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Histone Gene Transcription Factor Binding in Extracts of Normal Human Cells

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Transcriptional regulation of mammalian histone genes during S phase is achieved through activation of specific factors which interact with subtype-specific histone gene promoter sequences. It has previously been shown that in HeLa cells this induction is not mediated by obligatory changes in the DNA binding activity of histone gene transcription factors as cells progress through the cell cycle. Recently, it has been reported that the DNA binding properties of a putative histone gene transcription factor may be quite different in normal and transformed cells (J. Holthuis, T. A. Owen, A. J. van Wijnen, K. L. Wright, A. Ramsey-Ewing, M. B. Kennedy, R. Carter, S. C. Cosenza, K. J. Soprano, J. B. Lian, J. L. Stein, and G. S. Stein, Science 247:1454–1457, 1990). To determine whether the properties of well-characterized histone gene transcription factors are altered in transformed versus normal cells, we have examined the DNA binding activity of human histone transcription factors during the WI38 (a primary line of normal human fetal lung fibroblasts) cell cycle. The results demonstrate that the properties of Oct1, H4TF1, and H4TF2 are similar in WI38 and HeLa cells and that their DNA binding activities are constitutive during interphase of both normal and transformed cell lines. Although it remains possible that these factors are directly or indirectly perturbed as a result of cellular transformation, it appears unlikely that transformation results in gross changes in DNA binding activity as cells progress toward division.

The coordinate production of histones during the S phase of the eucaryotic cell cycle is a fundamental property of all somatic cells. The universality of this process suggests that the regulatory mechanisms which control histone production during S phase may be integral to orderly progression through the cell cycle and that any gross disturbance in the proper functioning of these mechanisms might result in failure to progress to division. Alternatively, unbalanced or unscheduled production of histones may have severe physiologic consequences, resulting in significant loss of viability (18). Our studies have been directed toward understanding the molecular mechanisms which regulate histone gene transcription during S phase as an avenue toward discovering the physiologic pathways which regulate the G_1 to S phase transition. One expectation of these endeavours has been that the immediate events which regulate histone gene expression during S phase would be similar in both normal and transformed cells, since the loss of growth control in transformed cells does not result in disruption of orderly progression through the cell cycle. Furthermore, no gross difference in the accumulation of histone mRNAs or transcriptional induction of histone genes during S phase has been noted in normal versus transformed cultured cells (1, 8, 11, 12). Despite these considerations, a recent report (13) that examines the interaction of a putative histone H4 transcription factor with the H4 promoter in extracts prepared from normal and transformed cultured cells challenges these assertions and contradicts previous experimental data concerning the properties of histone gene transcription factors in normal versus transformed cultured cells (15). The central conclusion of this work is that "the change from cell cycle-mediated to constitutive interaction of HiNF-D with the promoter of a cell growth-controlled gene is consistent with, and may be functionally related to, the loss of stringent cell growth regulation associated with neoplastic transformation." This article has provoked us to reexamine the binding of histone gene transcription factors in normal cells as they progress through the cell cycle, since any indication that these factors are deregulated upon cellular transformation would be important for future experimentation.

In the past several years, it has been established that histone gene transcription during S phase is mediated by highly conserved promoter sequences and their cognate transcription factors (for a review, see reference 12). In animal cells, these regulatory sequences have been shown to be conserved within the gene family encoding a specific histone subtype but distinct between subtypes (24). Thus, the vertebrate H4, H2b, and H1 gene families each have subtype-specific consensus elements at defined positions within their promoters, but there is no similarity between the elements present in the H4 versus the H2b or the H1 promoters. Transcription factors which interact with the subtype-specific consensus elements in each of these promoters have been purified and shown to be distinct (6, 9, 10). In this study, we have examined the properties of these factors (OTF1, H4TF1, H4TF2) in normal human cells during progression through the cell cycle and have failed to observe any changes in their DNA binding properties that are not evident in transformed cells.

MATERIALS AND METHODS

Cell culture and synchronization. Human primary WI38 cells were grown and maintained in Eagle minimal essential medium supplemented with glutamine and 10% fetal calf serum. At an early passage, cells were arrested at the G_1/S boundary by the double thymidine block precisely as described by Holthius et al. (13). Synchronization was monitored prior to release and at 4 and 11 h after release by fluorescence-activated cell sorting (FACS) analysis, thymidine incorporation, and histone mRNA abundance as previ-

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ously described (15). Contact-inhibited cells were obtained by maintaining cells in culture for 3 days beyond the time at which the cells became confluent.

