COORDINATE CONTROL AND SELECTIVE EXPRESSION OF THE FULL COMPLEMENT OF REPLICATION-DEPENDENT HISTONE H4 GENES IN NORMAL AND CANCER CELLS

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The replication of eukaryotic genomes necessitates the coordination of histone biosynthesis with DNA replication at the onset of S-phase. The multiple histone H4 genes encode identical proteins but their regulatory sequences differ. The contributions of these individual genes to histone H4 mRNA expression have not been described. We have determined by real time quantitative PCR and RNase protection that the human H4 genes are not equally expressed and that a subset contributes disproportionately to the total pool of H4 mRNA. Differences in histone H4 gene expression can be attributed to observed unequal activities of the H4 gene promoters, which exhibit variations in gene regulatory elements. The overall expression pattern of the histone H4 gene complement is similar in normal and cancer cells. However, H4 genes that are moderately expressed in normal cells are sporadically silenced in tumor cells with compensation of expression by other H4 gene copies. Chromatin immunoprecipitation analyses and in vitro DNA binding assays indicate that eleven of the fifteen histone H4 genes interact with the cell cycle regulatory factor HiNF-P, which forms a complex with the cyclin E/CDK2 responsive co-regulator p220NPAT. These eleven H4 genes account for 95% of the histone H4 mRNA pool. We conclude that the cyclin E/CDK2/p220NPAT/HiNF-P signaling pathway is the principal regulator of histone H4 biosynthesis.

INTRODUCTION

Histones have crucial roles in replication, transcription, repair and recombination (1-3). There is a fundamental requirement for coordinated de novo synthesis of the core histone proteins H2A, H2B, H3, and H4, as well as the linker H1 protein during S-phase to package nascent genomic DNA (1,2). Replication of a complete mammalian genome requires 10^8 of each of the individual histone proteins. Efficient production of this vast quantity of proteins necessitates that transcription of multiple histone genes at multiple loci be coordinately regulated with the onset and progression of genome replication during the cell cycle (4).

Histone biosynthesis is a unique process involving transcription initiation from compact promoters to form primary transcripts that lack introns and contain a highly conserved stem-loop structure that forms the 3’ end of the mature non-polyadenylated mRNA (4,5). Histone genes are organized into clusters and this organization has persisted throughout the course of evolution from yeast to human (2,4). The majority of the 74 known and characterized human histone genes are located in two major clusters at chromosomes 6p21 and 1q21, respectively (Table 1) (1,6-8).
is now known that the human genome contains 15 H4 genes that encode identical proteins. H4 genes in lower eukaryotes (e.g., sea urchin and Drosophila) are organized with the other histone gene types (i.e., H2A, H2B, H3 and H1) into units that are tandemly repeated, and all H4 genes in these organisms have virtually identical promoters and coding regions. Although the coding regions of human H4 genes are translated into identical proteins, there is surprising variation in the organization of the proximal promoters. Based on the availability of the complete human genome sequence, it is now possible to definitively assess the expression and regulation of the full complement of histone H4 genes.

Previous studies on a limited number of the H4 genes have suggested that the expression of many if not all of these H4 copies are coordinately controlled during the cell cycle (2;9-11). The human histone H4/n gene, which is temporally and functionally linked to DNA synthesis, provides a paradigm for cell cycle-dependent coordinate control of gene expression at the G1/S-phase transition. This H4 gene is regulated by multiple elements and cognate DNA binding activities, and one proximal promoter element (Site II) mediates cell cycle dependent transcription (12-17). A phylogenetically conserved H4 subtype-specific element is typically present in H4 genes within Site II. Site II is not responsive to the E2F class of transcription factors. Thus, Site II cell cycle regulatory mechanisms at the onset of S phase function independently of E2F (2;15;18). Three factors (HiNF-M, -D, and -P) interact with Site II to mediate cell cycle control of transcription at the onset of S phase (4;12-24). Recently, HiNF-P has been identified as the protein that, in conjunction with the cyclin E/CDK2/p220NPAT, controls H4 gene transcription via binding to its cognate H4 subtype specific element (25). It is necessary to assess whether all human histone H4 genes are coordinately controlled and whether HiNF-P is the key factor that synchronizes transcription of the histone H4 gene family with DNA replication.

