Purification of the human histone H4 gene-specific transcription factors H4TF-1 and H4TF-2

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Transcription of the cell-cycle-regulated human histone genes increases approximately fivefold during S phase. One step toward the elucidation of the biochemical mechanisms that govern cell-cycle-regulated expression of these genes is to purify and characterize the transcription factors that regulate these promoters. Here, we describe the purification of two previously identified factors, H4TF-1 and H4TF-2, which bind the human histone H4 promoter. Purification was achieved through a combination of ion-exchange and oligonucleotide affinity chromatography. On the basis of analysis of purified fractions by SDS–polyacrylamide gels and UV cross-linking, we believe that H4TF-1 is two polypeptides of 105 and 110 kD. This factor binds to a GC-rich DNA sequence required for maximal expression of the H4 gene but does not bind to any Sp1 consensus elements tested. H4TF-2 is a 65-kD protein that binds specifically to sequences within that highly conserved H4 subtype-specific consensus promoter element. Both highly purified factors activated transcription in vitro only from H4 promoters that contained their binding sequences, demonstrating that H4TF-1 and H4TF-2 are H4-specific transcription factors that potentiate expression of this gene.

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The central role played by transcriptional regulation in eukaryotic gene expression and thus many biological processes has become increasingly apparent in recent years. Numerous genes or gene subsets regulated at the transcriptional level are expressed in a tissue-specific, developmental, environmental, or temporal manner [for review, see Dynan and Tjian 1985; Jones et al. 1988]. One approach to understanding the biological basis of transcriptional changes is to dissect the pathways leading to production or activation of specific transcription factors. This strategy is particularly useful for studying gene expression in higher eukaryotes, because a genetic approach for defining regulatory pathways is not feasible. Accordingly, our efforts to understand the transcriptional control of human histone gene expression are aimed at elucidating the pathway[s] of induction during the transition from G1 to S phase of the cell cycle.

Transcription of replication variant histone genes increases at least three- to fivefold as cells enter S phase of the cell cycle (Heintz et al. 1983; Sittman et al. 1983) and is dependent upon active DNA synthesis (Heintz et al. 1983; Plumb et al. 1983). Because transcriptional induction of all five subtypes of histone genes occurs simultaneously, it was postulated that induction might result from the activity of a single DNA-binding protein in a manner analogous to the coordinate induction of the heat shock gene family (Pelham 1986). However, analysis of histone gene promoter DNA sequences and DNA-binding proteins does not support this simple model (Zhong et al. 1983; Hanly et al. 1985; Dailey et al. 1986; Sive and Roeder 1986; Sive et al. 1986). In particular, it has been established recently that induction of the human histone H2b gene in vivo is mediated by the H2b subtype-specific element (LaBella et al. 1987). Furthermore, a transcription factor (OTF-1), which binds to this element and very effectively stimulates transcription of the H2b gene in vitro, has been purified and characterized (Fletcher et al. 1987). OTF-1 is a ubiquitous 90-kD protein that interacts with the octanucleotide sequence 5'-ATTTGCAT-3' found in the H2b gene promoter and in several other cellular gene promoters (Sive and Roeder 1986; Fletcher et al. 1987). However, OTF-1 does not interact with the promoters of either the H4, H3, H2a, or H1 histone gene subtypes (H. Sive, unpubl.).

These results led to the proposal that transcriptional induction of histone genes during the cell cycle results from the activation of distinct subtype-specific transcription factors during the progression from G1 to S phase. Thus, identification and characterization of factors that regulate the expression of each histone gene subtype are important for understanding temporal regulation of transcription during the cell cycle.

Previous analysis of the human histone H4 promoter suggested that it is more complex than the H2b gene promoter (Hanly et al. 1985). DNA sequence analysis (Zhong et al. 1983; Dailey et al. 1987) revealed potential binding sites for two previously identified transcription factors, Sp1 (Kadonaga et al. 1986) and NFk-B (Sen and Baltimore 1986), ~80 bp upstream of the mRNA initiation site. Sequences corresponding to an H4 subtype-specific element (~45 bp upstream of the mRNA initiation site), which are highly conserved in both sequence and
position among H4 genes of many species [Wells 1986], were also identified. Thus, this latter promoter element is analogous to the H2b subtype-specific element. In vitro transcription analysis using HeLa nuclear extract identified DNA sequences between -106 and -73, which are required for maximal expression of the H4 gene, but failed to demonstrate a role for the subtype-specific element [Hanly et al. 1985]. However, in vivo footprinting and methylation protection experiments revealed the presence of a factor bound to the subtype-specific element in cultured cells [Pauli et al. 1987].

Previously we have identified two factors, H4TF-1 and H4TF-2, that bind specifically to a human histone H4 gene in vitro [Dailey et al. 1986]. H4TF-1 binds the H4 promoter ~95 bp from the mRNA initiation site in a region that is required for maximal transcription of the H4 gene in vitro [Hanly et al. 1985], whereas H4TF-2 binds to sequences that comprise the H4 subtype-specific element just upstream of the TATA box [Dailey et al. 1987]. Neither factor binds to other histone gene subtypes or the SV40 promoter, suggesting that H4TF-1 and H4TF-2 are H4 gene-specific factors that may regulate H4 gene expression.

In this paper, we describe the purification and initial characterization of H4TF-1 and H4TF-2 from HeLa nuclear extracts. Highly purified preparations of both proteins were obtained through a combination of ion-exchange and DNA affinity chromatography, and the factors were identified on silver-stained SDS-polyacrylamide gels and by UV cross-linking. G residues required for binding of each factor were identified by methylation interference experiments, and this information was used to construct mutant H4 promoters that were unable to bind each of the factors. The purified factors stimulated transcription in vitro only from H4 promoters that contain intact H4TF-1- and H4TF-2-binding sites. Our results suggest that both H4TF-1 and H4TF-2 play important roles in transcriptional regulation of the histone H4 gene.