Protein extract and DNA binding assay. Total proteins were extracted by a modified whole cell extract (WCE) protocol. Briefly, 10^7 cells in monolayer were washed, scraped with the help of a rubber policeman, and pelleted in phosphate-buffered saline. Cells were then resuspended in 10 pellet volumes of hypotonic buffer and immediately centrifuged, and the pellet was swollen for 10 min in 1 volume of hypotonic buffer at 4°C. Following the incubation period, cells were homogeneized with 20 strokes of a Dounce tissue grinder, and the resulting lysate was made 0.4 N KCl by adding the salt solution dropwise while constantly vortexing. The extract was prepared by tilting the high-salt lysate for 30 min at 4°C, centrifuging for 30 min, and dialyzing the supernatant against BC100. The pellet was used for RNA extraction and S1 analysis as previously described (3). Protein concentration of the WCE was measured by the Bradford method (2) and made uniform among the extracts.

DNA binding assays were performed essentially as previously described (6, 9, 10, 15). Each binding reaction mixture contained between 2 and 5 μg of extract, 100 ng of either specific or nonspecific DNA competitor, between 1 and 4 μg of salmon sperm DNA or dI-dC, and 1 ng of either DNA fragment or oligonucleotide as the probe. Most of the oligonucleotides used in this study have been previously described. Oligonucleotide OTF1 has been described by Fletcher et al. (9); H1TF2 has been described by Gallinari et al. (10); H4TF1, here called TF0.1, is similar to NAT 7.8 (6); H4TF2, here called SR3.4, is similar to NAT 9.10 (6); and its mutated counterpart, here called SR1.2, is similar to oligonucleotide 5 (6). The promoter DNA fragments F0108 H4 (23) and H4.A H4 (4) have also been described elsewhere. The binding buffer used to assay OTF1 activity contained 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 4% Ficoll, 40 mM KCl, and 2.5 mM $MgCl_2$; for H4TF1 and H4TF2, the same buffer minus Mg^{2+} or a buffer containing 12.5 mM HEPES (pH 7.5), 10% glycerol, 50 mM KCl, 0.005% Nonidet P-40, 0.1 mM EDTA, and 1 mM dithiothreitol worked equally well. The protein-DNA complexes were subject to polyacrylamide gel electrophoresis on a 4% acrylamide (acrylamide/bisacrylamide, 30:1.5) in low-ionic-strength buffer as previously described (10).

Western immunoblot analysis. A 20-μg sample of WCE was electrophoresed in denaturing conditions on an 8% acrylamide-sodium dodecyl sulfate (SDS) gel according to Laemmli (17). The gel was then electrically blotted to a nitrocellulose filter, using a Hoefer apparatus, in a 25 mM Tris–200 mM glycine–20% methanol buffer at 30 V for 3 h. The nitrocellulose was then treated and hybridized to high-titer antiserum raised in rabbit against OTF1 (19), and the OTF1 band was detected by the alkaline phosphatase method as recommended by the supplier (Vector).

RESULTS

To ensure that the results of these studies can be directly compared with those previously published by Holthuis and coworkers (13), we have chosen to examine the properties of OTF1, H4TF1, and H4TF2 in one of the cell lines chosen for analysis in their studies. Thus, we have employed normal diploid human WI38 cells synchronized by a double thymidine block exactly as described by those investigators (13).