Due to the extensive similarity of the histone H4 sequences, it has been difficult to determine the relative contributions of the 15 individual H4 genes to the total histone H4 mRNA pool by molecular approaches that require hybridization of relatively large nucleic acid probes (i.e., northern blotting and RNase protection). However we have circumvented this limitation by applying PCR-based approaches that discriminate between individual histone genes and transcripts. In this study we used quantitative PCR (qPCR) and chromatin immunoprecipitation (ChIP) to examine the expression and regulation of the 15 human histone H4 genes in normal and tumor-derived cells. One key finding of our study is that expression levels of the individual genes differ considerably, consistent with variations in H4 promoter organization and activity. Furthermore, our results firmly establish that eleven genes, which account for greater than 95% of histone H4 mRNAs, are coordinately controlled during the cell cycle and are responsive to the cyclin E/CDK2/p220NPAT/HiNF-P signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**

-Cos-7, HeLa, HCT116, IMR90, SaOS and T98G cells were obtained from the American Type Culture Collection (Rockville, MD). The Cos-7, HeLa and T98G stock cultures were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. HCT116 and SaOS cells were maintained in McCoy’s 5A medium (Invitrogen) supplemented with 10% or 15% FBS respectively, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. IMR90 were maintained in BME (Invitrogen) supplemented with 10% or 15% FBS, respectively, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. IMR90 were maintained in BME (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. IMR90 were maintained in BME (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. All cell lines were split at a ratio of 1:10 when 70% confluent in 100 mm plates (Corning Incorporated, Corning, NY). Culture conditions were 37°C in a 98% humidified, 5% CO2 incubator.

**RT-qPCR Analysis of H4 Gene Expression**

-RNA was extracted from specified cell lines using Trizol® reagent (Invitrogen) according to the manufacturer’s protocol. Purified total RNA was subjected to DNaseI digestion followed by column purification according to the DNA-free RNA kit™ (Zymo Research, Orange CA). Eluted total DNA-free RNA was quantitated by spectrophotometry and 1 µg added to a reverse
transcriptase reaction using the iScript™ cDNA Synthesis kit (BioRad, Hercules CA), with a mixture of random hexamers and oligo d(T) primers. Varying dilutions of cDNA were used as templates in qPCR reactions with oligonucleotides specific to the different histone H4 gene 5’ UTRs (Table 2). Relative quantitation was determined using a 7000 Sequence Detection System (Applied Biosystems) measuring real time Sybr Green (BioRad) fluorescence and calculated by the ΔΔCT method as recently described (26). Overall efficiencies of qPCR were calculated from the slopes of the standard curves of serial dilutions in steps of 2 \[\log (2)\] scale, and found to be nearly identical for each primer set. Expression profiles for H4/a mRNA were extrapolated by comparison of H4/a fluorescent MGB probe-containing qPCR reactions to both Sybr Green and MGB probes specific for H4/n mRNA.

**RNase Protection Assays (RPA)-**RPAs were performed as described in Koessler et al., 2003 (27). Briefly, \[^{32}P\]-labeled antisense RNA was in vitro transcribed from linearized template DNA using the MaxiScript T7/T3 Kit (Ambion, Austin, TX) in the presence of 3.3 mM \[^{32}P\]UTP (3000 Ci:mmol; 50 mCi) and 50 mM unlabeled UTP for use in RNase protection assays (RPA II Kit from Ambion). \[^{32}P\]UTP-labeled β-actin antisense RNA was added in each hybridization reaction to normalize RNA quantities. Protected RNA fragments were resolved in a denaturing 8 M urea/5% (v/v) polyacrylamide gel. A \[^{32}P\]-end labeled Sau3A1 digest of pUC19 DNA was used as a size marker. The PhosphorImager System (Molecular Dynamics, Sunnyvale, CA) was used to quantify protected RNA fragments. The relative expression of the individual histone H4 genes was calculated as the ratio of signal intensity standardized by the β-actin. To compare RPA data to RT-qPCR data the relative expression of H4/d was set to a value of 100% (max) and the relative expression levels of the other H4 mRNAs was determined as a percent of this maximal level.

**Reporter Gene Analysis-**In all experiments N-terminus activity was measured in whole cell extracts using a Luciferase assay kit (Promega, Madison WI) and the results were normalized by co-transfection with 1 µg of pCMV-β-gal. To determine the expression of histone H4 as a function of the HiNF-P/p220NPAT complex, cells were transiently transfected with either 150 ng wildtype HiNF-P or 300 ng of an expression vector containing wt p220NPAT or both with 200 ng of one of the histone H4 wildtype-promoter-LUC reporter constructs. All cDNA's and reporter constructs were mixed with FuGENE6 (Roche Molecular Biochemical) in 100 µl serum free medium for 20 min at room temperature then applied to cells. Cells were incubated overnight with DNA mixture in a final volume of 2 ml medium and then harvested 24 h later. Luciferase activity was measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

