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Identification of Promoter Elements Necessary for Transcriptional Regulation of a Human Histone H4 Gene In Vitro

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We have examined the nucleotide sequences necessary for transcription of a human histone H4 gene in vitro. Maximal transcription of the H4 promoter requires, in addition to the TATA box and cap site, promoter elements between 70 and 110 nucleotides upstream from the transcription initiation site. These distal promoter elements are recognized preferentially in extracts from synchronized S-phase HeLa cells. The inability of non-S-phase nuclear extracts to recognize the H4 upstream sequences reflects a specific lack of a transcription factor which interacts with those sequences. These results indicate that the cell cycle regulation of human histone gene expression involves both a specific transcription factor and distal transcription signals in the H4 promoter.

Since the original observation that histone protein synthesis and DNA replication are tightly coupled (18), extensive literature describing the regulation of histone gene expression during the mammalian cell cycle has been generated (for a review, see references 9 and 12). At least three important general conclusions can be reached from these studies. First, it is now clear that the histone gene families of higher eucaryotes are quite complex and that individual members of these families are regulated differently with respect to the cell cycle (24, 25). Furthermore, there is at this point no evidence to suggest that the rather unstructured genomic organization of mammalian histone genes (8, 20, 22) is directly involved in their regulated expression. Second, it has been established by direct measurements of the rates of transcription and decay (7, 23) of histone mRNA in synchronized mammalian cells that the production of histone mRNA is regulated at both the transcriptional and posttranscriptional levels. In HeLa cells, the rate of synthesis and the half-life of histone mRNAs each increase three- to fivefold during S phase (7). Third, several studies suggest that the regulation of histone gene expression in vivo is dynamic. For example, it has been shown that human histone gene expression can be turned on and off several times during a single cell cycle by inhibiting or allowing DNA synthesis to occur (7, 17). In addition, it has been shown that the increase in the rate of synthesis of histone mRNA upon entry into S phase occurs within 10 min after release from a block in DNA synthesis (3). These conclusions suggest to us that at least one step in the mechanism for controlling histone gene expression may involve a gene-specific transcription factor whose activity is regulated during the cell cycle.

One approach we have taken toward an understanding of the detailed molecular mechanisms for regulating transcription of histone genes during the cell cycle is to attempt to reproduce these events in vitro. Hence, it recently was demonstrated that transcription of a cloned human histone H4 gene in nuclear extracts prepared from synchronized HeLa cells appears to mimic the in vivo regulation of this gene (6). Specifically, it was shown that efficient transcription of this H4 gene in vitro is dependent on a gene-specific transcription activity which is maximal in extracts from S-phase cells and that this activity is not limiting for transcription of several other genes. Furthermore, competition experiments presented in that study support the conclusion that the limiting activity for H4 transcription in vitro can bind stably to the H4 template. These properties are consistent with our expectations of a protein which may be involved in histone gene regulation in vivo.

To determine whether the in vitro system is accurately reproducing the in vivo regulation of H4 transcription, we have begun to analyze the nucleotide sequences required for this process in vitro and in vivo. In this study we have constructed and used an extensive series of promoter mutants to determine the sequence elements responsible for in vitro transcription of the human histone H4 gene. We find that maximal transcription of this H4 gene in vitro requires distal promoter elements that are utilized preferentially in extracts from S-phase cells. Furthermore, we describe a complementation assay which can be used to isolate the transcription factor from S-phase nuclei which interacts with the histone H4 distal promoter elements.

MATERIALS AND METHODS

Construction of deletion mutants. The basic steps in the construction of the H4 promoter mutants are shown in Fig. 1 and are as follows. pHu4A plasmid DNA (10 μg) was linearized with either *Eco*RI or *Hin*dIII and diluted to 0.2 ml in 200 mM NaCl–20 mM Tris (pH 7.9)–12 mM $MgCl_2$–12 mM $CaCl_2$–1 mM EDTA. BAL 31 nuclease (2.5 U) was added, and every 15 s for 4 min an equal amount of the reaction mix was withdrawn into a tube containing 10 μl of 0.2 M EDTA. The pooled reactions were extracted with phenol and phenol-chloroform and concentrated by precipitation with ethanol. 3′ recessed ends were filled in with the Klenow fragment of DNA polymerase, *Bam*HI linkers were ligated onto free ends, and the samples were digested with a large excess of *Bam*HI and the appropriate second enzyme. The digested DNA was resolved by electrophoresis through a 2.5% agarose gel, and pools of deleted insert DNA covering ca. 100 base pairs (bp) were isolated by electrophoresis onto DEAE paper. All operations and manipulations involving M13 vectors and phage were essentially as described previously (16). Each pool of BAL 31-digested insert DNA was ligated into the appropriately cleaved M13 vector DNA, transformed into *Escherichia coli* JM103, and plated to produce individual phage plaques. Plaques were screened by

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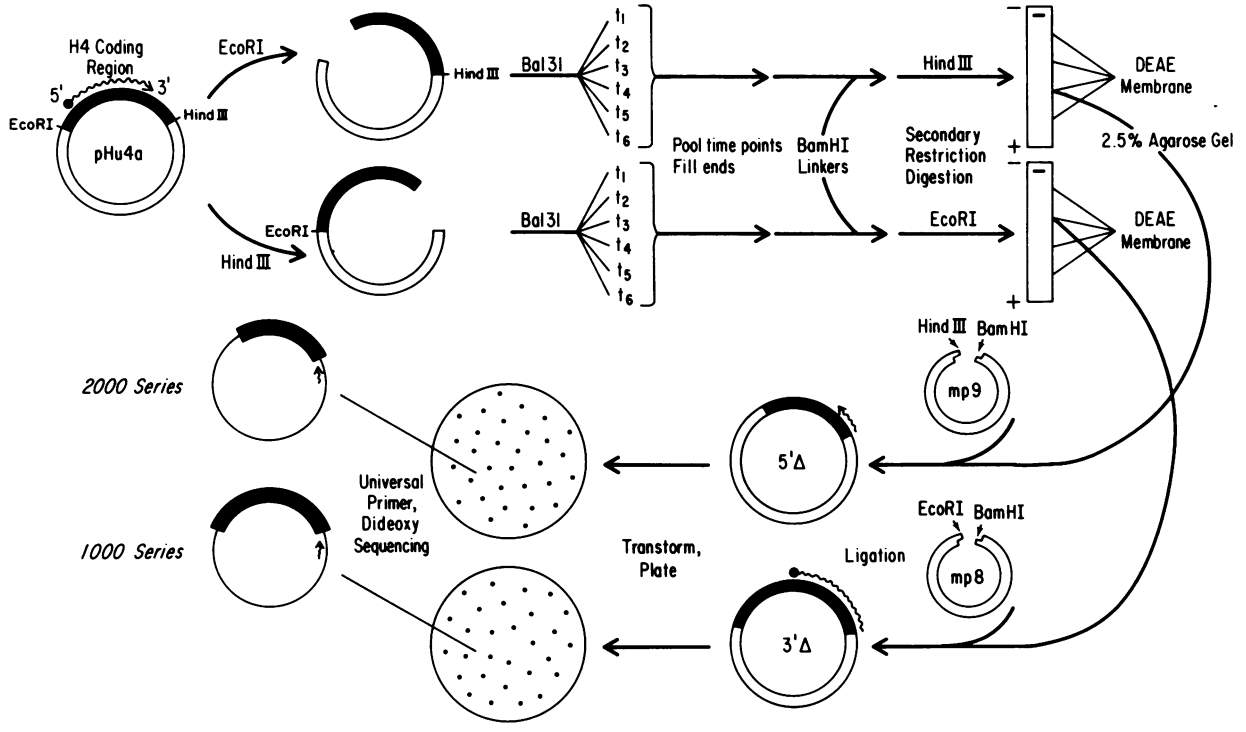