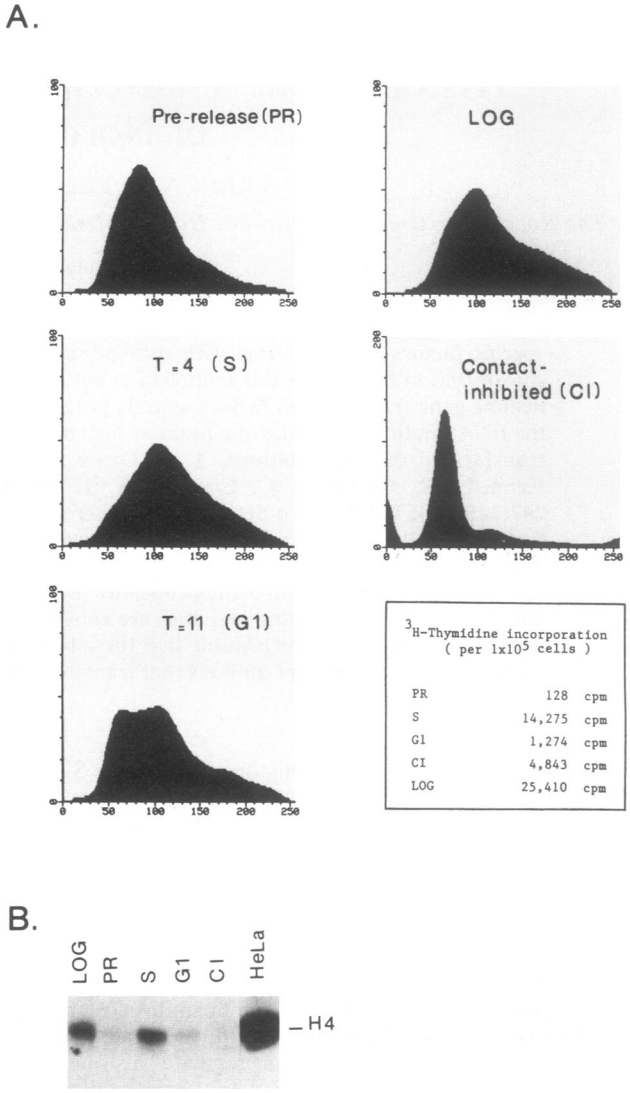

FIG. 1. Synchronization analysis of WI38 cells. (A) FACS analysis. Histograms of prerelease (PR), S, and G_1 samples refer to cells synchronized by two rounds of thymidine block; LOG and CI refer to independent parallel cultures of log-phase and contact-inhibited cells. The *x* axis denotes DNA content; the *y* axis denotes the number of cells. Trichloroacetic acid-precipitable counts obtained by pulse-labelling cells with 10 μCi of [^{3}H]thymidine per ml are also presented. (B) S1 analysis. Total RNA (2 μg) isolated from synchronized cells was hybridized to a human histone H4 probe (pHu4a) 3′ end labelled at the *Bst*EII site and subjected to S1 analysis.

The degree of synchrony achieved in the WI38 cell populations synchronized by the double thymidine block was assessed by three different methods. At 0, 4, and 11 h following release, parallel cultures were harvested for WCE preparation and RNA extraction, FACS analysis, and trichloroacetic acid precipitation of incorporated [^{3}H]thymidine counts. As shown in Fig. 1, all three methods of analysis demonstrate that cells synchronized by this protocol progress through the cell cycle in unison. Thus, progression through the cell cycle is evidenced by the shift of the position of the DNA content peak from prerelease to $t = 4$ and by an increase in tritiated thymidine incorporation of greater than 100-fold. Furthermore, direct measurement of

the accumulation of H4.A mRNA by S1 nuclease analysis demonstrates the accumulation of this mRNA during S phase in these synchronized cells, consistent with all previous studies. Two additional cell populations were collected and processed for analysis: cells which have not been treated with drugs but are logarithmically growing, and cells which have exited the cell cycle due to contact inhibition. As expected for normal cells, the contact-inhibited cell populations had a G_1 DNA content and depressed level of DNA synthesis relative to the logarithmically growing cells.

OTF1 abundance and binding activity are constant during interphase of the WI38 cell cycle. OTF1 (recently renamed Oct1) is a POU-homeodomain transcription factor which interacts with the H2b subtype-specific consensus element that has been shown to be necessary and sufficient for cell cycle regulation of H2b transcription in vivo (9, 16, 21). The demonstration that purified Oct1 can stimulate octamer-dependent H2b transcription in vitro at least 20-fold (9) provided strong evidence that it is the cognate transcription factor for H2b expression in vivo. Additional support for the involvement of Oct1 in cell cycle regulation of H2b transcription in vivo is provided by the recent demonstration that Oct1 is differentially phosphorylated as cells progress through the division cycle (19). Several studies to assess Oct1 DNA binding properties have demonstrated that Oct1 binding activity does not change in actively growing cells as they transit the cell cycle (7, 15) but is depressed in cells that are arrested by serum starvation (14, 15). In this study, we have measured Oct1 DNA binding activity and abundance in WI38 cells to assess whether gross differences in DNA binding are evident in normal human diploid cells versus transformed cells.

Data from three separate synchronization experiments of WI38 cells are shown in Fig. 2. In each extract, the specificity of the OTF1 gel shift is demonstrated by specific competition analysis. Comparison of the observed DNA binding activity for OTF1 in the WI38 extracts is consistent with our previous studies of this protein in transformed human cells and in the nontransformed rodent cell lines CHO and NIH3T3 (15; unpublished data). Thus, no significant change in DNA binding is observed in interphase extracts in any of the three independent experiments, whereas decreased OTF1 DNA binding activity is observed in contact-inhibited cells which have exited the cell cycle. Since the extracts shown in experiment III are from the cells characterized by FACS analysis, [^{3}H]thymidine incorporation, and histone mRNA content in Fig. 1, these results definitively demonstrate that even in normal human diploid cells, OTF1 DNA binding does not significantly vary during interphase. The decrease in DNA binding observed in contact-inhibited cells is very reproducible, although the magnitude of this effect somewhat variable. This is also true in CHO and NIH3T3 cells and may simply reflect whether the cells have completely entered G_0 due to the serum starvation or contact inhibition protocol used. One might expect, for example, that cells which have been maintained for very long periods in G_0 would exhibit an even larger effect on DNA binding.