**Chromatin Immunoprecipitation-**Adherent cells, growing synchronously or asynchronously, were washed twice with ice cold 1X PBS on the plate. Cells were immediately treated with 5 ml of 1% formaldehyde crosslinker in 1X PBS at room temperature for 10 min with gentle rotation. The crosslinking reaction was quenched by adding 5 ml of .25M glycine in 1X PBS at room temperature for 5 min. Cells were then washed with ice cold 1X PBS twice more, scraped in 5 ml of 1X PBS on ice, and harvested by centrifugation at 1000 xg for 5 min at 4°C. Crosslinked cell pellets were resuspended in lysis buffer (50 mM Tris-Cl pH 8.1, 150 mM NaCl, 1% v/v NP-40, 2X complete protease inhibitor (Roche Molecular Biochemical)) and incubated on ice for 20 min. Lysates were then sonicated to an average DNA size of 500-100 bp by agarose gel electrophoresis and the extracts were cleared by centrifugation at 14,000 xg for 15 min at 4°C. Cleared extracts were divided into sample and input aliquots to allow for subsequent quantitation. Sample aliquots were subjected to primary immunoprecipitation with 2 µg of purified immunoglobulin or 3 µl of crude serum for each appropriate antibody. Following primary antibody incubations, a 1/10 volume of protein-A/G agarose bead slurry (Santa Cruz, Santa Cruz, CA) was added and allowed to bind for 1-4 h. Immunocomplexes bound to the beads were harvested by centrifugation at 1000 xg for 3 min. Beads were then washed consecutively with the following buffers: low salt (20 mM Tris-Cl pH 8.1, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1X complete protease inhibitor), high salt (20 mM Tris-Cl pH 8.1, 500 mM NaCl, 1% Triton
X-100, 2 mM EDTA), LiCl (10 mM Tris-Cl pH 8.1, 250 mM LiCl, 1% deoxycholate, 1% NP-40, 1 mM EDTA) and 3 washes with TE (10 mM Tris-Cl pH 8.1, 1 mM EDTA). Crosslinked protein-DNA complexes were then eluted from antibodies and beads twice with 100 µl of elution buffer (1% SDS, 100 mM NaHCO₃). The pooled eluates were supplemented with 1/10 volume of 3 M sodium acetate pH 5.2 and the crosslinks were reversed at 65°C overnight. DNA was then purified via phenol/chloroform extraction and isopropanol precipitation with 5-20 µg glycogen carrier. Precipitated DNA was allowed to rehydrate in resuspension buffer (10 mM Tris-Cl pH 8.1) and material ratios between sample and inputs were carefully documented to allow for subsequent quantitation of locus immunoprecipitation. ChIP samples were then subjected to quantitative qPCR analysis on an ABI Sequence Detection System with locus-specific primers and probes.

**Electrophoretic mobility shift assay (EMSA)** – In vitro DNA binding of HiNF-P to selected histone H4 genes was analyzed as described (13;25). The radio-labeled probe we used represents an optimized HiNF-P binding site that is based on the HiNF-P recognition site in Site II of the H4/n gene (5’ CTT CAG GTT TTC AAT CTG GTC CGA TAC T). The probe was incubated with HiNF-P enriched nuclear extract from HeLa cells. Competition experiments were carried out with 50, 100, 200 or 400 fold excess of unlabeled oligonucleotides spanning the analogous regions in the promoters of distinct H4 genes.

### RESULTS

**mRNAs of Multiple Human Replication-Dependent Histone H4 Genes are Coordinately Induced in S-phase Cells.** We predicted temporal and/or quantitative differences in expression of the 15 human histone H4 genes because of the heterogeneity in 5’ flanking sequences of the individual genes. Therefore, we initially examined the relative levels of expression of distinct H4 mRNAs during the cell cycle. Human T98G glioblastoma cells were serum deprived for 72 hours to induce quiescence, and then stimulated to reenter the cell cycle with serum. Synchronously cycling cells were harvested at 4, 21 and 25 hours post-stimulation, corresponding respectively to the G₁, S and G₂ phases of the cell cycle as determined by FACS analysis (Fig. 1A). Total RNA was examined by reverse-transcriptase quantitative fluorescent PCR (RT-qPCR). Relative contributions of individual histone H4 genes to the total H4 mRNA pool were determined with primer pairs specific to the unique 5’ untranslated region of each H4 gene (Table 2). Primer pairs were tested against dilutions of genomic DNA to verify that the amplification efficiencies were similar. The mRNA levels of each histone H4 gene are upregulated in S-phase between 5 and 20 fold above levels in G₁ and G₂ phases (Fig. 1B). This result is the first demonstration that the complement of H4 genes in the human genome is temporally and coordinately controlled at the G1/S-phase transition and is consistent with earlier studies that examined a limited number of representative human H4 genes (9-11;13;20;22;25;28).

Our results also indicate that there are differences in the extent to which distinct H4 mRNA species are maximally expressed. We find that the H4/b, H4/d, H4/e, H4/j, H4/n and H4/o genes account for greater than 76% of total histone H4 mRNA during S-phase. H4/g, H4/h and H4/i are moderately expressed, together representing 20% of total histone H4 mRNA, while H4/a, H4/c, H4/k, H4/m and H4/p contribute minimally to the total H4 mRNA pool (less than 5%). Thus, nine of the histone H4 genes generate the majority of mRNAs that are required to accommodate DNA replication and nascent chromatin assembly.