**Results**

**Determination of the DNA contact sites of H4TF-1 and H4TF-2**

The DNA bases on both the coding and noncoding strands of H4 promoter DNA which are contacted by H4TF-1 and H4TF-2 were determined by methylation interference experiments, as described in Methods. As shown in Figure 1A, methylation of several G residues on both strands interferes with H4TF-2 binding. Significantly, these residues are included in the most highly conserved region of the H4 subtype-specific element 5’-TCAN4GGTCC-3’ [Wells 1986]. As also shown in Figure 1A, methylation of a cluster of G residues on the noncoding strand, ~95 bp from the transcription initiation site, interferes with H4TF-1 binding. No G residues on the opposite strand, including those within the Sp1-binding site, were essential for H4TF-1 binding. Thus, the core DNA sequence bound by H4TF-1 is 5’-GGGGGAGGG-3’.

**Purification of H4TF-1 and H4TF-2 from HeLa nuclear extracts**

The observation that nuclear extracts prepared from rapidly growing cells contain significant levels of H4TF-1 and H4TF-2 DNA-binding activities (data not shown)
obviated the need to use extracts prepared from synchronized (S phase) HeLa cells as starting material for purification of these proteins. Furthermore, nuclear extracts were suitable, as no significant amount of either factor fractionates into the cytoplasmic S100 fraction during extract preparation (L. Dailey, data not shown).

As described previously, the DNA-binding activities of H4TF-1 and H4TF-2 are sensitive to the presence of chelating agents in the chromatography buffers [Dailey et al. 1987]. The DNA-binding domains of both proteins probably require zinc ions as cofactors in a manner analogous to the *Xenopus laevis* TFIIA [Hanas et al. 1983; Miller et al. 1985] and Sp1 [Kadonaga et al. 1987] proteins. It was therefore necessary to eliminate EDTA from the chromatography buffers and to supplement the buffers with 10–20 μM ZnCl₂ to preserve H4TF-1 and H4TF-2-binding activities during the course of the purification.

The purification protocols developed for H4TF-1 and H4TF-2 are summarized in Figure 2. All fractions were assayed for factor-binding activity by gel-shift analysis [Fried and Crothers 1981; Garner and Revzin 1981], as described in Methods.

### Purification of H4TF-1

Previous work demonstrated that H4TF-1- and H4TF-2-binding activities can be separated by chromatography on phosphocellulose (P11) [Dailey et al. 1986]. H4TF-2 flows through P11, whereas H4TF-1 is retained and elutes at 0.5 M KCl. The strategy employed for subsequent purification steps relied on the differential binding affinity of each of the factors for nonspecific and specific DNA sequences.

The protein peak of the P11 0.5 M KCl fraction was pooled, dialyzed to 0.1 M KCl and applied to a column of sonicated salmon sperm DNA; H4TF-1 activity was recovered in the 0.4 M KCl step. The pooled fractions were applied to an oligo-affinity column that contained the distal portion of the H4 promoter (from −116 to −87), which is bound by H4TF-1 but which contains no known binding sites for other proteins.

The oligo-affinity column was washed and a six column volume 0.2 M KCl step was applied to minimize trailing of nonspecific material into H4TF-1-containing fractions. A linear salt gradient from 0.2 to 1.0 M KCl was used to elute H4TF-1 from the oligonucleotide resin. No protein could be detected in the gradient fractions by Bradford [Bradford 1976] assay.

All fractions were assayed by gel-shift analysis for H4TF-1-binding activity. As shown in Figure 3A, a shift corresponding to H4TF-1 protein/DNA complexes was detected in the gradient fractions along with several faster-migrating species. The amounts of these faster-migrating binding activities varied in different preparations, and they tended to appear later during the fractionation procedure. At least a subset of these faster-migrating species may bind specifically, as indicated in competition experiments (L. Dailey, data not shown).

Aliquots of the gradient fractions were analyzed by SDS–PAGE [Laemmli 1970] and silver staining to identify polypeptides that coelute with H4TF-1-binding activity. As shown in Figure 3B, there is no single protein that coelutes quantitatively with H4TF-1 binding activity, but a 110-kD protein is present in all fractions that contain the H4TF-1/DNA complex. Several lower-molecular-weight proteins were also present in the earlier gradient fractions, but their elution profiles are clearly distinct from that of the H4TF-1/DNA complex identified in the gel-shift analysis. A summary of H4TF-1 purification is presented in Table 1A.

#### Identification of H4TF-1 by UV cross-linking

To identify the polypeptide that corresponds to H4TF-1, UV cross-linking studies were performed using gradient fractions from the oligonucleotide columns that contain H4TF-1 activity. These experiments employed an oligonucleotide probe corresponding to H4 promoter DNA between −53 and −132 and a second oligonucleotide probe [mutant 2] that only differs from the wild-type sequence at four positions within the H4TF-1-binding site (see Methods). The mutant probe is unable to bind H4TF-1 efficiently in gel shift assays [L. Dailey, data not shown] or compete for H4TF-1 binding in footprinting assays (see Fig. 5B). Thus, proteins specifically bound to the H4TF-1-binding site can be distinguished from any that associate with the oligonucleotide probes nonspecifically by comparing the pattern of cross-linking obtained with each probe. Oligonucleotide probe was mixed with an aliquot of an H4TF-1 gradient fraction, and the sample was UV irradiated. After nuclelease digestion, the proteins were electrophoresed on an SDS gel,
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Figure 3. Analysis of fractions from the H4TF-l-specific oligonucleotide affinity column. (A) Gel-shift analysis. Pooled peak fractions from the nonspecific DNA column were loaded onto an H4TF-l-specific oligonucleotide-affinity column, and H4TF-1 was eluted as described in Methods. Aliquots of 1 µl from every third fraction were analyzed by gel-shift assay. Fraction numbers are indicated at the top of each lane; (-----) The shift corresponding to H4TF-1/DNA complexes. (B) SDS-PAGE analysis. Aliquots (6 µl) of the oligonucleotide gradient fractions were loaded on a 7% SDS-polyacrylamide gel and stained with silver. Numbers at top of each lane indicate fraction number; arrows indicate the bands corresponding to H4TF-1 (see Fig. 4); (M) protein molecular weight markers (kD).

which was then exposed to X-ray film. As shown in Figure 4, several protein species in oligonucleotide column gradient fraction 15 were radiolabeled by this procedure when the wild-type probe was used. To determine which of these species corresponds to H4TF-1, cold oligonucleotide competitor was included in the binding reaction. Cold specific competitor essentially eliminated labeling of the 105- and 110-kD polypeptides, as well as some smaller-molecular-weight species, whereas these bands were still labeled in the presence of nonspecific competitor (Fig. 4). Furthermore, labeling of the 105- and 110-kD species was much less efficient when the mutant 2 probe was used, even though nonspecific DNA-binding proteins were labeled to a similar extent with either probe. H4TF-1 was identified from these experiments as two polypeptides of 105 and 110 kD.