To assess whether the DNA binding activity that we have observed can be directly correlated with the abundance of OTF1 in these extracts, Western blot analysis was used to detect the quantity of OTF1 present in each extract. As shown in Fig. 2C, the amount of OTF1 present in the WI38 extracts is quite constant throughout the cell cycle. One interesting observation from these experiments is that the amount of OTF1 present in the HeLa nuclear extract is similar to that seen for the WI38 cell extracts, although the OTF1 DNA binding activity in the HeLa extract is significantly higher. This can be partially explained by the fact that the HeLa extract is prepared from isolated nuclei and the WI38 extract is prepared from whole cells, which should enrich for any DNA binding activity when normalized for equivalent amounts of extract protein. However, the large difference in DNA binding observed in the case of OTF1 may also signify a difference in the state of OTF1 in HeLa versus WI38 cells, which is significant. Although this result is quite reproducible, it is not yet clear whether it reflects an aspect of transformation, the adaptation of HeLa cells to rapid growth, or a difference between cells grown in suspension versus adherent cell growth. The important point that is established here is that there is no qualitative difference in the regulation of Oct1 DNA binding activity as W138 or HeLa cells progress through the division cycle.

H4 transcription factor binding during the WI38 cell cycle. Several activities which bind human histone H4 genes in vitro have been reported. Two of these (H4TF1 and H4TF2) have been very highly purified and shown to interact specifically with several human histone H4 promoters (6). Both of these proteins require Zn^{2+} for both DNA binding and transcription activity, suggesting that they belong to the zinc finger class of transcriptional activator proteins (5). H4TF2 is a 65-kDa protein which is present at approximately 10% of the OTF1 level in several different cell types that specifically interacts with the subtype-specific consensus elements present in a large number of mammalian histone H4 promoters. These properties suggest that its function might be restricted to histone gene expression. The high conservation of the H4TF2 binding site in H4 promoters and the similarity in its position relative to the TATA box strongly suggest that it is the functional equivalent of the H2b and H1 cell cycle regulatory sequences, although it has not yet been directly demonstrated that the H4 subtype-specific element mediates cell cycle control in vivo. H4TF1 has been identified as two polypeptides of 105 and 110 kDa which bind to a GC-rich sequence approximately 100 bp upstream of the transcription initiation site in the H4.A promoter (4, 5). However, it is quite clear that this factor does not interact with several of the cloned human histone H4 promoters, suggesting that it probably is not critical for cell cycle regulation of the H4 gene family (unpublished data). A third DNA binding activity (HiNFD) which binds in the region of the H4 subtype-specific consensus sequence has been identified by van Wijnen and colleagues (22). A variety of its properties suggest that it is not directly related to H4TF2, although it has not been purified and it remains possible that H4TF2 is a component of HiNFD. It has been directly implicated in cell cycle regulation on the basis of its failure to bind in extracts prepared from differentiated cells in culture (23) and normal cells which are not in S phase (13). Our previous investigations of H4TF1 and H4TF2 DNA binding during the cell cycle have failed to reveal a change in DNA binding properties of these proteins during S phase. However, the reported fluctuation in HiNFD during S phase in normal cells (13) and the possibility that H4TF2 might in some way be related to HiNFD have prompted us to reassess binding of H4TF1 and H4TF2 during the cell cycle and to try to detect cell cycle-dependent HiNFD binding in normal cells.

Our past studies of the histone H4.A promoter using a variety of DNA binding assays have not revealed a specific DNA-binding complex with the characteristics of HiNFD. Although the reported binding site for HiNFD is present in the H4.A promoter, one possible explanation of this result is