**Differential Regulation of Histone H4 Gene Expression in Normal and Transformed Human Cells.** The promoter elements of H4 genes interact with a multiplicity of transcription factors that respond to cell growth-related signaling pathways that may be differentially activated in normal versus tumor cells. Thus, we assessed whether the deregulation of signaling pathways in transformed and tumor cells affects the composition of H4 mRNA pools. We compared the expression patterns of individual human histone H4 genes in fetal liver, fetal colon and IMR90 (normal diploid lung fibroblast) cells with those in four different tumor-derived human cell lines (i.e., HCT116 colorectal carcinoma cells, T98G glioblastoma cells, SaOS osteosarcoma cells and HeLa cervical carcinoma cells).

We initially compared RNase protection assay data to that of quantitative RT-qPCR in two
human carcinoma lines (data not shown). The RPAs and the RT-qPCRs were performed in completely independent experiments. The two assays show consistent quantitative differences in the relative expression of individual histone H4 genes in asynchronous HeLa and SaOS cell lines. In further studies we used RT-qPCR as the primary method to determine the relative contributions of the individual histone H4 genes in various cell lines and tissues.

The most striking result is that the expression levels of the individual H4 genes are substantially different regardless of the cell type, and that the relative levels are distinct from the theoretically expected contribution of ~7% per gene copy (dashed lines in Figs. 2A and B; 15 genes is 100%). In three normal and four tumor-derived cell types, we find that the five most highly expressed genes (H4/d, H4/e, H4/j, H4/n and H4/o) contribute the majority of H4 mRNAs to the total pool. The only quantitative difference is that these highly expressed genes contribute a disproportionate percentage of the total H4 mRNAs in tumor-derived cells as compared to normal cells. For example, the above five highly expressed genes and six modestly expressed H4 genes (e.g., H4/a, H4/b, H4/c, H4/k, H4/m and H4/p) contribute respectively 55% and 18% in normal cells, whereas these contributions are 80% and 9% in tumor-derived cells. These differences can primarily be attributed to undetectable mRNA levels of a subset of H4 genes in tumor-derived cells as compared to normal cells. For example, the above five highly expressed genes and six modestly expressed H4 genes (e.g., H4/a, H4/b, H4/c, H4/k, H4/m and H4/p) contribute respectively 55% and 18% in normal cells, whereas these contributions are 80% and 9% in tumor-derived cells. These differences can primarily be attributed to undetectable mRNA levels of a subset of H4 genes in tumor-derived cells as compared to normal cells.

**Basal H4 Promoter Activity Dictates H4 mRNA Accumulation.** It is well documented that all histone H4 mRNAs contain a 3’ stem-loop structure (hairpin) that is necessary for 3’-end processing and mRNA stability (8). Because the hairpin sequences are completely conserved among all H4 genes, it is unlikely that the differential expression of H4 gene mRNAs is due to these post-transcriptional mechanisms. Rather, quantitative differences and temporal similarities in the expression of H4 gene copies may reflect the divergence and conservation of distinct 5’ regulatory elements within H4 promoters. Therefore, we tested whether the observed changes in expression of H4 gene copies are related to differences in H4 gene promoter activity. We monitored luciferase reporter gene expression using promoters of all H4 genes located within the two major histone gene superclusters (human chromosomes 1 and 6) in multiple cell types. The promoters with highest transcriptional activity in normal and tumor-derived cells include H4/d, H4/j and H4/n, and by inference also H4/e and H4/o as their corresponding promoters are essentially duplicates of H4/d and H4/n, respectively (Fig. 3). These are the same H4 genes that exhibit high endogenous levels of expression (Fig. 2). Furthermore, in normal cells the activities of individual H4 promoters are more similar to the average than in tumor-derived cells, mirroring the endogenous H4 mRNA expression patterns (Figs. 2 and 3). Thus, differential expression of H4 genes is transcriptionally mediated as expected from the divergence in the overall organization of regulatory elements within H4 promoters.

The H4/n gene has been shown to be regulated by a highly conserved cell cycle regulatory domain (Site II) and an auxiliary module (Site I) that augments the transcription rate. Regions analogous to Site II of the H4/n gene are present in the other H4 genes and can easily be aligned (Fig. 4). However, there is considerable heterogeneity in promoter organization beyond Site II. Alignment of the fifteen human Site II sequences reveals that there are four H4 genes (i.e., H4/a, H4/c, H4/k and/or H4/m) that exhibit clear mismatches with the H4 subtype specific consensus element, while a fifth H4 gene (i.e., H4/m) exhibits a single nucleotide deviation in the TATA box. These five genes generally have lower than average expression and promoter activity in different cell types. This finding is consistent with our previous results that Site II is a positive element that mediates the cell cycle dependent activation of H4 gene transcription (15;25).

