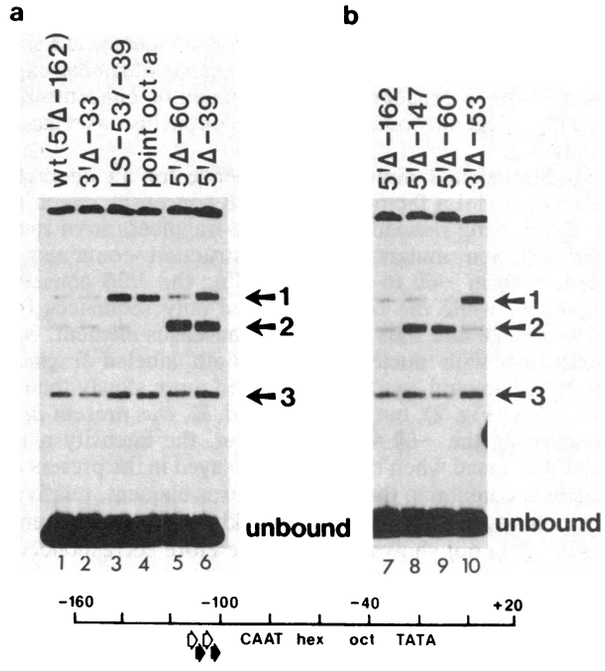

FIG. 1. Analysis of *H2B* promoter–factor interactions. A human *H2B* promoter fragment extending from a *Pst* I site at −162 to a *Hin*dIII site at +20 (relative to the cap site) was 3′ end-labeled and used to detect factor–DNA complexes as described in *Materials and Methods*. Sequences required for maximal *in vitro* transcription extend from −118 to −21. Important sequence elements (see text and ref. 1) and their relative positions are indicated on the probe diagram. These include (5′ to 3′) a series of direct repeats (arrows, −114 to −100), a CCAAT sequence (−82 to −78), a hexamer element (GACTTC) conserved in human histone genes (−68 to −63), an *H2B* consensus element (−53 to −39) containing the octamer (see text and Fig. 4), and a TATA box (−30 to −24). (*a*) Analysis of sequences required for band 1 complex formation. The positions of labeled complexes 1, 2, and 3 and unbound DNA are indicated. Unlabeled competitors (0.5 μg each, corresponding to a 25-fold molar excess of competitor to labeled insert) are indicated above the corresponding lanes. The 5′-deletion plasmids (lanes 1, 5, and 6) contained sequence extending from the position indicated above the appropriate lane to +230; the 3′ deletion (lane 2) extended from −33 to −162. In the linker-substitution mutant (LS −53/−39, lane 3), 14 bp of *H2B* sequence between −53 and −39 was replaced by a *Hin*dIII linker, with no other perturbation of the wild-type *H2B* promoter. The point mutant oct·a (lane 4) contained two transversions in the *H2B* consensus octamer, altering this sequence from ATTTGCAT to AGTTGAAT, in an otherwise wild-type gene extending from −162 to +1200. The intensity of band 1 (quantitated by densitometry) was lower by a factor of 3.5 with the wild-type (wt) competitor than when the point, linker-substitution, or −39 mutants were used as competitors. The intensity of band 2 was lower by a factor of 6 with the wild-type (−162) plasmid than with −39 or −60 mutant competitors. Autoradiographic exposures were in the linear range of both the film and densitometer. Titrations over a range of *H2B* competitor concentrations (0.25–2.0 μg) resulted in the same relative decreases in the intensities of bands 1 and 2 as are shown here. (*b*) Analysis of sequences required for band 2 complex formation. Competitor plasmids (0.5 μg each) are indicated above the appropriate lane. 5′ deletions extended from the point indicated to +230 (lanes 7–9); the 3′ deletion extended from −53 to −162 (lane 10). The intensity of band 2 decreased by a factor of 5 in the presence of the −162 5′- or −53 3′-deletion competitors, relative to the level seen with either the −147 or the −60 5′-deletion mutants as competitor. Data in *a* (lanes 1–6) and *b* (lanes 7–10) are from two separate experiments.

The most rapidly migrating complex (indicated by band 3) was apparently due to interaction with a nonspecific DNA-binding protein, since its intensity was not significantly altered by competition with any specific template but could be strongly reduced by preincubation of the nuclear extract

with unlabeled nonspecific competitor DNA (see Fig. 5). Although the experiments of Fig. 1 were conducted at a single competitor DNA concentration, the results of similar experiments with a broader range of competitor concentrations (see Fig. 1 legend) confirmed the above conclusions regarding bands 1–3.