It is apparent from the elution profile of the proteins from the gradient that the peak of elution of the 110-kD protein occurs at higher salt than that of the 105-kD polypeptide. UV cross-linking analysis of one of these later fractions (fraction 27), using the wild-type probe, only labeled the 110-kD protein (Fig. 4). Labeling of this polypeptide was inhibited by specific but not by nonspecific competitor present in the binding reactions. In addition, labeling of the 110-kD protein in fraction 27 was reduced drastically when the mutant 2 probe was used (Fig. 4). Thus, the 110-kD protein present in higher KCl gradient fractions is also H4TF-1 and can bind independently of the 105-kD polypeptide.

Table 1. Purification table of H4TF-1 and H4TF-2

| Fraction* | Total protein (mg) | Total activity (unit)b | Specific activity (unit/mg) | Fold purification | Yield (%) |
|-----------|--------------------|------------------------|-----------------------------|-------------------|-----------|
| A. H4TF-1 |                    |                        |                             |                   |           |
| Nuclear extract | 1775               | 1,183,000              | 666                         | 100               | 100       |
| Phosphocellulose | 136               | 946,400                | 6959                        | 10                | 80        |
| dsDNA-Sepharose | 24                | 435,344                | 18,139                      | 2.6               | 37        |
| Oligo affinity-Sepharose | 0.015 | 35,490                | 2,366,000                   | 130               | 3         |
| B. H4TF-2 |                    |                        |                             |                   |           |
| Nuclear extract | 2273               | 66,666                 | 29.4                        | 100               | 100       |
| Phosphocellulose/DEAE-Sepharose | 682 | 33,333                | 48.9                        | 2.6               | 50        |
| S Sepharose | 19.9               | 11,500                 | 578                         | 11.8              | 17        |
| dsDNA-Sepharose/ssDNA-Sepharose/oligo affinity-Sepharose | 0.006 | 2,666                | 444,444                     | 768               | 3.9       |

* dsDNA and ssDNA indicate double-stranded and single-stranded DNA, respectively.
* One unit is defined as that amount of fraction required to bind 10 fmole of DNA probe.
proteins were fractionated by SDS-PAGE (L. Dailey, not shown). Thus, the H4TF-1/DNA complex seen in previous experiments, competition for H4TF-1 binding to the H4 promoter using a 25-fold molar excess of SV40 promoter DNA, but even this amount of SV40 DNA did not compete for H4TF-1 binding to the H4 promoter (L. Dailey, data not shown). The H4TF-1 binding sequence and a putative ‘medium affinity’ Spl-binding sequence [5’-GGGGCGGGAC-3’] are juxtaposed on the H4 promoter (Dailey et al. 1987). H4TF-1 binding to the Spl sequence has never been observed in DNase I footprinting experiments from gel-shifted H4TF-1/DNA complexes (Dailey et al. 1986).

When fractions containing both the 105- and 110-kD species were assayed by gel shift (Fig. 3A), only a single shifted band was observed. In other experiments, the shifted complex resolved on a native gel after UV cross-linking and digested with nuclease within the gel results in a doublet of radiolabeled proteins when the eluted proteins were fractionated by SDS–PAGE (L. Dailey, data not shown). Thus, the H4TF-1/DNA complex seen in the gel shift assay reflects a mixture of probe bound to either the 105- or 110-kD species. This may explain why the elution profile of H4TF-1 DNA-binding activity does not correspond well with that of any single protein on the silver-stained SDS gel.

Together, these results show that most of the H4TF-1 activity elutes from the oligo-affinity column as a doublet of 105 and 110 kD and that the latter species elutes at higher salt.

**Distinguishing H4TF-1 from Sp1**

Several of the properties of H4TF-1 are strikingly similar to those of Sp1. The molecular weight of Sp1 is 105 kD (Kadonaga et al. 1987), and the H4TF-1 polypeptides are 105 and 110 kD. Both Sp1 and H4TF-1 require Zn$^{2+}$ as cofactor for DNA binding (Dailey et al. 1987; Kadonaga et al. 1987). Furthermore, the DNA sequence required for H4TF-1 binding (5’-GGGGGAGGG-3’) is similar to the Sp1 consensus binding sequence, 5’-GGGGAGGG-3’ (Kadonaga et al. 1986). Highly purified Sp1 has also been shown to bind to a DNA sequence in the HIV promoter, 5’-GGGAATGAGGC-3’ (Jones et al. 1986), which is very similar to the H4TF-1 binding sequence. These striking similarities prompted us to examine whether H4TF-1 is, in fact, Sp1.

In previous experiments, competition for H4TF-1 binding to the H4 promoter using a 25-fold molar excess to either histone DNAs, which contain Sp1 sites, or SV40 plasmid [pSV2CAT] was not observed (Dailey et al. 1986). Although this quantity of H4 plasmid DNA is sufficient to compete for H4TF-1 binding to the H4 promoter, if the H4 promoter contained a very high-affinity Sp1 site, it is possible that the sites in the competitor DNAs may not have been present in sufficient molar excess to compete. These experiments were repeated using a 200-fold molar excess of SV40 promoter DNA, but even this amount of SV40 DNA did not compete for H4TF-1 binding to the H4 promoter (L. Dailey, data not shown).

The H4TF-1 binding sequence and a putative ‘medium affinity’ Spl-binding sequence [5’-GGGGCGGGAC-3’] are juxtaposed on the H4 promoter (Dailey et al. 1987). H4TF-1 binding to the Spl sequence has never been observed in DNase I footprinting experiments from gel-shifted H4TF-1/DNA complexes (Dailey et al. 1986). However, if H4TF-1 binds the Sp1 site with low affinity, a footprint over the Sp1 sequence might not be observed, as the design of these experiments only allows visualization of binding to the site of highest affinity. To examine this possibility, a standard DNase I footprinting reaction was performed, using increasing amounts of highly purified H4TF-1 to determine whether the Sp1 site could be occupied under these conditions. An ordered filling of Sp1 sites of differing affinity has been demonstrated for Sp1 on the SV40 promoter (Dyand Tjian 1983b).