FIG. 1. Diagram showing strategy for construction of deletion mutants in the human histone H4 gene.

the in situ plaque hybridization procedure with nick-translated insert DNA from pHu4A. Positive plaques were sequenced by the chain termination procedure as described by Sanger et al. (19).

Extract preparation and in vitro transcription. Cell culture and synchronization were exactly as described by Heintz et al. (7). Preparation of transcription extracts and runoff transcription assays were performed in nuclear extracts as previously described (6). S-phase nuclear extracts were prepared from synchronized cells 2 to 4 h after release from the synchronization protocol. Non-S-phase nuclear extracts were prepared from unsynchronized cells which had grown to saturation in Spinner culture. Extract complementation experiments were performed as 25-μl reactions with the standard reaction conditions. Each assay contained 9 μl of the non-S-phase nuclear extract and 6 μl of the complementing fraction.

S1 nuclease analysis of in vitro transcription products was as follows. Purified in vitro transcribed RNA was hybridized overnight at 45°C in 80% formamide–0.4 M NaCl–10 mM PIPES (piperazine-*N*,*N*'-bis2-ethanesulfonic acid; pH 6.4)–1 mM EDTA to 0.5 μg of single-stranded M13 phage DNA prepared from mutant 21803, which contains the coding strand of the pHu4A human histone H4 gene between positions −693 and +74 (see Fig. 5A). The reaction (20 μl) was diluted to 200 μl with 0.25 M NaCl–0.05 M sodium acetate (pH 4.6)–4.5 mM $ZnSO_4$ and digested with 500 U of S1 nuclease for 1 h at 37°C. The reaction products were precipitated and analyzed by electrophoresis in 8% polyacrylamide urea gels. Primer extension analysis of in vitro synthesized RNA products was essentially as described by Maniatis et al. (11) and used the M13 universal primer to distinguish newly made histone transcripts from endogenous H4 mRNA. Densitometric quantitation was done with a Beckman Du8 spectrophotometer equipped with a gel-scanning accessory. Multiple exposures were processed and scanned to ensure accuracy.

RESULTS

Construction of H4 promoter mutants. The H4 gene used in this study (pHu4a) was originally isolated by Heintz et al. (8) and has been completely sequenced and shown to encode an mRNA whose abundance is regulated during the cell cycle (26). Since our interest is in defining the nucleotide sequences important for posttranscriptional processing as well as for transcriptional regulation of this gene, we prepared an extensive series of both 5' and 3' deletion mutants throughout the 1.3-kilobase (kb) human DNA insert carrying the H4 gene. The strategy for producing these mutants is outlined in Fig. 1. Due to the large size of region we wished to cover with these mutants, each set of BAL 31 reactions was ligated to *Bam*HI linkers and digested with the appropriate second enzyme, and the resulting fragments were resolved by agarose gel electrophoresis. Separate pools of insert DNA, each spanning ca. 100 bp, were isolated and ligated into M13 vectors. In this way, several hundred individual phage containing human DNA inserts were produced from each pool. Phage DNA was purified from 10 to 20 single plaques from each pool, and the nucleotide sequence was determined by the chain termination method of Sanger et al. (19).

The primary sequence of the pHu4a histone H4 promoter and the locations of the various deletion mutants used in this analysis are shown in Fig. 2. As has been previously noted (26), in addition to the consensus sequences which appear to be conserved in a wide variety of eucaryotic promoters (9), this gene contains a histone H4 specific sequence element ca. 50 bp upstream from the transcription initiation site (CAP) that is required for transcription of a *Xenopus leavis* H4 gene in *Xenopus* oocytes (2). Furthermore, immediately

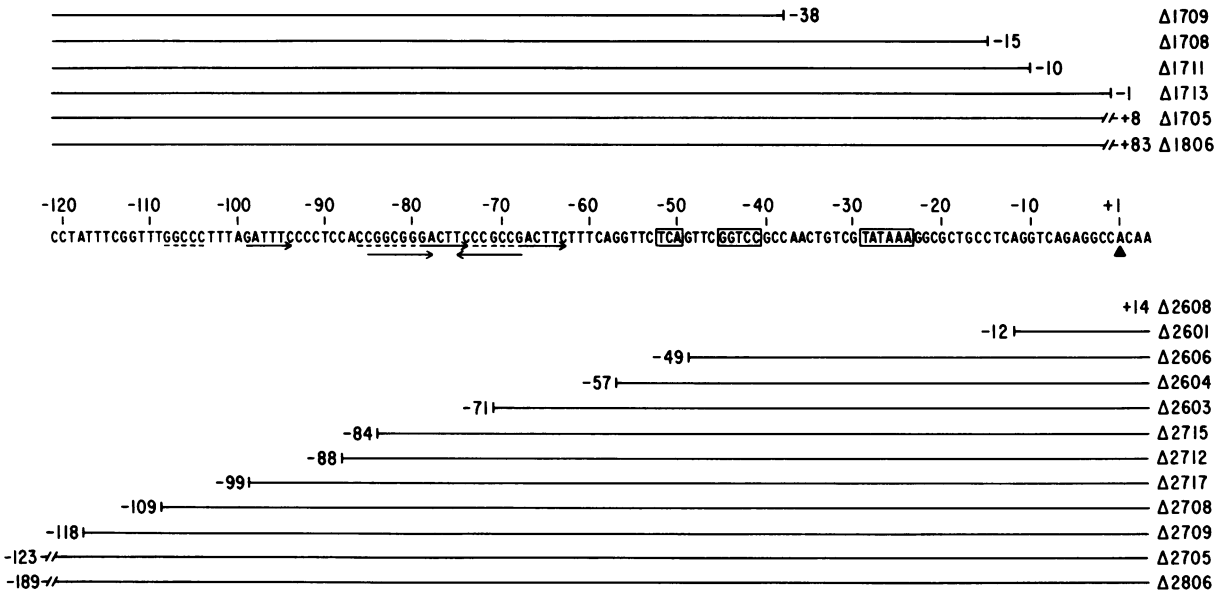


FIG. 2. Diagram showing primary structure of the histone H4 promoter and location of the 5′ and 3′ deletion mutant alleles.