Additional and more direct evidence for an interaction between a HeLa factor and the *H2B* consensus region was obtained using radioactively labeled fragments from two 5′ *H2B* deletion mutants. One construction contained sequences from −60 to +230, including the *H2B* consensus sequence, while the other contained only sequences from −39 to +230 and thus lacked the consensus element. After incubation with nuclear extract, both labeled fragments generated several bands that migrated more slowly than the free DNA (Fig. 2), but an extra band, B, was present in the assay using the −60 mutant. Further, the intensity of this band decreased when binding was assayed in the presence of plasmids containing the *H2B* consensus element, relative to the intensity seen with plasmids lacking an intact consensus region (data not shown). Band B therefore corresponded to the *H2B* consensus region–factor complex.

Band A in Fig. 2 corresponded to a complex requiring sequences between +100 and +230 for its formation, as demonstrated by competition analyses using various 5′- and 3′-deletion mutants (data not shown). Bands C corresponded to non-*H2B*-specific DNA–protein complexes, since their intensities were reduced equally by specific and nonspecific competitor DNAs.

Using a DNase I protection ("footprint") assay, we examined the region of DNA that was protected from nuclease digestion by the *H2B* consensus region-binding factor. Factor–DNA complexes in the initial incubation mixture were treated with DNase I before electrophoresis. Subsequently, labeled bands corresponding to bound (band 1 in Fig. 1) and unbound DNA fragments were excised from the gel. After protein removal (see *Materials and Methods*) the DNase cleavage patterns of each population were examined. Fig. 3 shows the results of one such analysis. A region of the *H2B* promoter between approximately −40 and −53 (solid bar) was protected from nuclease cleavage in the band 1 complex, whereas a nuclease-hypersensitive site was visible at −56 (arrow, see figure legend). This region encompassed the *H2B* consensus element including the core octanucleotide

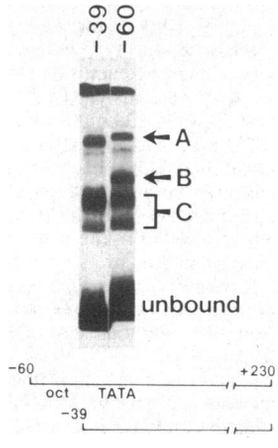

FIG. 2. Analysis of sequences required for *H2B* consensus complex formation. The 3′ end-labeled probes used are diagrammed; they consisted of *Hin*dIII–*Eco*RI fragments of 5′-deletion mutants either containing or lacking the consensus element. DNA–factor complex formation was as detailed in *Materials and Methods*. The probe used is indicated above the appropriate lane. Complexes A, B, and C and unbound DNA are indicated.

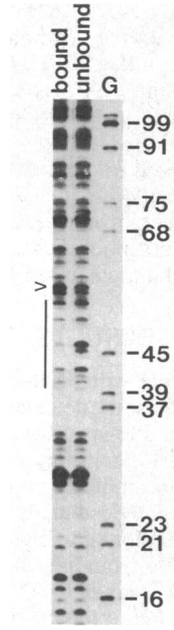

FIG. 3. DNase I "footprint" analysis of band 1 (*H2B* consensus complex). The *H2B* promoter fragment used for the analyses shown in Fig. 1, and extending from −162 to +20, was 3′ end-labeled at +20. DNase I protection analysis of DNA–factor complexes was as described in *Materials and Methods*. After nuclease digestion, purified DNA fragments were run in a sequencing gel alongside the same fragment cleaved at guanine residues (lane G). Numbers indicate G positions 5′ to the *H2B* cap site. DNA isolated from the *H2B* consensus complex (bound) and unbound DNA are indicated. The protected region (−40 to −53) is shown by a bar, and the hypersensitive site (−56), which was more readily apparent at a lower autoradiographic exposure, is indicated by the arrowhead.

(see Fig. 4) and unequivocally confirmed the DNA binding specificity of the *H2B* consensus region-binding factor.

Immunoglobulin and U2 snRNA Genes Bind the *H2B* Consensus Region-Binding Factor(s). Since a variety of nonhistone genes contain the octamer sequence, we determined whether a subset of these genes could interact with the *H2B* consensus factor. Incubation/competition experiments were carried out with the labeled *H2B* promoter fragment used in Fig. 1 and with unlabeled human U2 snRNA promoter, mouse immunoglobulin heavy chain enhancer, and mouse κ light chain immunoglobulin promoter competitors. A comparison of the octamer and flanking sequences in these genes is presented in Fig. 4. To maximize potential competitor DNA–protein interactions, specific competitor and carrier DNAs were preincubated with nuclear extract before the addition of the labeled fragment. This protocol greatly decreased the intensity of band 3 (which we therefore deduce to be due to nonspecific DNA–protein interaction) but did not otherwise alter the positions or intensities of bands 1 and 2.