To confirm that H4TF-1 does not bind to the Sp1 sequence, different H4 promoter oligonucleotides were used to compete for H4TF-1 binding in footprinting assays. Each oligonucleotide contained H4 promoter DNA from positions –132 to –53. The wild-type oligonucleotide contains both H4TF-1 and Sp1 sites and a 75-fold molar excess of this oligonucleotide completely abolishes the H4TF-1 footprint (Fig. 5B). Inactivation of the Sp1 site in mutant 3 has no effect on the ability of this oligonucleotide to compete for H4TF-1 binding. In contrast, mutant oligo 2, which has an intact Sp1-binding sequence, but an altered H4TF-1-binding site [from 5’-GGGGAGGGG-3’ to 5’-GGGAATGAGGC-3’], is unable to compete for H4TF-1 binding even at a molar concentration 600-fold greater than the probe. These results indicate that H4TF-1 specifically recognizes only the sequence 5’-GGGGAGGGG-3’ and is unable to bind to sequences corresponding to an Sp1 consensus site. Therefore, despite their similarities, H4TF-1 is a distinct factor from Sp1.

**Purification of H4TF-2**

HeLa nuclear extract was also used for the purification of H4TF-2. Flowthrough material from the phosphocellulose column was loaded onto a DEAE–Sepharose column, and H4TF-2 was eluted at 0.35 M KCl. The peak fractions of the 0.35 M KCl step were pooled, dialyzed, and applied to a S Sepharose column. H4TF-2 activity was eluted from the S Sepharose column with 0.25 M KCl.
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KCl, and pooled fractions from this step were dialyzed to 0.1 M KCl. This material was loaded onto serial columns of double-stranded DNA, single-stranded DNA, and an oligo-affinity column containing H4TF-2 DNA-binding sequences. H4TF-2 flowed through both double- and single-stranded DNA columns at 0.1 M KCl, whereas many other DNA-binding proteins were retained on these nonspecific DNA columns and were not loaded onto the affinity matrix. The oligo-affinity column was loaded and washed extensively, and proteins that bind to the column with low affinity were eluted with 0.18 M KCl. H4TF-2 activity was recovered using a linear gradient from 0.18 M to 0.5 M KCl, and the gradient fractions were analyzed by gel-shift assay and SDS gels. As shown in Figure 6, A and B, a 65-kD polypeptide is the only protein species that coelutes with H4TF-2 DNA-binding activity.

Identification of H4TF-2 by UV cross-linking

The identity of H4TF-2 was confirmed by UV cross-linking experiments. An aliquot of the pooled peak fractions from the oligonucleotide column was incubated with an oligonucleotide probe containing the H4TF-2-binding site. After UV irradiation and nuclease digestion, the products were fractionated on an SDS-polyacrylamide gel. As shown in Figure 6C, a single radiolabeled species of 65 kD was apparent. As a control, a mutant probe containing 4-bp changes within the H4TF-2-binding site was used to analyze the H4TF-2 pooled gradient fractions [mutant 5, see Methods]. As shown in Figure 6C, no radiolabeled polypeptides were observed when this mutant probe was used in cross-linking. Thus, the 65-kD species that can be cross-linked only to the wild-type probe is H4TF-2. A summary of H4TF-2 purification is shown in Table 1B.

Transcription analysis of H4 templates with mutations in factor-binding sites

The previous analysis of transcription of H4 promoter—deletion mutant templates in HeLa nuclear extract identified regions of the promoter required for maximum transcriptional activity (Hanly et al. 1985). To determine the individual effects of H4TF-1, H4TF-2, and Sp1 binding sequences on transcription of the H4 gene in vitro, mutant promoters with point mutations in each of the factor binding sites were constructed. Wild-type and mutant promoters were inserted upstream of the CAT cassette sequence developed by Sawadogo and Roeder (1985). The base changes chosen to disrupt binding of H4TF-1 and H4TF-2 were based on the methylation interference data of Figure 1; changes designed to disrupt potential Sp1 binding were based on published results (Gidoni et al. 1985).

The ability of each of the templates to direct transcription in vitro using HeLa nuclear extracts was compared [Fig. 7]. Alteration of the H4TF-1-binding site in mutant 2.4 (Fig. 7, lane 2) resulted in an ~4.5-fold reduction in transcription compared with wild-type levels, whereas mutation of the Sp1-binding sequence reduced transcription twofold [mutant 3.4; Fig. 7, lane 3]. The −50 deletion mutant template, which lacks binding sites for all three factors, was transcribed ninefold less efficiently than the wild-type template. In contrast, mutation of the H4TF-2-binding site in the 1.5 mutant template had no apparent effect on H4 transcription [Fig. 7, lane 4]. From these experiments we conclude that the sequences required for binding H4TF-1 and Sp1 clearly affected H4 promoter utilization in vitro, whereas no effect was demonstrated for the H4TF-2 sequences. These results agree with previously described deletion mutant analysis of the H4 promoter which showed a gradual drop in H4 promoter transcription efficiency when se-
sequences between −100 and −70 were deleted and no effect of deleting the H4 subtype-specific element (Hanly et al. 1985).

Purified H4TF-1 and H4TF-2 stimulate H4 gene expression in vitro

It was important to establish whether highly purified preparations of H4TF-1 and H4TF-2 are able to stimulate transcription from the H4 promoter in vitro, particularly because no transcriptional role for H4TF-2-binding sequences could be demonstrated using crude nuclear extract (above results and Hanly et al. 1985). The inability to demonstrate an effect of the H4TF-2-binding site could result from the fact that the in vitro assay is performed under conditions of template excess and the amount of H4TF-2 may be limiting in the reaction. Therefore, H4TF-2 activity was assayed by adding highly concentrated purified factor to HeLa nuclear extract. To demonstrate that transcriptional stimulation requires DNA sequences that are recognized by H4TF-2, we compared the effect of H4TF-2 addition on transcription from wild-type and mutant templates.