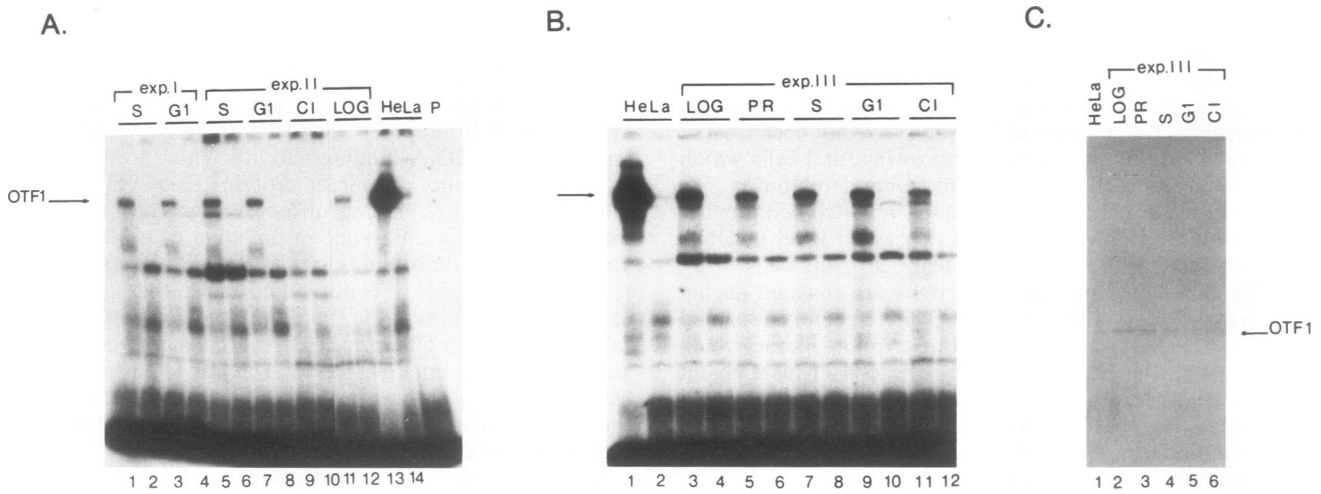

FIG. 2. Cell cycle analysis of human histone H2b transcription factor OTF1 in synchronized WI38 cells. (A and B) DNA binding assays. Results from three separate synchronization experiments are shown. Extracts used are indicated above the gels. Odd-numbered lanes contain 100 ng of nonspecific oligonucleotide as nonspecific competitor (H1TF2 binding site); even-numbered lanes contain 100 ng of specific competitor (OTF1 binding site). All reactions were carried out in the presence of 3 μg of protein extract, 4 μg of dI-dC, and 1 ng of 3′-end-labelled OTF1 oligonucleotide. HeLa unsynchronized nuclear extract is included as a reference. Arrows point to the specific OTF1-DNA complex. Lane P contains probe alone. (C) Western blot analysis. Twenty micrograms of protein from synchronized WI38 WCE was loaded per lane of an SDS-gel, blotted to nitrocellulose filter, and hybridized to high-titer polyvalent anti-OTF1 antibodies. An HeLa sample is included as a control. The arrow point to the OTF1 protein. Extracts are designated as for Fig. 1.

that the F0108 H4 promoter has a significantly higher affinity for this factor than does H4.A. To determine whether this might explain the discrepancy between our results and those of Holthuis and colleagues (13), we have repeated these experiments using the F0108 promoter. As shown in Fig. 3A, a complex similar in mobility to HiNFD can be formed on the F0108 DNA under the conditions reported for HiNFD binding. However, repeated attempts to demonstrate that this complex is specific by using competition analysis with unlabeled F0108 DNA, other H4 promoter sequences, or oligonucleotides encompassing the HiNFD binding site have failed. On the other hand, increasing the concentration of nonspecific dI-dC DNA (Fig. 3A, lanes 2 and 3) or salmon sperm DNA in these binding reactions eliminates the slow-mobility nonspecific DNA complexes, allowing visualization of two discrete faster-migrating complexes (lanes 2, 3, 6, and 7). Competition analysis using purified promoter DNA fragments from either the F0108 or H4.A promoter or oligonucleotides that have been previously shown to specifically bind to H4TF1 and H4TF2 establishes that these two complexes are due to binding of these well-characterized H4 transcription factors. Thus, the SR3.4 oligonucleotide competes for only the faster-migrating H4TF2 complex (lanes 4), whereas the TF0.1 oligonucleotide competes for specific binding of H4TF1 (lanes 5). These results are completely consistent with our previous published data (15) and extend this work to demonstrate that both of these factors also interact with the F0108 promoter. Under no conditions, including precisely those reported by Holthuis and colleagues (13), have we been able to detect a specific DNA binding complex that resembled HiNFD in extracts from either HeLa or WI38 cells.