distal to the H4 specific consensus element there are three copies of a hexanucleotide consensus element which we have previously noted in the promoter region of several human histone genes (26). Each of these elements is associated with a short guanosine-cytosine (G-C)-rich sequence which, in the case of the promoter proximal repeats, is itself an inverted repeat.

The H4 CAP site is required for efficient in vitro transcription. The role of DNA sequences immediately surrounding the transcription initiation site of eucaryotic genes remains unclear. Initial studies in which these sequences were perturbed (5) indicated that this region of the promoter served only to position the exact start of transcription and had very little effect on the quantity of RNA produced. However, subsequent analyses demonstrated that in some cases changes in the sequence of the CAP site could have very large quantitative effects. The uncertainty in the role of the CAP site sequences, and the possibility that sequences internal to the transcribed region of this gene could affect transcription, prompted us to reexamine this problem for the human H4 gene.

Deletion mutants which progressively remove sequences from the 3′ end of this promoter were assayed by runoff transcription to a *Bgl*II restriction site ca. 0.7 kb from the CAP site. Mutants in which the body of the H4 gene has been removed but the wild-type H4 promoter (1806; see Table 1) is retained are fully active (Fig. 3). In contrast, mutants in which the wild-type CAP site has been replaced are severely reduced in transcriptional activity. Some residual transcriptional activity is evident from mutant promoters which retain wild-type sequences through the TATA box (1713, 1711, and 1708). Hence, primer extension analysis was used to determine the initiation sites for each of these mutant promoters (data not shown). As expected, initiation from these promoters occurred approximately the same distance from the TATA box, and the nucleotide sequence in that region determined the precise position and number of initiation sites (Fig. 3B). In this case, therefore, it is quite clear that the histone H4 CAP site is important for determining both the position of transcription initiation and the efficiency of utilization of this promoter in vitro.

Distal transcription signals are essential for maximal transcription of the H4 promoter in vitro. As mentioned above, a previous analysis of this H4 gene in vitro (6) demonstrated that the wild-type gene was transcribed severalfold more efficiently in nuclear extracts from S-phase cells. Since our interest is in defining the nature of those sequences required for maximal expression of this gene in vitro, our initial transcription studies have been performed in S-phase nuclear extracts.

The experiment shown in Fig. 4 presents runoff transcription analysis of several 5′ deletion mutants in the human histone H4 promoter. Two important points can be made from these data. First, it is clear that the level of transcription is strongly dependent on the H4 promoter concentration, rather than on the DNA concentration, as evidenced by comparison of transcription from the pHu4A and 2705 templates. In this case, equivalent transcription is achieved when the pHu4A template is present at a lower DNA concentration than the 2705 DNA template. Since the pHu4A vector is pBr322, whereas the 2705 vector is M13, equimolar promoter concentration in the assay occurs when the pHu4A template is present at ca. 65% of the 2705 DNA concentration. The fact that transcription of the H4 gene is dependent on the molarity of the promoter, rather than on the absolute DNA concentration in the assay, indicates that the limiting step under these conditions is promoter specific. Second, the analysis of even this limited set of 5′ deletion mutants in the H4 promoter demonstrates that the efficient transcription of this histone gene in vitro requires sequences distal to the TATA box but probably not extending beyond 123 bp upstream from the CAP site.

To confirm these results and to further define the human histone H4 promoter, transcription of a more complete set of 5′ deletion mutants was done. In this case, closed circular supercoiled M13 DNAs were used as templates in the assay, and the transcripts were analyzed by the S1 nuclease procedure first described by Berk and Sharp (1). It was necessary to use single-stranded M13 phage DNA to protect the in vitro synthesized radioactively labeled RNA transcripts because significant amounts of human histone mRNAs are present in the transcription extracts. In addition, a small