As shown in Figure 8A, transcription from the wild-type (−128) promoter was stimulated ~2.5- to 3-fold upon addition of H4TF-2. Transcription from the −92 and −73 5′-deletion mutant templates also increased ~2.5-fold in the presence of H4TF-2. The −50 mutant had only a partial H4TF-2-binding site and was severely impaired in its ability to bind the factor (Dailey et al. 1986).

Significantly, transcription from the −50 mutant template was not stimulated by H4TF-2. Furthermore, transcription from the 1.5 mutant whose promoter is identical to that of the wild-type, except for 4-bp changes in the H4TF-2-binding sequences, also did not respond to H4TF-2 addition, whereas transcription from the wild-type template was stimulated ~2.5-fold (Fig. 8B).

The transcriptional activity of highly purified H4TF-1 was assayed as well. To establish a complementation system to test H4TF-1 transcription activity, HeLa nuclear extract was chromatographed on a heparin–agarose column; bound proteins were eluted with 0.4 M KCl and dialyzed against BC100. As demonstrated previously, this fraction contains all of the basic RNA polymerase II transcription factors as well as RNA polymerase II (Dynan and Tjian 1983a). Because H4TF-1 also elutes from heparin–agarose at this salt concentration, it was removed selectively by batch adsorbing the fraction with H4TF-1-specific oligonucleotide resin, as described in Methods.

Figure 8C shows that in contrast to the 4.5-fold difference observed in nuclear extract (Fig. 7), wild-type and mutant 2.4 templates were transcribed at comparable levels in the H4TF-1-depleted heparin fraction. Addition of 0.5 and 1.0 µl of highly purified H4TF-1 stimulated transcription from the wild-type template proportionately [2.5-fold and 5-fold, respectively], whereas transcription from the 2.4 template was not stimulated. This assay system was saturated for H4TF-1 by addition of 1 µl of the H4TF-1 fraction because addition of more
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Discussion

To elucidate the mechanisms responsible for transcriptional regulation of the human histone H4 genes, we have purified two transcription factors, H4TF-1 and H4TF-2, that bind specifically to the promoter region of a human histone H4 gene. Two polypeptides of 105 and 110 kD were shown to possess H4TF-1-binding activity. These two polypeptides appeared reproducibly in different affinity-purified preparations of H4TF-1, although several smaller polypeptides, which also specifically bound to the H4TF-1 site, could be seen frequently as well. That these smaller proteins were not evident in all preparations of H4TF-1, that they tend to appear in the later steps of H4TF-1 purification, that in situ UV cross-linking experiment of the specific H4TF-1, complex yielded only the 105- and 110-kD species [see Results], and the H4TF-1 preparations lacking the faster migrating complexes remained transcriptionally active support the conclusion that the 105- and 110-kD proteins are responsible for H4TF-1 activity. For these reasons, we believe that the faster-migrating species present in Figure 3 and the low-molecular-weight proteins evident in the UV cross-linking experiments in Figure 4 are the result of proteolytic digestion of H4TF-1.

The relationship between these two polypeptides is not yet known. It is possible that the 105-kD protein is a proteolytic digestion product of the 110-kD species; alternatively, they may be differentially modified forms of one polypeptide or may be distinct proteins that possess the same binding specificity. We do not know whether both forms of H4TF-1 can activate transcription of the H4 promoter; but because the 110-kD form can be separated from the 105-kD species by differential elution from the oligonucleotide column, it should be possible to address this question.

Methylation interference experiments demonstrated that the H4TF-1-binding site includes the sequence 5'-GGGGGAGGG-3' on the noncoding strand of the H4 promoter. Base changes within this sequence prevent

Figure 7. In vitro transcription analysis of wild-type and mutant H4 promoters in HeLa nuclear extract. (Top) The sequence of the coding strand of the H4 promoter. Numbers above the sequence indicate distance (in bp) from the mRNA initiation site. The base changes made in each of the mutant templates are indicated above the sequence for the mutants 2.4, 3.4, and 1.5. Numbers below the sequence indicate the end points of the 5' deletion mutant templates used in the transcription analysis of Fig. 8. Horizontal lines under the sequence show the region of H4TF-1 and H4TF-2 footprints [the H4TF-1 footprint extends beyond the sequence shown here to -113]. (Bottom) Autoradiogram from an in vitro transcription analysis of wild-type and mutant templates in HeLa nuclear extract. (Lane 1) Wild-type template; (lane 2) mutant 2.4; (lane 3) mutant 3.4; (lane 4) mutant 1.5; (lane 5) mutant -50. All reactions also contained as internal control the pML/C2AT/A-53sh template derived from the adenovirus major late promoter. All templates contain promoters inserted upstream of the C2AT cassette sequence [Sawadogo and Roeder 1985], as described in Methods. (H4) The position of the transcript derived from the H4 promoters, (ML) position of the transcript derived from the major late promoter.
binding by H4TF-1, as shown by gel-shift analysis using mutant probes (data not shown), and the mutant oligonucleotide is unable to compete for H4TF-1 binding in footprinting experiments (mutant 2, Fig. 5B).

Although these initial results suggested similarities between Sp1 and H4TF-1, the results of several experiments indicate that H4TF-1 is distinct from Sp1: (1) None of the Sp1-binding sites that were tested competed for H4TF-1 binding to the H4 promoter, even when a large molar excess of Sp1 competitor was used (Fig. 5B; Dailey et al. 1986); (2) binding of H4TF-1 to the Sp1 site on the H4 promoter in DNase I footprinting experiments was not observed (Fig. 5A). Thus, H4TF-1 and Sp1 may be closely related factors that differ in their specific binding properties or may be entirely distinct factors whose superficial binding properties are similar.

We do not know the extent of the H4TF-1 recognition sequence because the methylation interference experiments only identify G residues involved; thus, it is possible that the actual recognition sequence includes base pairs that flank the GC-rich core. DNA sequence comparison of H4 promoters that bind H4TF-1 suggest that the binding sequence may extend beyond the GC core (S.M. Hanly et al., in prep.). Determination of effects of mutation of these sequences on H4TF-1 binding should clarify the extent of the H4TF-1-binding site. At the same time, none of the above results rules out the possibility that Sp1 can bind to the H4TF-1-binding site. The transcription factor that interacts with the H4 promoter in vivo may be determined by the relative affinity of each of these factors for the binding site which could, in turn, be influenced by flanking DNA sequences and/or other promoter DNA-binding factors.