H4TF1 and H4TF2 binding activities are not cell cycle regulated. Our previous examination of both H4TF1 and H4TF2 DNA binding in extracts from synchronized HeLa, CHO, and NIH3T3 cells showed that the binding of these factors is not regulated during interphase (unpublished data). To determine whether they might be regulated at this level in normal human cells, the WI38 cell extracts used to measure OTF1 activity in Fig. 2 were assayed for the H4 DNA binding activities. As shown in Fig. 4, the activities of these factors can be revealed by using either the F0108 or H4.A promoter DNA fragment as a probe and oligonucleotides containing the H4TF1 and H4TF2 binding sites as specific competitors. For comparative purposes, binding assays using highly purified preparations of both H4TF1 and H4TF2 are included.

H4TF1 activity is readily detected in the WI38 extracts as a discrete complex which comigrates with the H4TF1 complex from HeLa cells or the purified protein. The specificity of binding is demonstrated by the fact that this complex is competed for only by the H1TF1 binding site oligonucleotide (Fig. 4, lanes 3, 6, 9, 12, 15, and 19). Consistent with our previous studies, this DNA binding activity does not vary significantly during the cell cycle. A second specific DNA-binding complex which migrates slightly faster that H4TF1 is also evident in the WI38 extracts. The identity of the protein responsible for this complex has not been established, although it is a very minor contaminant in the purified HeLa cell H4TF1 preparations, suggesting that it is not directly related to H1TF1. The level of activity for this DNA-binding protein also does not vary appreciably during the cell cycle.

H4TF2 DNA binding activity is more difficult to detect in crude cell extracts because of its very low abundance and the presence of a nonspecific DNA-binding complex which migrates only slightly faster than H4TF2 in this assay. However, comparison of the complexes observed in the presence of specific (Fig. 4, lanes 2, 5, 8, 11, 14, and 17) and nonspecific competitor oligonucleotides with the complex formed by highly purified H4TF2 preparations identifies the slower-migrating species in this difficult to resolve doublet as H4TF2 activity. Immediately below this doublet is a major nonspecific band which is present irrespective of the probe or protein preparation used in the assay. As shown in Fig. 4, neither H4TF2 DNA binding activity to the F0108 and H4.A promoters nor the nonspecific DNA binding activities ob-

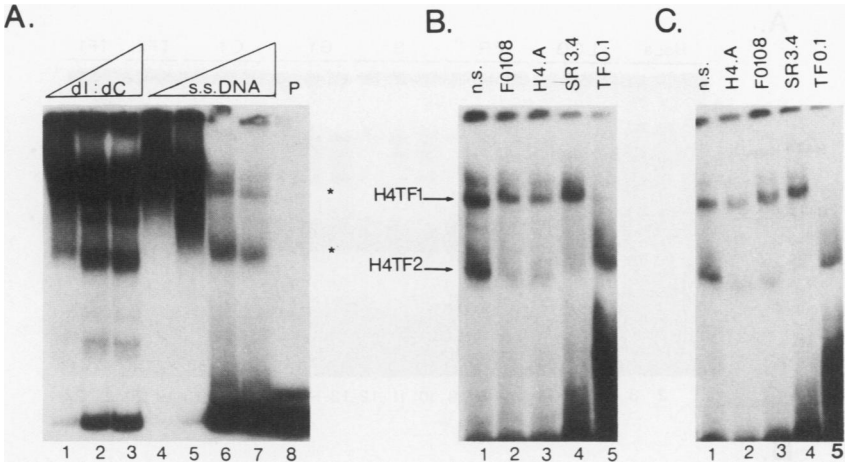

FIG. 3. Specific DNA-protein interactions in human histone H4 promoters. (A) Titration of dI-dC and salmon sperm DNA (S.S.DNA). In this experiment, a *Hin*dIII-*Xba*I fragment spanning from −130 to +20 of the F0108 human H4 promoter was 3′ end labelled and used as a gel shift probe in a binding reaction containing 7.5 μg of HeLa nuclear extract. Lanes 1 through 3 contain, respectively, 2, 3, and 4 μg of dI-dC; lanes 4 through 7 contain, respectively, 1, 2, 3, and 4 μg of salmon sperm DNA. Asterisks on the right mark the positions of two discrete gel-shifted bands. P indicates a lane with probe alone. (B) DNA binding assay and competitions. F0108 fragment DNA probe was allowed to complex with proteins under the conditions used for lane 6 in panel A. Either a DNA fragment (F0108 or H4.A) or an oligonucleotide (SR3.4 or TF0.1) was used as the competitor. n.s. refers to a 200-nucleotide DNA fragment derived from a TATA box-containing H1-chloramphenicol acetyltransferase construct (16). Arrows point to the specific shifts due to complexes with the distal transcription factor H4TF1 and the proximal H4TF2. (C) Same as panel B except that the *Hin*dIII-*Xba*I DNA fragment probe derives from human histone H4.A promoter.

served in these experiments vary significantly during the WI38 cell cycle. Although we have presented data only from a single experiment (experiment III), results were identical in the three WI38 cell extract experiments that we have performed.