The principal factor that interacts with Site II of the H4/n gene is HiNF-P (25). Because deviations of the H4 subtype consensus sequence correlate with reduced expression, we examined whether nucleotide changes decrease the level of HiNF-P binding. Competition EMSAs were performed with the HiNF-P consensus oligo as probe and increasing amounts of unlabeled Site II
competitors spanning a select set of H4 genes. Some of these oligos contain critical mutations that are predicted to decrease or abolish HiNF-P binding (Fig.5). Lack of binding is reflected by absence of competition at 400 fold molar excess as is observed for an H4/n derived oligonucleotide with critical mutations in the HiNF-P contact points (13). Our results show that the Site II sequences of the H4/a and H4/k genes do not bind HiNF-P, whereas the H4/c element binds very weakly (Fig. 5). In contrast, the Site II sequences of the H4/g and H4/p genes interact, as expected, very efficiently with HiNF-P. Together with previous results that compared interactions of HiNF-P with the H4/n & o genes and the H4/d & e genes (13;25), our studies establish a strong relationship between matches in the Site II/HiNF-P consensus and binding of HiNF-P.

**Histone H4 Gene Expression as a Function of Promoter Occupancy.** Having established the effects of Site II mutations on HiNF-P binding in vitro, we addressed whether differential regulation of H4 genes is due to differences in RNA polymerase II (RNAP II) association and the ability of HiNF-P to bind to H4 loci in vivo. Gene specific promoter occupancy by these two proteins was assayed in asynchronous T98G cells by chromatin immunoprecipitation and quantitative fluorescent PCR (ChIP-qPCR). Promoter occupancy was determined by the specific presence of various H4 promoter sequences in immunoprecipitates obtained using antibodies to HiNF-P and RNAP II (Fig. 6).

We find that there is a positive correlation between HiNF-P and RNAP II recruitment to the H4 promoters and the corresponding mRNA levels, although the presence of HiNF-P and/or RNAP II does not guarantee high transcript levels. The genomic promoters of all H4 genes examined, with the exception of H4/a, H4/c and H4/m, interact with HiNF-P in vivo. The genes that do not interact with HiNF-P are minimally expressed and do not contribute appreciably (<3%) to the overall H4 mRNA pool. This finding supports our previous and current studies that HiNF-P occupancy of H4 gene loci is necessary for optimal expression and that HiNF-P is a primary regulator of H4 gene transcription (25).

**Highly Expressed H4 Genes are Responsive to the Cyclin E/CDK2/p220NPAT/HiNF-P Signaling Pathway.** HiNF-P activation of the H4/n gene depends critically on co-activation by the cyclin E/CDK2 responsive p220NPAT protein, and endogenous HiNF-P and p220NPAT levels are limiting for H4/n gene transcription (25). We assessed which of the multiple histone H4 promoters are regulated by this signaling pathway using reporter gene assays in which HiNF-P and p220NPAT are co-expressed (Fig. 7). For example, the H4/n gene is upregulated 3-fold by HiNF-P or p220NPAT alone (data not shown), and is synergistically activated 10-fold or more when both proteins are coexpressed (Fig. 7), consistent with our previous observations (25). As expected, the H4/a, H4/c and H4/m genes, which do not bind HiNF-P as determined by EMSA and ChIP-qPCR analysis (Figs. 5 and 6), do not respond to the HiNF-P/p220NPAT signaling pathway in HCT116, T98G and SaOS cells. H4/c and H4/m genes respond modestly in IMR90 cells, which appear to be the exception. However, the induced activities of these two promoters remain quite low in IMR90 cells, perhaps reflecting an indirect effect of HiNF-P/p220NPAT. More importantly, of the eleven HiNF-P-dependent genes that we analyzed, seven are robustly upregulated by p220NPAT signaling in all cell types and three in at least two cell types. The eleventh gene (H4/g) responds qualitatively, but exhibits very low induced promoter activity. We conclude that all eleven HiNF-P-responsive H4 genes are also co-responsive to p220NPAT. Because these genes contribute to greater than 95% of the total H4 mRNA pool (Fig. 2), it appears that the HiNF-P/p220NPAT signaling pathway is essential for coordinate control of histone H4 gene expression.

**DISCUSSION**

In this manuscript, we examined the similarities and differences among the 15 human histone H4 genes in cell cycle control, expression in normal and tumor-derived cells, proximal promoter activity and promoter binding of transcription factors. Our studies firmly establish coordinate cell cycle control of the majority of histone H4 gene expression as a result of the cyclin E/CDK2/p220NPAT/HiNF-P signaling pathway. Furthermore, a subset of these genes is sporadically silenced in tumor cell lines in comparison to normal cells. The unequal
expression of H4 genes may reflect differences in position within nuclear architecture and/or distinctive promoter element organization. We have observed that histone H4 proximal promoter activities are consistent with the expression levels of endogenous H4 mRNAs. Finally, we establish that all highly expressed H4 genes are regulated by the cell cycle dependent transcription factor HiNF-P.