The importance of the H4TF-1-binding site on transcription of the H4 gene was demonstrated by in vitro transcription assays of templates containing either a wild-type or mutant (2.4) H4TF-1-binding site using HeLa nuclear extract. In these experiments, the 2.4 mutant was transcribed with 4.5-fold less efficiency than the wild type. Furthermore, stimulation of transcription upon addition of purified H4TF-1 to an H4TF-1-depleted transcription system was observed with the wild-type template only. This latter result demonstrates that the purified protein that binds specifically to the H4TF-1 site is active for transcription in vitro.

A second H4-specific factor, H4TF-2, was also purified from HeLa cells, and UV cross-linking and SDS gels demonstrated that this factor is a single 65-kD polypeptide species (Fig. 6). Competition experiments described previously had shown that sequences binding H4TF-2 are present in almost all of the human histone H4 genes assayed (Dailey et al. 1986). Accordingly, H4TF-2-binding sequences, identified by methylation interference, correspond to the most highly conserved bases of the H4 subtype-specific promoter element (Wells 1986). In vivo footprinting and methylation protection studies using an H4 gene revealed that a factor is bound to this region (Pauli et al. 1987) and the G residues identified correspond to those that have been identified in vitro. Together, these results indicate that H4TF-2 is the factor that binds to the highly conserved H4 subtype-specific DNA sequences both in vivo and in vitro.

The results of in vitro transcription assays using H4 promoter deletion mutant templates demonstrated the ability of purified H4TF-2 to stimulate transcription.
from only those promoters that contain specific DNA-binding sequences. The amount of H4TF-2 activity added was ~22 times more concentrated than that found in the extract. Thus, the previous inability to detect an effect on transcription upon deletion of the H4 subtype-specific element was most likely due to the very low concentration of this factor in nuclear extract. Although the magnitude of transcriptional stimulation by H4TF-2 was small (2.5- to 3-fold), it was reproducible. This stimulation was entirely dependent on H4TF-2-binding because transcription of templates that were unable to bind H4TF-2 (the −50 and 1.5 mutant templates) were not stimulated by factor addition [Fig. 8]. As demonstrated by the −73 mutant [Fig. 8], transcription stimulation by H4TF-2 can occur independently of binding of H4TF-1 or Sp1 because this mutant lacks both of these sites. Furthermore, neither Sp1 nor H4TF-1 can be responsible for the observed stimulation because we have determined by DNase I footprinting assay that neither of these proteins is present in the purified H4TF-2 fractions [L. Dailey, data not shown]. Therefore, transcriptional stimulation in these experiments can only be attributed to H4TF-2, and these results demonstrate a previously undetected transcriptional activity of H4TF-2 on the human histone H4 promoter. Together, these data demonstrate that the factor that binds the H4 subtype-specific element is active in transcription of the H4 gene and suggest that it may play an important role in transcriptional regulation of this gene during the cell cycle. The H4 promoter employed in this analysis represents only 1 of ~20 copies present in the cell. Four other H4 promoters that have been analyzed contain different combinations of the binding sequences described here [S.M. Hanly et al., in prep.]. H4TF-1- and Sp1-binding sites are each present in other H4 promoters but have been found together only in the promoter analyzed here. In contrast, H4TF-2-binding sequences are present in all but one H4 gene promoter. Differences in the relative transcription rates of these genes may be due, therefore, to the different combinations of factor-binding sequences present in each of the H4 promoters. The observation that the presence of H4TF-1 and Sp1 binding varies in the different H4 genes suggests that they may determine a basal level of transcription. In contrast, H4TF-2-binding sequences are almost always found in these genes and, by analogy to the OTF-1-binding site of the H2B gene [Fletcher et al. 1987; LaBella et al. 1987], it is possible that this subtype-specific element mediates cell-cycle-regulated transcription of the H4 gene. In vivo experiments to analyze expression of the different H4 genes and H4 promoter deletion mutants will clarify the role of each of these elements in the regulation of histone H4 transcription during the cell cycle.

Methods

Gel-shift assay

The conditions used to assay H4TF-1 and H4TF-2 binding have been described previously [Fried and Crothers 1981; Garner and Revzin 1981, Dailey et al. 1986], using an end-labeled EcoRI–SstII promoter DNA fragment [−196 to +46] from the H4 mutant 2806 [Hanly et al. 1985]. NP-40 (0.1%) was added to binding reactions, gels, and electrophoresis buffers for analysis of highly purified H4TF-1 to prevent aggregation.

Methylation interference

The coding strand of the EcoRI–SstII fragment of the H4 mutant 2806 [Hanly et al. 1985] was end-labeled at the 3' end with [α-32P]dATP and the Klenow fragment of DNA polymerase I. The 5' ends were labeled with [α-32P]dATP and T4 polynucleotide kinase; only labeling of the coding strand [at −196] was retained after digestion of the DNA at position +1 with HinfI. The 3' and 5' end-labeled probes were each partially methylated by dimethylsulfate [Maxam and Gilbert 1980] and used for gel-shift experiments with H4TF-1 or H4TF-2 fractions, as described above. The positions of free and bound probe were identified by autoradiography of wet gels and purified using an Elutrap [Schleicher and Schuell]. The isolated DNA was cleaved at modified G residues with 10% piperidine and precipitated with ethanol. The dried pellets were resuspended in an amount of loading buffer such that each sample contained a comparable amount of radioactivity (3 × 10^6 cpm) per 4–6 μL. Samples were analyzed on 8% polyacrylamide sequencing gels.

Synthesis of oligonucleotides and preparation of DNA columns

Oligonucleotides that bind either H4TF-1 or H4TF-2 were synthesized on an Applied Biosystems DNA synthesizer. The double-stranded portion of the H4TF-1-specific oligonucleotide contained H4 promoter sequences between positions −116 and −87, with an 8-nucleotide single-stranded extension on the bottom strand [NAT 7.8]. The H4TF-2-specific oligonucleotide spanned H4 promoter DNA between position −70 and −41 with a 12-nucleotide extension on the top strand [NAT 9.10]. These DNAs were coupled to the resin (0.3 mg/ml resin) according to the manufacturer’s specifications. CNBr-activated Sepharose CL4B [Pharmacia] was coupled with sonicated salmon sperm DNA [Pharmacia], with ~200 μg of DNA/ml of resin.