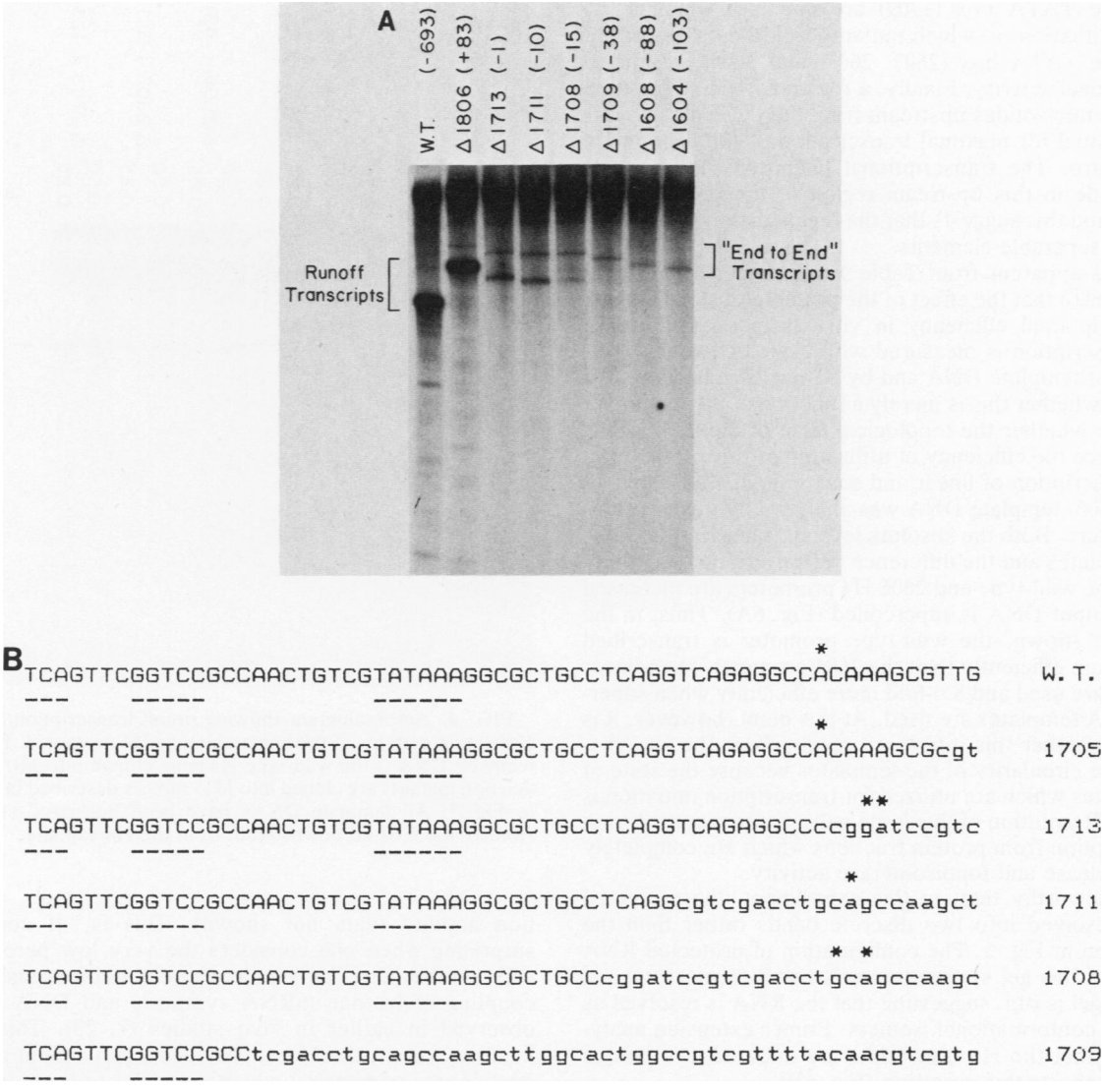


FIG. 3. Analysis of H4 3′ deletion mutants near the cap site. (A) Autoradiograph showing runoff transcripts generated by in vitro transcription of *Bgl*II-cleaved 3′ deletion mutant DNA templates. Runoff transcripts result from initiations at the cap site in the promoter region and transcription to the end of the *Bgl*II fragment ca. 0.7 kb downstream. The wild-type template was cleaved with *Hin*dIII and generates a 0.62-kb runoff transcript. End-to-end transcripts initiate at either end of the fragment released by digestion of the template DNA. (B) Nucleotide sequences of the mutant histone H4 promoters and initiation sites for transcription as established by primer extension analysis (data not shown). Symbol: *, initiating nucleotides for in vitro transcripts of these H4 promoters.

constant amount of a wild-type promoter which would generate a slightly larger protected RNA than the mutant template DNAs was included as an internal control for each individual assay (Fig. 5A).

The activities of the mutant promoters used in this assay (Fig. 5B) suggest that the human histone H4 promoter may contain several distal elements which contribute to its transcriptional activity in vitro. This is most apparent in examining the transcriptional activities of mutant H4 promoters in which the deletion endpoints are between ca. −70 and −100 upstream from the transcription initiation site. Promoters containing progressively more wild-type sequence between these positions are transcribed with progressively increased efficiencies. Thus, there is not a single discrete position at which this promoter is restored to wild-type function but rather a series of positions at which the promoter is incrementally restored to full transcriptional efficiency.

The quantitative results we have obtained from several repetitions of these assays, with both linear and closed circular DNA templates in the in vitro transcription assays, are presented in Table 1. This tabulation supports the general conclusions stated above and reveals several more subtle features of this promoter which were not immediately apparent. The boundaries of the H4 promoter for in vitro function extend between ca. 8 bp downstream and 100 bp upstream from the transcription initiation site. Within this domain, there are at least three different regions which contribute to efficient expression in vitro. The sequences immediately surrounding the start site for transcription function not only form correct positioning of the CAP site but also can profoundly influence the quantity of RNA produced. As expected, the TATA homology appears to be absolutely essential for in vitro function. Hence, mutants which contain the full upstream sequences but do not

include the TATA box (1709) are inactive, whereas the converse situation, in which mutants lack the upstream but contain the TATA box (2603, 2604, and 2606), results in transcriptional activity. Finally, a region extending between 70 and 110 nucleotides upstream from the CAP site appears to be essential for maximal transcription of the histone H4 gene in vitro. The transcriptional phenotypes of mutants which reside in this upstream region of the promoter are complex, and this suggests that the region may be composed of several separable elements.

It also is apparent from Table 1 (especially for mutants 2603 and 2606) that the effect of the promoter distal elements on transcriptional efficiency in vitro is most pronounced when transcription is measured with closed circular supercoiled input template DNA and by S1 nuclease analysis. To determine whether this is merely a function of the method of analysis or whether the topological form of the input DNA can influence the efficiency of utilization of this promoter in vitro, transcription of linear and supercoiled, wild-type and mutant (2606) template DNA was analyzed by the S1 nuclease procedure. Both the absolute level of transcription of the DNA templates and the difference in transcriptional activity between the wild-type and 2606 H4 promoters are increased when the input DNA is supercoiled (Fig. 6A). Thus, in the experiment shown, the wild-type promoter is transcribed 2.5-fold more efficiently than the 2606 promoter when linear templates are used and 8.0-fold more efficiently when supercoiled DNA templates are used. At this point, however, it is not clear whether this difference results from the superhelicity or the circularity of the templates because the state of the templates which are utilized for transcription initiation is unknown. Resolution of this issue will require reconstitution of transcription from protein fractions which are completely free of nuclease and topoisomerase activity.

It is noteworthy that, in this experiment, the protected RNA is resolved into two discrete bands rather than the several seen in Fig. 5. The configuration of protected RNA observed in this gel system depends on the temperature at which the gel is run, suggesting that the RNA is resolved as a series of conformational isomers. Primer extension analysis proves that the H4 transcripts are initiated at a unique site and supports this assertion (Fig. 6B).

Histone H4 promoter distal transcription elements are preferentially recognized in S-phase nuclear extracts. As stated above, all of the preceding transcriptional analyses have been performed in nuclear extracts prepared from synchronized S-phase HeLa cells. Since our interest is in identifying and characterizing the activity which recognizes the H4 upstream sequence elements and in determining whether that activity is required for the cell cycle-regulated transcription of the H4 gene, we were interested to investigate whether the upstream sequence elements are recognized preferentially in S-phase nuclear extracts.