Coordinate Induction of H4 Gene Expression during the G1/S-phase Transition. Many previous studies have focused on the cell cycle dependent expression of total histone mRNAs as determined by northern blot analysis. A subset of these studies included assays capable of distinguishing one or more histone gene copies. However, as these studies were performed before the human genome project was completed, none of the previous findings permitted a complete analysis of the individual contribution of all members of the histone gene family.

It has been well established that biosynthesis of histone H4 is mediated by multiple functionally expressed H4 genes (2;9-11). By comprehensively analyzing the expression of the human H4 gene family in synchronized cells, one major finding of this study is that all mRNAs derived from the multiple human histone H4 genes are indeed simultaneously upregulated when cells progress into S-phase. Because post-transcriptional mechanisms operating on distinct H4 mRNAs are expected to be identical, this coordinate regulation is directly attributable to transcriptional mechanisms. While our data now conclusively establish that coordinate regulation of the full complement of histone H4 genes does indeed occur, the results indicate that a surprisingly small number of genes accounts for the majority of H4 gene expression. We find that the promoters of the histone H4 genes are not regulated in an equivalent manner by cell cycle driven signaling events. Interestingly, two of the histone H4 (n and o) genes that are most responsive to signals at the G1/S-phase transition are recent duplications in the human genome (6). This recent duplication may reflect a preferred preservation and expansion of H4 genes with the requisite regulatory organization to support DNA replication and cell survival.

Histone H4 Genes Contribute Differentially to the Replication Dependent Pool of Total Histone H4 mRNA. Carcinoma cells, due to dysfunctional regulation of many cell cycle related factors, will differentially activate replication-dependent promoter elements of various genes. We have determined that this differential activation extends to histone H4 genes and may not be a function of tissue specificity. Comparing the expression of histone H4 genes in normal cells with the expression in human carcinoma cells reveals that a subset of individual H4 genes is differentially expressed distinct from any theoretically expected contribution to the total H4 mRNA pool. The disparity in the relative levels of this subset of H4 mRNAs is even greater in carcinoma cell lines, substantiating that aberrant cells have dysfunctional replication-dependent signals.

The Promoters of Histone H4 Genes Exhibit Variation in Promoter Element Organization. The burden of coordinate control of mRNA levels is shared by two main mechanisms, transcriptional induction and increased mRNA stability by 3’ endonucleolytic processing. However, it is well documented that all histone H4 3’ stem-loop structures are completely conserved. Therefore, the variation in endogenous expression of histone H4 mRNAs in normal and carcinoma cells is directly attributable to the relative activities of the individual H4 gene promoters. Alignment of the H4 subtype-specific cell cycle elements reveals that some residues are necessary for HiNF-P binding and consequently cell cycle coordinate control of H4 expression at the G1/S phase transition. Furthermore, variation in the H4 subtype element sequences causes some H4 genes to be poorly responsive to HiNF-P and results in lower contribution to the total pool of H4 mRNA. Thus, the variation in the contributions of the individual H4 genes is not likely due to mRNA stability but rather to divergence of promoter elements and/or overall chromatin organization of histone gene clusters.

Highly Expressed H4 Genes are Responsive to the HiNF-P Signaling Pathway. HiNF-P and RNAP II occupancy of histone H4 promoters does not completely correlate with H4 gene mRNA contribution to the total H4 mRNA pool. Recruitment of RNAP II to low expressing genes may indicate a stalled RNAP II complex.
Lack of promoter occupancy by HiNF-P is typical for low expressing H4 genes, albeit that Site II occupancy by HiNF-P does not assure high transcription rates. This finding suggests that HiNF-P is required for high level expression but insufficient for maximal activation of cell cycle dependent H4 gene transcription. HiNF-P activation of these genes depends on co-activation by the cyclin E/CDK2 responsive p220NPAT protein (25;28), and we find that the H4 genes that are most responsive to the HiNF-P/p220NPAT complex are also the most highly upregulated at the G1/S-phase transition. Furthermore, our study shows that there is a correlation between HiNF-P occupancy, responsiveness of the histone H4 promoter and maximal induction at the G1/S-phase transition. Taken together these findings support the concept that the HiNF-P/p220NPAT complex is the principal regulatory module that functionally links the coordinate regulation of histone H4 genes with the onset of DNA replication at the G1/S-phase transition.