Oligonucleotides were also prepared for use as probes and construction of mutant H4 promoters. Oligonucleotides 1, 2, and 3 contained a coding strand of H4 promoter DNA from −132 to −66 and a noncoding strand from −132 to −53. Oligonucleotide 1 contained wild-type H4 DNA sequences; the H4TF-1-binding site of oligonucleotide 2 was changed from 5′-GGGGGAGGGG-3′ to 5′-GGGAGTGCA-3′; the Spl sequences were changed from 5′-GGGGGCGGAC-3′ to 5′-GGTACCTGAC-3′ in oligonucleotide 3. Oligonucleotides 4 and 5 contained a coding strand of H4 DNA from −65 to −12 and a noncoding strand from −52 to −12. Oligonucleotide 4 had wild-type H4 DNA, whereas oligonucleotide 5 contains altered H4TF-2-binding sequences (from 5′-CGGTCCCG-3′ to 5′-CAC-TACG-3′).

Chromatography

HeLa cell nuclear extracts were prepared essentially as described by Dignam et al. [1983]. All chromatography buffers [BCNZ buffers] contained 20 mM Tris-HCl [pH 7.9 or 7.0, as indicated], 20% glycerol, 0.02% NP-40, 10 μM ZnCl2, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and various concentrations of KCl. Protein concentrations were determined by Bradford assay [Bradford 1976], and H4TF-1 and H4TF-2 DNA-binding activities were determined by gel-shift assay.
Typically, 200 ml of HeLa nuclear extract (10 mg/ml protein from ~4 x 10^10 nuclei) in BC100NZ was applied to a 100-ml phosphocellulose [P11, Whatman] and a 50-ml DEAE-Sepharose Fast Flow (Pharmacia) column linked in tandem at flow rates of 0.5–1.0 and two column volumes per hour, respectively. Steps of 0.3 M KCl, 0.5 M KCl, and 0.8 M KCl were used to elute proteins from the P11 column. H4TF-1 was eluted in the 0.5 M KCl step; the peak fractions were pooled and dialyzed to 0.1 M KCl in BCNZ buffer. The dialyzed material was loaded at a rate of 2.5–3 column volumes per hour onto a 15-ml double-stranded DNA-Sepharose column. Bound H4TF-1 was eluted with BC100NZ, the peak fractions from this step were dialyzed against BC100NZ and applied to a 0.5-ml oligonucleotide column at a flow rate of five column volumes per hour. After washing with six column volumes of BC100NZ, proteins bound with low affinity were eluted from the column with six column volumes of 0.2 M KCl. A six-column-volume gradient from 0.2 to 1.0 M KCl was then applied to elute H4TF-1.

H4TF-2 activity loaded at 0.1 M KCl flows through the P11 column and binds to the DEAE-Sepharose column. H4TF-2 was eluted with 0.35 M KCl, dialyzed against BC50NZ (pH 7.0), and then applied to a 12-ml S Sepharose Fast Flow (Pharmacia) column at two to three column volumes per hour. After washing, H4TF-2 was recovered with 0.25 M KCl and protein from this step was dialyzed against BC100NZ pH 7.9. The dialyzed material was applied to a 1-ml double-stranded DNA, a 1-ml single-stranded DNA (BRL), and a 0.4-ml H4TF-2-specific oligonucleotide column linked in tandem. The non-specific DNA columns were loaded at a flow rate of two column volumes per hour, whereas the oligonucleotide column was loaded at five column volumes per hour. The columns were washed in tandem with BC100NZ and then detached. A six-column-volume 0.18 M KCl step was applied to the oligonucleotide column, and subsequently H4TF-2 activity was recovered with a linear gradient (six column volumes) from 0.18 to 0.5 M KCl. Aliquots of oligonucleotide fractions from both the H4TF-2 and H4TF-1 columns were analyzed by silver staining [ICN] of SDS–polyacrylamide gels (Laemmli 1970). Quantitation of specific binding activities was accomplished by densitometric determination (using a Beckman Du-8 spectrophotometer) of the amount of probe shifted by different volumes of fraction or by determining the amount of radioactivity present in the shifted bands that had been excised from the gel.

UV cross-linking

A modified UV cross-linking protocol was developed based on those of Chodosh et al. (1986) and Wu et al. (1987). Wild-type and mutant oligonucleotides were synthesized to use as probes in the cross-linking experiments. The probe for H4TF-1 was made from the noncoding strand of the H4 promoter, positions -53 to -132, hybridized to a complementary oligonucleotide, positions -113 to -132. The probe for H4TF-2 was prepared by hybridizing an oligonucleotide of the coding strand of the H4 promoter, positions -65 to -12, to a complementary oligonucleotide that contained sequences -36 to -12. These oligonucleotides were made completely double stranded by incubating with Klenow fragment of DNA polymerase I in the presence of 100 µM each of BUDr, dGTP, and dATP and 40 µCi of [α-32P]dCTP. The reactions were chased with 100 µM of cold dCTP.

A mutant H4 DNA-noncoding strand [positions -53 to -132] containing the sequence 5'-GGGAGGG-3' instead of the wild-type 5'-ACTACGA-3' instead of the H4TF-1-binding site was also synthesized. Similarly, an H4TF-2 mutant probe was made with a coding strand containing the sequence 5'-ACTAC-3' in place of the wild-type sequence 5'-GGTCC-3' within the H4TF-2-binding site.

Ten-microliter binding reactions containing 0.5 ng of either mutant or wild-type probe, 100 ng poly(dI-dC), and a 2-µl aliquot from a peak fraction of an H4TF-1 or H4TF-2 oligonucleotide column were incubated for 10 min at room temperature. In some experiments with H4TF-1, a 100-fold molar excess of specific [NAT 7.8] or non-specific [RP1, derived from the human fos promoter and generously provided by Ron Prywes] oligonucleotide was included in the binding reactions. The samples were UV irradiated (1600 watts, 260 nm) for 20 min at 40°C. CaCl_2 and MgCl_2 were added to final concentrations of 10 µM and 1 mM, respectively, and DNase I (50 µg/ml, Sigma) and micrococcal nuclease (10 U/ml, BRL) digestions were carried out at 37°C for 10 min. The samples were heated and applied, along with size markers [Bio-Rad and Sigma], to SDS gels containing either 10% [H4TF-2] or 7% [H4TF-1] polyacrylamide. After electrophoresis, the gels were silver stained to visualize the marker proteins, dried, and exposed to X-ray film.