In a previous study of transcription of the wild-type H4 gene in extracts prepared from synchronized HeLa cells, it was demonstrated that the relatively inefficient transcription of the H4 gene in non-S-phase cells resulted from a specific lack of the limiting activity for H4 transcription in vitro (6). It further was noted that in extracts from unsynchronized cells the relative efficiency of transcription of the H4 and adenovirus major late promoters can vary significantly. Subsequent studies indicate that preparation of transcription extracts from cells which are no longer in the exponential growth phase does not result in a general loss of transcription activity of the adenovirus major late promoter but frequently results in a relative loss of histone H4 transcription activity (data not shown). This is, of course, not surprising when one considers the very low percentage of cells synthesizing DNA under these conditions and the tight coupling of histone mRNA synthesis and DNA synthesis observed in earlier in vivo studies (7, 23). This type of nuclear extract, therefore, provides a convenient and abundant source of material which is similar to the non-S-phase nuclear extracts prepared from synchronized cells because it is specifically deficient in the activity which limits transcription of the H4 gene in vitro.

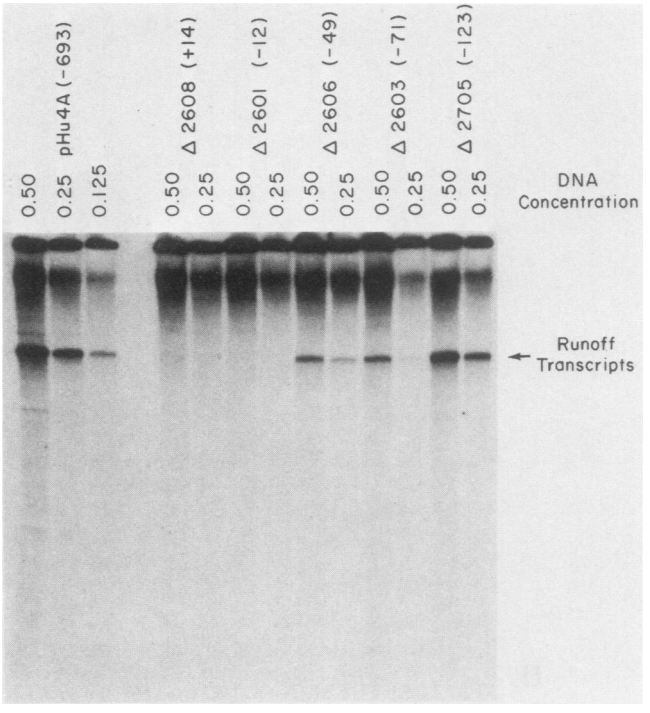
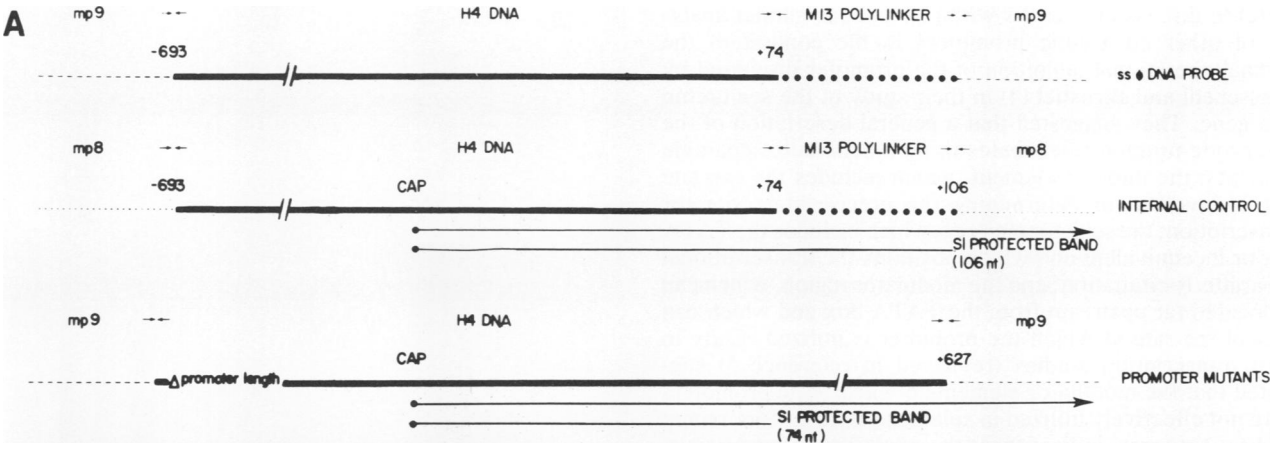


FIG. 4. Autoradiogram showing runoff transcription analysis of 5′ deletion mutants in the human histone H4 promoter. The pHu4A template DNA is the wild-type H4 gene cloned into pBr322 (8); all deletion mutants are cloned into M13 mp9 as described in the legend to Fig. 1. All template DNAs have been linearized at a unique *Hin*dIII site 0.62 kb downstream from the H4 cap site.

Dramatic differences between the transcription efficiencies of several of the template DNAs were observed in the S- and non-S-phase nuclear extracts (Fig. 7). In confirmation of the results presented above, transcription of the H4 promoter in the S extract was absolutely dependent on the TATA sequence and was increased severalfold when sequences between 70 and 110 bp upstream from the CAP site were present. In contrast, although in vitro transcription in the non-S extract is also dependent on the TATA homology, inclusion of the distal sequence elements had no effect on activity. These results indicate, therefore, that the increased transcription of this human H4 gene in S-phase nuclear extracts is due to an activity which exerts its action through the histone H4 distal promoter sequences.