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Reference List


FIGURE LEGENDS

Fig. 1. **Cell cycle dependent coordinate control of histone H4 gene expression.** (A) FACS profiles of T98G cells synchronized by serum deprivation and harvested at 4, 21 or 25 hours post serum-stimulation, which corresponds to G₁, S and G₂ phases respectively. (B) All H4 gene mRNA levels are induced from G₁ to early S-phase between 5 and 20 fold. In G₂ cells, all H4 gene mRNA levels are similar to those in G₁ cells. H4/d and H4/e (◊), as well as H4/n and H4/o (◇), are respective genomic duplicates and cannot be distinguished from one another by qPCR. Therefore the relative contribution of each of these duplicated genes was estimated as half of the total when these symbols are used (◊◇).

Fig. 2. **Expression patterns of the human replication dependent histone H4 genes in various cell types.** Relative histone H4 gene expression profiles were determined by RT-qPCR in two normal fetal tissues (A), normal diploid-derived cells (A), and four tumor-derived cells (B). The profiles are presented as percent contribution to total H4 mRNA pools. A dashed line indicates the theoretical level of contribution if each H4 gene were equally expressed (15 = 100%). The relative contribution of each duplicated gene was estimated as half of the total (◊◇). ND = not determined. Error bars represent the standard deviation of triplicate experiments.

Fig. 3. **Basal promoter activity of the individual H4 genes.** Transient transfection of the individual H4 proximal promoters driving luciferase expression in normal diploid IMR90 cells, as well as T98G, SaOS and HCT116 tumor-derived cell lines. Luciferase activities were measured as relative light units (RLU) and normalized with co-transfected pCMV-β-galactosidase. A dashed line indicates the average level of normalized expression of all analyzed H4 reporter constructs. H4/d and H4/e (◊), as well as H4/n and H4/o (◇), respective promoters cannot be distinguished, and activity from a single proximal promoter construct is representative for both genes (◊◇). Error bars represent the standard deviation of triplicate experiments.

Fig. 4. **Alignment of the Site II cell cycle regulatory element in the full complement of human histone H4 genes.** Mismatches in Site II, which spans both the H4 subtype specific element (cons) and the TATA-box, are examined in relation to H4 mRNA expression and H4 promoter strength. HiNF-P binds to DNA by interacting with the conserved residues of the H4 subtype-specific cell cycle element to induce transcriptional activation of H4 genes in concert with G₁/S-phase transition signals (13, 14, 16, 17, 22, 25, 28). The sites of HiNF-P contact within the consensus element are indicated (*). Mismatches with the consensus are indicated in bold lower case. The alphabet of redundant nucleotides is as follows: D = A or G or T, H = A or C or T, M = A or C, R = A or G, W = A or T, Y = C or T. The columns to the right correlate sequence data with H4 gene expression and transcription. H4 mRNA expression data are summarized as follows: - = not expressed, + = below average levels in most or all cell lines, ++ = intermediate levels in several cell lines, +++ = above average expression in all cell lines, ND = not determined. The following symbols reflect H4 promoter strength: - = no significant activity, + = low activity in most or all cell lines, ++ = intermediate activity in several cell lines, +++ = strong activity in most or all cell lines, ND = not determined.

Fig. 5. **In vitro interactions between HiNF-P and Site II equivalents in selected H4 genes.** (A) EMSAs were performed with HeLa nuclear protein and a standard radio-labeled HiNF-P binding site oligonucleotide in the absence (control) or presence of increasing amounts of unlabeled competitor oligonucleotides (50, 100, 200, and 400 fold molar excess, representing a total binding site concentration of 25, 50, 100 and 200 nM) spanning variant Site II sequences derived from the genes indicated to the left. Arrowheads indicate two protein/DNA complexes (P = HiNF-P; u = unrelated complex). (B) Table summarizing matches of Site II equivalents in the indicated H4 genes (left column: + = 100% match; - =
mismatch) with binding of HiNF-P to Site II in vitro by EMSA (second column; data presented in Fig. 5A) and in vivo by ChIP (third column; data presented in Fig. 6). The fourth column indicates the sensitivity of the distinct H4 genes to treatment with HiNF-P anti-sense oligonucleotides (+ = HiNF-P dependent and H4 mRNA decreased; - = not HiNF-P dependent) as previously documented (28).

Footnotes are as follows: * = the H4/n and H4/o genes are recent duplicates and results are based on the same oligonucleotide; ** same for the H4/d and H4/e genes, data comparing the H4/n (F0108) and H4/d (HuH4a) were published previously under different gene names (13, 25); # = exceptions to the rule that HiNF-P binding in vitro predicts in vivo interactions and HiNF-P dependency (note: there may be uncharacterized HiNF-P sites elsewhere in the H4/k gene, while the H4/m gene has a deviant TATA-box as shown in Fig. 4).