DISCUSSION

In this study, we have reexamined the DNA binding properties of transcription factors thought to be important for cell cycle regulation of histone H2b and H4 genes in normal human WI38 cells as they progress through the cell cycle. The results demonstrate that the DNA binding activities of these factors do not significantly vary during interphase in WI38 cells, although we have not analyzed a highly synchronous population of metaphase WI38 cells to determine whether activity might decrease during mitosis as has been observed for OTF1 activity in HeLa cells (19). These results are entirely consistent with our previous studies using centrifugally elutriated HeLa cell extracts (15), suggesting that the DNA binding properties of histone gene transcription factors are qualitatively similar as both normal and transformed cells progress through the cell cycle. This conclusion directly contradicts that reached by Holthuis and coworkers (13) in a recent study of HiNFD DNA binding activity in normal versus transformed cells, which led them to conclude that the properties of this putative H4 transcription factor are quite different in these two types of cells. In particular, they asserted that HiNFD DNA binding is constitutive in transformed cells but that this activity is very strongly cell cycle regulated in a variety of normal cell types. Resolution of this discrepancy is an important issue, because a definitive demonstration that these cell cycle-dependent transcription factors are direct targets of cellular transformation would be an important biological insight concerning the molecular consequences of transformation.

The first major point that we wish to make with regard to this issue is that the difference that we have observed cannot be due to the cell type analyzed or the method of synchronization, since we have used the same cell type and synchronization protocol as reported by Holthius et al. (13). The presence of significant DNA binding activity for histone gene transcription factors in G_1 extracts is also not explained as a result of this strain of WI38 cells being transformed, since these cells display appropriate G_0 arrest when allowed to grow to confluence (Fig. 1A). Furthermore, as evidenced by the specific accumulation of histone H4 mRNA during S phase (Fig. 1B), histone gene regulation is observed in these cells. These considerations rule out the most obvious biological explanations for the discrepancy between our results and those of Holthius and colleagues.

A second possible explanation for the differences that we have observed could reside in the nature of the activities analyzed in the two studies. Thus, we have measured the binding activities of three very well characterized histone gene transcription factors as discrete species in crude cellular extracts. As expected from our previous work, the activities of these factors are constitutive during interphase. In contrast, no information is available regarding the biochemical identity of HiNFD because it has not been purified or characterized in any significant detail. Our attempts to measure HiNFD activity in both HeLa and WI38 extracts under a variety of conditions, including precisely those reported previously (13), have failed to reveal a specific DNA-binding complex with the properties of HiNFD. The only conditions in which a complex that migrates as expected for HiNFD was observed in our studies were at very low nonspecific competitor polynucleotide concentrations in the binding reaction. However, the slowly migrating complexes observed under these conditions could not be competed for by the HiNFD binding site at any lower concentrations than when an irrelevant nonspecific DNA was used. If HiNFD activity is relevant for histone gene expression, then it must be extremely labile to have gone undetected in