Two additional points are illustrated in this experiment (Fig. 7). First, comparison of the transcriptional activities of the wild-type internal control and the test DNA in each reaction reveals that in the S-phase nuclear extract, significant competition with the wild-type promoter is not achieved unless the distal transcriptional control elements are present on the test DNA (compare transcription from the control H4



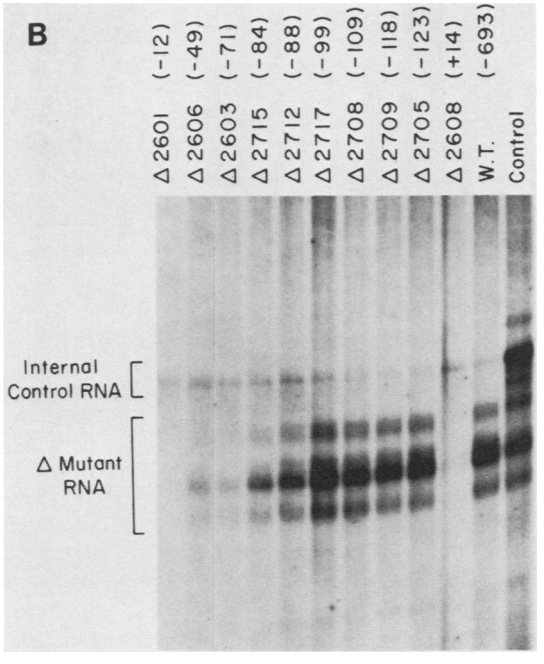


FIG. 5. (A) Diagram depicting expected S1 protection of in vitro RNA transcripts. RNA transcripts from test DNA templates are protected from digestion by single-stranded M13 phage DNA from mutant 21803, which contains the coding strand of the H4 gene between −693 and +74. Transcripts from the control DNA template (1803) are protected from digestion to position +106 (74 nucleotides of H4 sequence plus 32 nucleotides of M13 polylinker sequence) by hybridization to 21803 phage DNA. (B) Autoradiogram showing in vitro synthesized RNA products protected from S1 nuclease digestion by the 21803 single-stranded DNA probe. The assays displayed in the first 11 lanes each included 0.5 μg of the test DNA and 0.1 μg of the control DNA. The reaction shown in lane 12 contained 0.5 μg of wild-type H4 DNA and 0.5 μg of the control DNA.

gene in reactions containing, for example, 2603 and 2708 DNA). This supports earlier competition studies (6) from which it was concluded that an H4 specific transcription activity could be sequestered only by the H4 histone template DNA. Second, transcription of these templates in the non-S-phase nuclear extract results in the generation of an additional transcript ca. 0.7 kb in length. Mapping experiments indicate that this transcript originates in the M13 vector DNA at a fortuitous TATA sequence 730 bp from the *Hin*dIII site used to linearize the test DNA (data not shown). Since the previous characterization of transcription of this H4 gene in extracts from synchronized cells (6) used a pBr322 vector for the H4 gene, such a transcript was not apparent. We do not know why the M13 pseudopromoter is not utilized in the S-phase nuclear extract.

A complementation assay for the H4 distal transcription activity. The results presented thus far indicate that S-phase nuclear extracts are enriched in a *trans*-acting positive transcription factor which effects its activity through interaction with the distal transcription signals in the human histone H4 promoter. It is of obvious significance to purify this activity and characterize its mode of action. Furthermore, the results presented in Fig. 7 suggested that the non-S-phase nuclear extract, in which the H4 specific activity was depleted, may provide a convenient complementation assay for purification of the H4 distal transcription factor. To demonstrate that such a complementation system is useful and that the inability of the non-S-phase nuclear extract to recognize the H4 distal promoter elements does not result from the presence of a dominant negative transcription activity, we performed a mixing experiment similar to that previously described (6). In this case, however, we analyzed the transcription of closed circular supercoiled transcription templates (Fig. 6) because the difference in specific transcription of the mutant and wild-type H4 promoters is maximal under these conditions.

The addition of the S-phase nuclear extract to the assay did not result in an increase in the transcriptional efficiency of the mutant histone H4 promoter (Fig. 8). However, as expected from the results presented in Fig. 7, the presence of the S-phase extract in the assay caused a dramatic increase in transcription of the wild-type histone H4 promoter. It is evident, therefore, that the non-S-phase nuclear extract, when used in combination with the recombinant histone H4 promoters described above, can be utilized for the isolation of the H4 distal transcription factor.

DISCUSSION

The initial issue we have addressed in this study is the identification of DNA sequences required for maximal expression of a human histone H4 gene in vitro. It is most

useful to discuss our results with reference to similar analyses of other eucaryotic promoters in the context of the original operational definition of the promoter discussed by Grosschedl and Birnstiel (4) in their study of the sea urchin H2a gene. They suggested that a general description of the eucaryotic promoter delineates three functionally separable elements: the initiator element, which includes the cap site and is involved in determining the precise start site for transcription; the selector element, which includes the TATA box or its equivalent and which positions the transcriptional apparatus for initiation; and the modulator region, which can be located far upstream from the TATA box and which can control the rate at which the promoter is utilized. Early in vitro transcription studies (reviewed in reference 5) suggested that the modulator elements of eucaryotic promoters were not effectively utilized in soluble systems. More recent studies, however, indicate that the modulator segments can be recognized in vitro and that the discrepancies between those sequences utilized for transcription in vivo and in vitro are becoming less significant (see below).

In this study of the human histone H4 promoter, the role of the initiator and selector elements of this promoter are consistent with the functions proposed for these sequences from previous analysis of a variety of mammalian mRNA encoding genes. The H4 cap site is important for the exact positioning of the transcription initiation site and can have severe effects on the efficiency of utilization of this promoter in vitro. The TATA sequence is absolutely required for transcription of the H4 gene in vitro and is capable, in the presence of the wild-type cap site, of directing significant transcription from the correct initiation site in the crude nuclear extract. The suggestion of a core promoter (10) composed of the initiator and selector elements and which determines the basal level of transcription in the absence of modulator elements or activities which interact with those elements may be useful. It is certainly reasonable to consider that the exact sequence of these proximal regions determines the efficiency with which the general transcription factors can utilize the promoter in vitro in the absence of appropriate gene-specific factors.

TABLE 1. In vitro transcription of pHu4A deletion mutants[a]

Mutant	Position	% Transcription		
		Runoff	S1	Primer extension
5′ Deletions				
Wild type	−693	100	100	
2806	−189	100 (5)	100 (3)	
2705	−123	100 (5)	100 (3)	
2709	−118	100 (5)	100 (3)	
2708	−109	91 (3)	100 (3)	
2717	−99	100 (3)	120 (3)	
2712	−88	71 (3)	63 (3)	
2715	−84	42 (3)	37 (3)	
2603	−71	28 (3)	16 (3)	
2604	−57	26 (1)	ND	
2606	−49	27 (3)	13 (3)	
2601	−12	0 (3)	0 (3)	
2608	+14	0 (2)	0 (1)	
3′ Deletions				
1806	+83	100 (3)		100 (2)
1705	+8	ND		100 (2)*
1713	−1	13 (3)		8 (2)
1711	−10	8 (3)		6 (2)
1708	−15	<5 (3)		<5 (2)
1709	−38	0 (3)		0 (2)

[a] Transcriptional efficiencies of mutant histone H4 promoters in vitro are shown. Positions of the endpoints of each deletion are relative to the transcriptional initiation site. The number of independent assays used to quantify transcription of each promoter in vitro is within parentheses. ND, Not done; *, data not shown.