Fig. 6. **Histone H4 gene expression as a function of promoter occupancy by a crucial H4 transcription factor and RNA polymerase II.** In T98G cells, ChIP was performed with antibodies to HiNF-P, and RNAP II. qPCR for the promoter/5’ region of each histone H4 gene (X-axis) was performed on the ChIP samples and compared to input to establish the percentage of each locus that is immunoprecipitated (Y axis = % input). The relative contribution of each duplicated gene was estimated as half of the total (◊—). The inset shows a sample chromatin immunoprecipitation experiment using the H4/n gene with pertinent controls for specificity. Negative controls include non-specific IgG, and immunoprecipitates with HiNF-P and RNA pol II antibodies that were performed in the presence of the corresponding immunogenic peptide. Error bars represent the standard deviation of triplicate experiments. Additional negative control experiments included analysis of precipitates with qPCR primers against functionally expressed non-histone H4 genes (positive for RNA polymerase II and negative for HiNF-P), as well as non-expressed genomic segments (negative for both RNA polymerase II and HiNF-P) (data not shown). In all cases, we observed background values for negative controls. The qPCR results shown here were consistent with and more quantitative than results from gel-based ChIP assays for the H4/n (FO108) gene that we previously presented (Mitra et al., 2003).

Fig. 7. **The majority of H4 gene promoters are synergistically induced by HiNF-P and p220NPAT.** Normal and tumor-derived cell lines were transiently co-transfected with the individual H4 proximal promoters with (black bars) or without (white bars) expression vectors for HiNF-P and p220NPAT. Fold change in expression is indicated for the promoters of H4 genes that have basal activity above average (dashed line in Fig. 2). H4/d and H4/e (◊), as well as H4/n and H4/o (◇), respective promoters cannot be distinguished from one another and activity from a single proximal promoter construct is representative for both genes (◊◇). Error bars represent the standard deviation of triplicate experiments.
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<tr>
<th>Chromosomal Location</th>
<th>Accession number</th>
<th>Reference</th>
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<td>H4/a 6p21</td>
<td>X60481</td>
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<td>Albig and Doenecke</td>
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<td>Albig et al. 1997</td>
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<td>H4/p 12p13.1</td>
<td>AY128653</td>
<td>Marzluff et al. 2002</td>
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* H4/f is a pseudogene (Albig and Doenecke, 1997)
Table 2: Specific primers for the individual histone H4 transcripts

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<tr>
<th>Gene</th>
<th>Forward primer (5’→ 3’)</th>
<th>Reverse primer (5’→ 3’)</th>
<th>Sybr/Fam-MGB probe</th>
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<td>CCGTTCCCAGCCCTTACCC</td>
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<td>CCACTGTTCGCTCTATCCSAGA</td>
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<tr>
<td>H4/d &amp; H4/e</td>
<td>TGGGTGAGACTCCTCCTTGGCT</td>
<td>AAGACCCTTCCCGCCTTTT</td>
<td>Sybr-Green</td>
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<tr>
<td>H4/g</td>
<td>TACCTCAGCTGCCATAGGA</td>
<td>AACCTTTTCCGCTTGTG</td>
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<td>H4/h</td>
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<td>CTGGGTCAGACCCCTTTCC</td>
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<td>H4/m</td>
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<td>H4/p</td>
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Asynchronous cells

0 hrs \( (G_0) \)
4 hrs \( (G_1) \)
21 hrs \( (S) \)
25 hrs \( (G_2) \)

Quantitative RT-PCR

T98G

normalized expression

hrs post-serum

25 hrs \( (G_2) \)
4 hrs \( (G_1) \)
0 hrs \( (G_0) \)

H4: /a /b /c /d /e /g /h /i /j /k /l /m /n /o /p

11 8 4 17 2 1 0 1

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### Organization of the Histone H4 Cell Cycle Element & Relative Expression of the Complement of H4 mRNAs

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<tr>
<th>H4 subtype consensus element</th>
<th>TATA-box</th>
<th>H4 mRNA strength</th>
<th>H4 promoter strength</th>
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<tr>
<td>-105 CC; CTTCA- CATGA GGT TTTCAAACAAGGTCCGTC</td>
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**Site II 5’ end**

| cons=DGTYYTCADTYHGGTCCGMH | cons=TATWWVDD | Site II 3’ end |

HiNF-P contact sites
### Section A

**Fold Excess**

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**32P= HiNF-P consensus oligo**

### Section B

**HiNF-P consensus motif**

```
DGTYYTCDTGYHGGTCCGMH
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<thead>
<tr>
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<th>Control Excess (EMSA)</th>
<th>In Vivo (ChIP)</th>
<th>H4 mRNA Levels (HiNF-P Antisense)</th>
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**DNA binding**

- ND: Not detected
- +: Detected
- ++: Strong detection
- #: Indeterminate
- **: Strongest detection
Coordinate control and selective expression of the full complement of replication-dependent histone H4 genes in normal and cancer cells
William F. Holmes, Corey D. Braastad, Partha Mitra, Cornelia Hampe, Detlef Doenecke, Werner Albig, Janet L. Stein, Andre J. van Wijnen and Gary S. Stein

J. Biol. Chem. published online August 29, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M506995200

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