Fig. 5*a* shows the results of competition with plasmids containing either of two 5′ deletions of a human U2 snRNA gene. The mutant that contained 256 bp 5′ to the cap site included the octamer homology, whereas in the −198 mutant this homology had been deleted. The deleted region has been shown (11, 12) to be essential for efficient transcription of this gene by *Xenopus laevis* oocyte injection and mammalian *in vitro* transcription assays. Although incubation with increasing concentrations of the two competitors decreased the intensity of all labeled complexes, the −256 mutant reduced the intensity of the band corresponding to the *H2B* consensus region-binding factor 3.5-fold more than did the −198 mutant. This demonstrated that a sequence between −256 and −198

H2B PROMOTER
-50 -40
ACCTTATTTGCATAAGCG

U2 PROMOTER
-222 -212
GGGGCATGCAAATTCGAA

-212 -222
TTCGAATTTGCATGCCCC (TRANSCRIBED STRAND)

Ig_k PROMOTER
-69 -59
CAATGATTTGCATGCTCT

Ig_H ENHANCER
+1540 +1550
GGGTAATTTGCATTTCTA

FIG. 4. Comparison of octamer homologies in histone H2B, immunoglobulin, and U2 snRNA genes. Nucleotide sequences are those of the human histone H2B gene from the plasmid pHh4C (22), the human U2 snRNA gene described by Westin *et al.* (11), the T1 κ light chain immunoglobulin promoter (4), and the immunoglobulin heavy chain enhancer isolated from a clone containing the γ_{2b} gene (9). Sequences are written in the 5′ to 3′ direction, with the nontranscribed strand shown, unless otherwise noted. Numbers indicate nucleotide position relative to the cap site.

in the U2 promoter interacted with a factor(s) in the band 1 complex.

To address whether a mouse immunoglobulin κ-chain promoter could stably interact with the *H2B* consensus region-binding factor, we used DNA fragments containing 130 or 60 bp of sequence 5′ to the cap site in competition assays. Sequences between these points contain the consensus octanucleotide and are required for the B-cell-specific expression of this gene (3, 4). Fig. 5*b* shows that the −130 mutant decreased the intensity of the *H2B* consensus band 3-fold more than equivalent amounts of the −60 mutant, suggesting that the octamer homology in this gene could bind to the *H2B* consensus region-binding factor(s).

Fig. 5*c* shows that a mouse immunoglobulin heavy chain enhancer, active primarily in cells of lymphoid lineages (9), also competed effectively for the H2B consensus region-binding factor. A plasmid containing the enhancer decreased the amount of labeled complex 7-fold more than an equivalent amount of control plasmid.

DISCUSSION

Using DNA–protein mobility-shift and DNase I protection assays, we have shown that several regions of a cell cycle-regulated human histone H2B gene interacted with factors in HeLa cell nuclear extracts. These included a region between −162 and −147 (bp relative to the cap site), a region between +100 and +230, and an *H2B* consensus element centered at −45. Non-histone genes that contain homologies to this latter element could interact with the *H2B* consensus region-binding factor(s), suggesting that a common transcription factor can bind to and may be used by these genes.

The significance of the complexes in the −162 to −115 and +100 to +230 regions remains unclear, since sequences in these regions are not required for maximal levels of transcription *in vitro* (1). However, factors interacting with these regions might contribute to transcription efficiency *in vivo* or they might have some other function; for example, the coding region complex might contribute to mRNA stability.

The complex whose significance is most clear is that involving an *H2B* consensus element. This sequence is required for effective function of promoter elements that are further upstream; its perturbation reduces transcription as