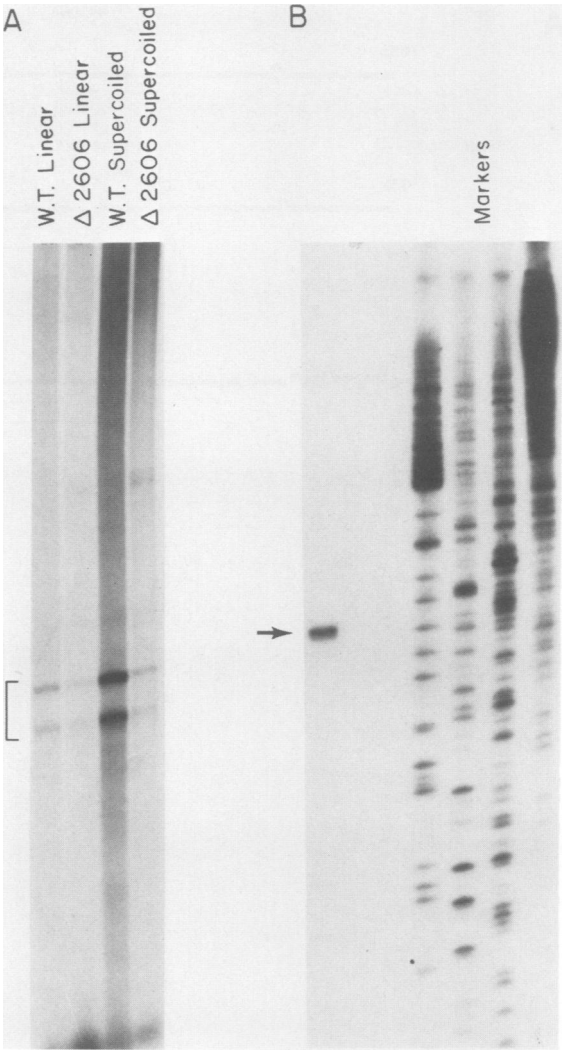


FIG. 6. Comparison of the activity of linear and circular DNA templates in the S-phase nuclear extract. (A) In vitro transcription of linear and circular wild-type and mutant H4 promoters as analyzed by S1 nuclease protection of the reaction products (Fig. 5A). (B) Primer extension analysis of in vitro synthesized RNA. H4 template DNA 1806 (+84) was transcribed in vitro, and the initiation site of the RNA was established by primer extension analysis, using the M13 universal primer. Marker DNA products are chain-terminated DNAs from control DNA sequencing reactions.

The definition of the promoter distal upstream sequences which are required for maximal transcription of the human histone H4 gene in vitro is more complex. In this case, a productive context for this discussion is found in the very detailed analyses of the herpesvirus *tk* gene accomplished by McKnight and co-workers (13–15) in the past several years. These studies resulted in the identification of two distal transcription signals located between 37 and 109 nucleotides upstream from the cap site which are important for the transcription of the *tk* gene. Their most recent studies of the function of these distal transcription signals suggest that,

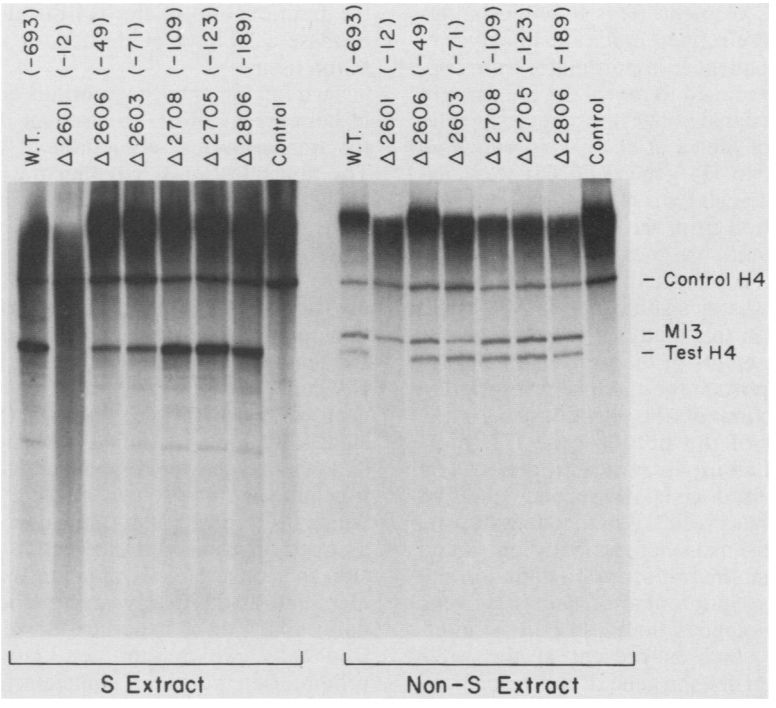


FIG. 7. Autoradiogram showing runoff transcription products from various H4 promoters with S- and non-S-phase nuclear extracts. The M13 test DNA (0.5-μg) templates were linearized at the unique *Hin*dIII site to generate a 0.62-kb RNA product. The control DNA (0.1 μg) was pHu4A linearized at a unique *Bam*HI site 0.97 kb from the H4 cap site. Control lanes indicate reactions containing only the pHu4A control DNA templates. M13 indicates an RNA transcript of 0.73 kb which is initiated in the M13 template DNA (see the text).

although they can function in either orientation and at variable distances from the *tk* TATA box, both the placement and polarity of these elements are important in determining their quantitative effect on transcription. They further suggest that these signals utilize the same transcription factor to effect maximal expression of the *tk* gene. Finally, it is quite clear that, although these elements are theoretically capable of forming an intrastrand base-paired stem, this property does not determine their function.

Our initial analysis of the histone H4 gene has revealed several features of the upstream region of this promoter which are similar to the *tk* gene. Two features are immediately apparent. First, there is a G-C-rich inverted repeat located between 66 and 83 nucleotides upstream from the cap site. Second, three direct repeats are present between −60 and −105. Since these features are overlapping in this region of the H4 promoter, we have not yet been able to distinguish which of these elements is responsible for modulating H4 transcription. It is clear, however, that the presence of the inverted repeat does not impart full activity on the H4 promoter. Hence, mutant 2712, which contains wild-type sequences through position −88, is transcribed at only ca. 65% of the wild-type rate. Full transcriptional activity is not restored until sequences ca. 100 to 110 nucleotides upstream from the cap site are included. It also is clear that the presence of the hexanucleotide repeat 5′ GACTTC 3′, in the absence of its associated G-C-rich element, does not provide function. Thus, mutant 2603 is transcribed in vitro with the same efficiency as mutants lacking this element. At present, therefore, it seems that both of these sequence elements may contribute to the transcriptional activity of the H4 promoter.