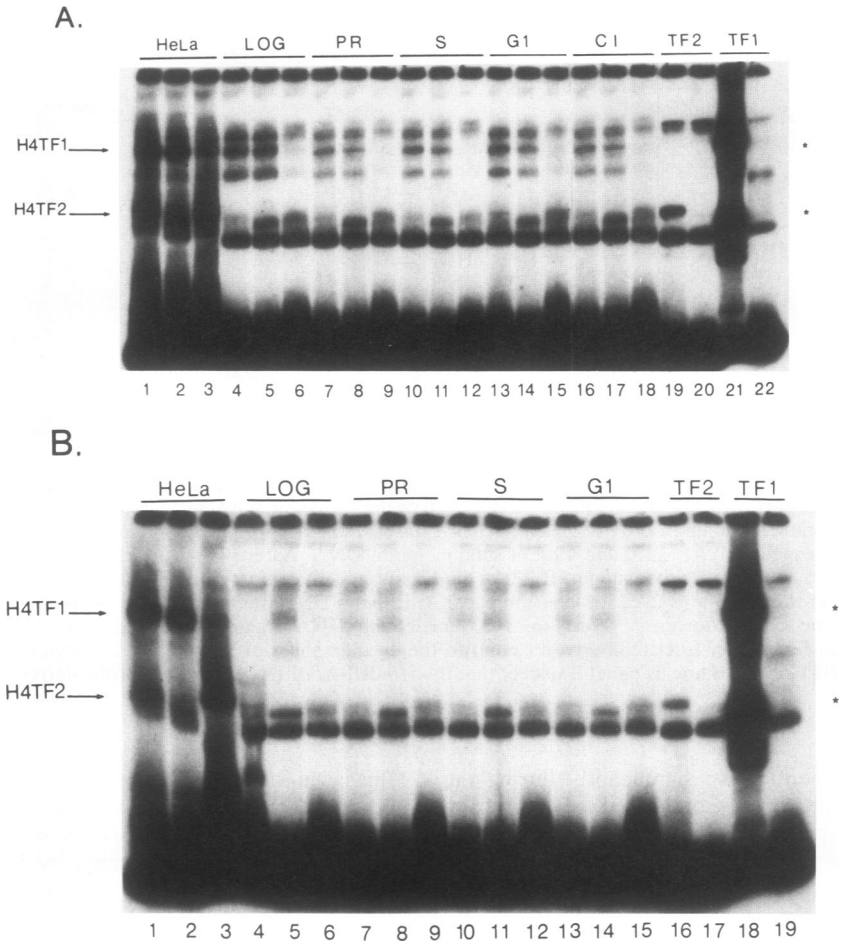

FIG. 4. DNA binding analysis of human histone H4 transcription factors H4TF1 and H4TF2 in synchronized WI38 cells. (A) Gel shift assay using the F0108 H4 DNA fragment probe. The extracts used (see legend to Fig. 1) are indicated at the top; TF2 and TF1 refer to highly purified preparations of H4TF2 and H4TF1 proteins added to the binding reactions in lieu of extract. Lanes 1, 4, 7, 10, 13, 16, 19, and 21 contain 100 ng of a nonspecific oligonucleotide competitor (SR1.2, a point mutant of SR3.4 that abolishes binding); lanes 2, 5, 8, 11, 14, 17, and 20 contain 100 ng of an oligonucleotide competitor specific for H4TF2 (SR3.4); lanes 3, 6, 9, 12, 15, 18, and 22 contain 100 ng of an oligonucleotide competitor specific for H4TF1 (TF0.1). Arrows and asterisks indicate specific protein-DNA complexes. (B) Same as panel A except that the probe used was H4.A and the contact-inhibited extract was not analyzed. Oligonucleotide competitors used: SR1.2 in lanes 1, 4, 7, 10, 13, 16, and 18; SR3.4 in lanes 2, 5, 8, 11, 14, and 17; TF0.1 in lanes 3, 6, 9, 12, 15, and 19.

our assays under conditions which allow detection of a variety of specific transcription factors. It remains possible that an unstable specific DNA complex with the properties of HiNFD could behave differently in normal versus transformed cell types, although our failure to reproduce those results suggest that much more information regarding the biochemical nature of this putative histone gene transcription factor must be obtained before this assertion can be seriously considered.

A third explanation for these results is simply that the experiments leading to the conclusion that there is a fundamental difference in the DNA binding properties of histone gene transcription factors in normal and transformed cells (13) are in error. The results presented herein demonstrate that such a difference is not observed for several well-characterized histone gene transcription factors (OTF1, H4TF1, and H4TF2). As mentioned above, whether this pertains in the case of HiNFD must await further experimentation. It is relevant to point out, however, that no competition analysis to demonstrate the specificity of the putative HiNFD binding complex was included in the analysis of HiNFD activity in the various normal and transformed cell extracts reported by Holthuis and coworkers (13). Furthermore, no internal control measurement of a specific DNA-binding complex which is not sensitive to this difference in cell types was analyzed to ensure that the extracts were generally of equal activity. Until these experimental issues are addressed, the observed differences in HiNFD binding activity during the cell cycle in normal cells must be considered provisional.

Finally, it is important to emphasize that our experiments do not address the fundamental issue raised by these studies. It remains possible that the mechanisms regulating transcription of histone genes in transformed cells are not normal, having been perturbed as a consequence of transformation or as a direct target of transformation. For example, the different specific activity of DNA binding by Oct1 in extracts from W138 versus HeLa cells may be directly relevant to this issue. A variety of properties of OTF1 suggest that its role in histone gene expression may be only one of its

important functions in the cell and that perturbations in the functional activity of this molecule might have quite pleiotropic effects on cellular phenotype (12). These considerations suggest that a thorough analysis of the chemical and functional properties of this and other histone gene transcription factors in normal versus transformed cells is warranted and could be quite revealing.

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