DNase I footprinting

H4 promoter DNA probe [−198 to +45] was 3' end-labeled at the EcoRI site [−198] with the Klenow fragment of DNA polymerase I. One nanogram of probe was mixed with protein in the presence of 0.3 µg of poly(dI-dC) in a 20-µl volume. The samples were incubated for 10 min at room temperature before addition of 1 µl of 50 mM CaCl_2, 0.1 mM MgCl_2, and 1 µl of a 5 µg/ml solution of DNase I (Sigma). After 30 sec of digestion at room temperature, 20 µl of a DNase stop solution (1% SDS, 200 mM NaCl, 10 µg/ml yeast RNA) was added; the samples were phenol/chloroform-extracted, chloroform-extracted, and ethanolic-precipitated. After centrifugation, the dried pellets were resuspended in 8 µl of formamide loading buffer and electrohoresed on an 8% polyacrylamide sequencing gel. The gel was then exposed to X-ray film.

Preparation of templates for in vitro transcription

H4 promoter deletion mutant templates cloned in pUC were digested with HindIII for runoff assays, as described previously (Hanly et al. 1985). Altered H4 promoters that contain specific base changes were constructed from oligonucleotides 1–5 synthesized on an Applied Biosystems DNA synthesizer, as described above [Synthesis of oligonucleotides]. Each strand of the H4 promoter from -132 to -12 [a StuI site] was synthesized in halves that overlap in the region around -60. Distal oligonucleotides 1–3 were synthesized with an EcoRI overhang at the -132 end. The strands of each oligonucleotide were phosphorylated with T4 polynucleotide kinase [BRL] and annealed to its complement. Appropriate combinations of the double-stranded hybridized oligonucleotides were ligated to construct a wild-type promoter [promoter 1.4 produced from oligonucleotides 1 and 4], and mutant promoters for binding H4TF-1 [mutant 2.4], Sp1 [mutant 3.4], or H4TF-2 [mutant 1.5]. The specific sequence changes introduced in each of the mutant promoters are indicated in Figure 7 and earlier in synthesis of oligonucleotide.

Another construct was made (H4C2AT) that contained the H4 promoter [−650 to +8] upstream of the C_A7 cassette sequence (Sawadogo and Roeder 1985) in pUC13. Two G residues in the H4 sequence were changed at +6 to A and +8 to T by oligonucleotide mutagenesis. H4C2AT was digested with EcoRI and Stul at [−650 and −5, respectively] to remove the H4 DNA upstream of the cap site. The H4 oligonucleotide segments [which contained a 5' EcoRI end and a 5' Stul end] were inserted by ligation upstream of the C_A7 cassette. The circular tem-
plates produced a discrete 390-nucleotide RNA species devoid of G residues when transcribed in the absence of GTP (Sawadogo and Roeder 1985). An additional template (–50 mutant) contained the EcoRI-StuI fragment from the H4 S′ deletion mutant 2606 (Hanly et al. 1985) ligated into the EcoRI and StuI sites of H4-C2AT. The –53 deletion mutant of the adenovirus major late promoter, pML(C2AT)Δ–53sh, was generously provided by Michele Sawadogo and Mike Van Dyke. The promoter DNA sequences were ligated to a C2AT cassette sequence that had been shortened with Bal31 (BRL). Thus, the –53 ML mutant produces a transcript smaller than 390 nucleotides, that can be distinguished from full-length cassette transcripts.

In vitro transcription of H4 templates using HeLa nuclear extract

Transcription reactions (25 μl) were performed as described previously (Sawadogo and Roeder 1985) and contained 80 μg of HeLa nuclear extract, 10 μg/ml of circular H4-C2AT DNA, and 5 μg/ml of pML(C2AT)Δ–53sh template. The samples were electrophoresed on a 4.5% polyacrylamide, 50% urea gel at 50 mA. The dried gel was exposed to X-ray film.

Depletion of H4TF-1 from a heparin 0.4 M KCl fraction

Twenty milliliters (200 mg) of HeLa nuclear extract was applied to a 10-ml column of heparin-agarose, and the bound proteins were eluted at 0.4 m KCl. This fraction is transcriptionally competent for class II genes (Dynan and Tjian 1983a). H4TF-1 was removed selectively by batch-adsorbing 1 ml of fraction to 200 μl of H4TF-1-specific oligonucleotide resin for 1 hr at 4°C, essentially as described by Scheidereit et al. (1987). After centrifugation at 12,000 g for 2 min, the supernatant was mixed with another 200 μl of oligonucleotide resin and the procedure was repeated. Gel-shift analysis of the supernatant before and after this treatment showed that >80% of H4TF-1 activity had been removed.

In vitro transcription assays for H4TF-1 activity

Transcription reactions (25 μl) were performed as described (Sawadogo and Roeder 1985), using 10 μg/ml of wild-type or mutant 2.4 DNA template. Each reaction contained 32 μg of the heparin–0.4 m KCl fraction and from 0 to 2 μl of the pooled peak fractions that had been recovered from an H4TF-1-specific oligonucleotide column. Transcription reactions and electrophoresis were performed as described above.

In vitro transcription assays for H4TF-2 activity

Transcription reactions (25 μl) contained 80 μg of HeLa nuclear extract, 10 μg/ml of HindIII-digested H4 DNA templates, and either 0 or 2 μl of an H4TF-2 oligonucleotide column fraction, which showed a peak of factor binding activity by gel-shift analysis. Protein and template were incubated for 10–15 min on ice prior to the addition of nucleotides and MgCl₂ to facilitate H4TF-2-binding to the DNA templates; no stimulation by H4TF-2 was observed without this preincubation. After addition of nucleotides and MgCl₂, the samples were incubated at 30°C for 45 min and processed and electrophoresed as above.

Quantitation of all transcription reactions was accomplished by densitometric scanning of several exposures of films from each experiment.

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