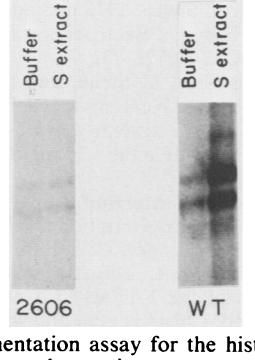


FIG. 8. Complementation assay for the histone H4 distal transcription activity. In each reaction, a constant amount of non-S-phase nuclear extract is complemented with either buffer or the S-phase nuclear extract as indicated. 2606 and WT (wild type) refer to the DNA templates present in each reaction.

An interpretation of the transcriptional phenotypes of the distal promoter mutants which is consistent with this idea is that the three hexanucleotide repeats with their associated G-C-rich sequences serve as discrete elements which can function additively. For example, inclusion of sequences upstream of the TATA box does not result in increased transcriptional activity until the first of these elements is completely included (2715). A further increase is observed in mutant 2712, which contains a complete copy of the two most proximal of these repetitive elements. Finally, full transcriptional efficiency is obtained when all three copies of the repeat are present (2708). The phenotype of mutant 2717, which does not contain full copies of all three wild-type elements but which is transcribed as well as or better than the wild-type gene in vitro, can be understood in this context by considering its exact nucleotide sequence. In this case, the G-C-rich domain, which is separated from the GATTTC sequence in the wild-type gene by four nucleotides, has been replaced by a G-C-rich sequence in the vector which imme-

diately flanks the GATTTC element. This hypothesis currently is being tested by more refined mutational analysis.

Two previous studies of sequences important for transcription of histone H4 genes have failed to reveal the presence of similar distal transcription signals. The most directly relevant of these is the study of Sierra et al. (21), in which an independently isolated human H4 gene (pF0108) was analyzed in vitro, using a whole-cell extract from unsynchronized HeLa cells. Transcription from the pF0108 H4 gene in vitro was very inefficient and was not dependent on sequences distal to the TATA box. Since the primary sequence of the pF0108 H4 promoter shares significant homology with the H4 promoter analyzed in this study, it seems probable that the analogous distal elements of the pF0108 human H4 gene will be shown to be important for maximal transcription in vivo. The lack of function of the distal transcription signals in the initial study of the pF0108 gene (21) most probably reflects the use of an in vitro transcription system prepared from unsynchronized cells. A second study of histone H4 promoter sequences resulted in the identification of a histone H4 specific consensus element between 40 and 50 nucleotides upstream from the transcription initiation site which is important for transcription of an *X. leavis* H4 gene after injection into the homologous oocytes (2). It is interesting that this element, which is present at the same position in the pHu4A human histone gene, is not recognized in the S-phase nuclear extracts. In fact, our preliminary tests of the transcription of a gene in which the only change is an insertion of 3 bp within the GGTCC element at −40 support this conclusion. These data suggest the interesting possibility that specific histone promoter elements may function preferentially or differentially depending on the specific cell type in which a given gene is expressed. Hence, it seems plausible that the utilization of the H4 specific consensus element between −40 and −50 may obviate the need for more distal transcription signals during oogenesis or early development. The idea of a complex histone promoter, in which the components can function independently in specific biological contexts, is particularly appealing for higher eucaryotes in which the demand for histone protein during growth and development is met by a small number of genes.

The second issue we wished to address in this study is whether sequences in the H4 promoter are responsible for the regulated transcription of this gene during the cell cycle. In a previous study (6), we described the preparation and characterization of nuclear extracts from synchronized HeLa cells in which the transcription of this human H4 gene mimicked its in vivo regulation. In this study, similar extracts were used to show that the histone H4 upstream promoter sequences are recognized preferentially in extracts prepared from synchronized S-phase HeLa cells. Maximally efficient transcription of the human histone H4 gene in vitro, therefore, involves both a specific transcription factor and distal sequence elements in the H4 promoter.

The third subject covered in this study is the development of a useful assay for the purification of the transcription factor which interacts with the H4 distal promoter elements. We have demonstrated that the inability of non-S-phase nuclear extracts to utilize the histone H4 distal transcription signals results from a relative lack of the H4 distal transcription factor in those extracts rather than from the presence of a dominant inhibitor. The use of the crude non-S-phase extract as the basis of a complementation system for purification of the H4 distal transcription factor is of considerable utility because of the quantity of material available and its ease of preparation. In fact, preliminary studies concerning the fractionation of the H4 distal transcription factor from S-phase cells have confirmed the utility of this complementation assay.

Two fundamentally important questions provide the focus of our current efforts to discover the molecular mechanisms for transcriptional regulation of histone gene expression. The first question is whether the putative regulatory event we have been studying in vitro is operating in vivo to control the transcription of histone genes. We wish to reiterate that, although the in vitro results we have presented to date suggest that transcriptional regulation of histone gene expression in vivo requires both a soluble H4 specific transcription factor and distal elements in the H4 promoter, these elements may not be sufficient for periodic expression of the H4 gene in vivo. The most accessible approach to this problem is to determine both whether the distal transcription signals we have identified in this study are utilized in vivo and whether they are responsible for the increased transcription rate of this H4 gene during S phase. Experiments in which the 5′ deletion mutants described in this analysis were assayed for transcriptional activity during transient expression in mouse L cells indicate that the sequence elements identified in this study are functional in vivo (unpublished data). Since these experiments were done with unsynchronized cells, we do not yet know whether the H4 distal promoter elements are sufficient for cell cycle regulation of transcription in vivo. The second question deals with the nature of the H4 distal transcription factor. It is of obvious importance to determine whether this is truly a gene-specific activity, whether it is involved in the expression of other histone genes, or whether it is important for transcription of other cellular genes. A definitive answer to this question will require the isolation of the H4 distal transcription factor from other general transcription activities present in the crude extracts we have analyzed thus far. However, comparison of the primary sequence of the H4 distal transcription signals with the upstream regions of other well-characterized promoters strongly supports the suggestion that this activity is involved in the transcription of a rather limited set of genes in vivo.

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