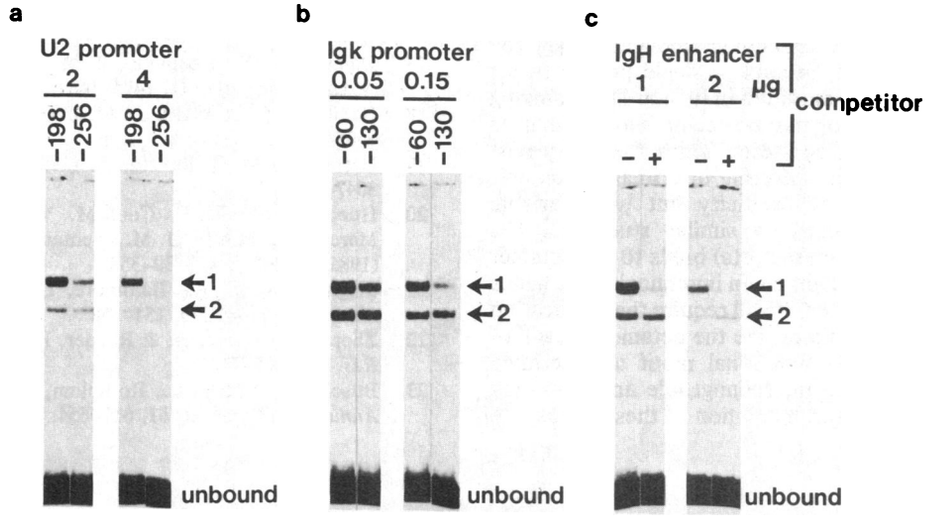

FIG. 5. Interaction of non-histone genes with the *H2B* consensus region-binding factor(s). Mobility-shift assays were performed with the labeled *H2B* promoter fragment described in Fig. 1 and with unlabeled specific competitors as detailed below and in Fig. 4. Unlabeled competitor DNAs were preincubated with nuclear extract before addition of the labeled fragment. The amount of specific competitor is indicated above the appropriate lanes. The lower levels of competitors indicated in *a*, *b*, and *c* correspond respectively to 125-, 30-, and 100-fold molar excesses of competitor to labeled insert. Data shown here were part of more extensive titrations, extending over an 8-fold range of competitors, whose overall results were consistent with those shown here. Bands 1 and 2 are the same as designated in Fig. 1. Relative band intensities were determined by densitometric scanning, with the autoradiographic exposure in the linear range of both film and densitometer. (*a*) U2 snRNA 5′-deletion mutant plasmids, containing the octamer homology (−256) or lacking this sequence (−198) and extending to +442, were used as specific competitors. The intensity of band 1 was lower by a factor of 3.5 with −256 than with −198 mutant competitor. (*b*) Specific competitors were 1-kbp fragments isolated from an immunoglobulin κ chain promoter and contained either 130 or 60 bp 5′ and 900 bp 3′ to the cap site, as indicated. The −130 mutant includes an octamer homology which is deleted in the −60 mutant. The intensity of band 1 was lower by a factor of 3 in the presence of the −130 mutant than with −60 mutant competitor. (*c*) Specific competitors were plasmids that either contained (+) or lacked (−) an immunoglobulin heavy chain enhancer that contains the octamer homology. The relative intensity of band 1 was 7-fold greater without than with the enhancer present.

much as 90% *in vitro* (1). A discrete labeled DNA–factor complex was seen only in the presence of an intact *H2B* consensus element. The amount of labeled complex was greatly reduced by competitor DNAs that contained this element. Further, the identity of this complex was confirmed by "footprint" analysis showing that a region including the highly conserved octamer core of the *H2B* consensus sequence was protected from digestion by DNase I. Since functional promoter elements 5′ or 3′ to the consensus sequence were not required for interaction with the specific binding factor(s), and since the *H2B* consensus sequence was the only promoter sequence protected from DNase I digestion, we conclude that this complex contained a single factor.

The *H2B* consensus element shares at least 8 nucleotides with functional elements in immunoglobulin promoters, heavy chain enhancers, and U2 snRNA promoters (Fig. 4). Transcription of these promoters is regulated quite differently. The *H2B* promoter is preferentially transcribed during the S phase of the cell cycle (22), immunoglobulin genes are transcribed exclusively in lymphoid tissues (3, 8, 9), and U2 snRNA genes are apparently expressed in all tissues (reviewed in ref. 23). It was therefore of great interest to determine whether these sequences could be recognized by a common factor in HeLa cell nuclear extracts. Using the gel shift/competition assay, we showed that a mouse heavy chain enhancer and those regions of a human U2 snRNA promoter and mouse light chain promoter containing the octamer effectively bound the *H2B* consensus region-binding factor(s).

There are several plausible interpretations of these data. The first is that a common factor interacts with the octamer element in all these genes and in all different cell types and thereby enhances their transcription. Such a factor could not be solely responsible for the lymphoid-specific or S phase-potentiated transcription of immunoglobulin or histone H2B genes but might contribute to their regulation through interactions with other more specific factors. For example, in the *H2B* promoter the octamer lies downstream of a histone-specific hexamer (see Fig. 1) that could bind a necessary cell cycle-regulatory factor (1). Second, a single protein factor may be posttranslationally modified in its non-DNA-binding domain(s) in a cell cycle- or tissue-specific way, so that its functional specificity would be altered. Third, there may exist a family of related proteins, differing in primary structure, with similar DNA binding specificity but with variable functional activities. Recently, a similar study (21) has demonstrated that a common factor(s) binds to the octamer element in both heavy and light chain immunoglobulin genes and in a heavy chain enhancer. It will require the purification of stimulatory factors that recognize the octamer in each of the genes in which it is a functional motif to determine whether these activities are interchangeable and how they contribute to the regulated transcription of these genes.

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