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Involvement of the cell cycle-regulated nuclear factor HiNF-D in cell growth control of a human H4 histone gene during hepatic development in transgenic mice

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ABSTRACT **Regulation of the cell cycle-controlled histone gene promoter factor HiNF-D was examined *in vivo*. Proliferative activity was measured by DNA replication-dependent histone mRNA levels, and HiNF-D binding activity was found to correlate with cell proliferation in most tissues. Furthermore, HiNF-D is down-regulated during hepatic development, reflecting the onset of differentiation and quiescence. The contribution of transcription to histone gene expression was directly addressed in transgenic mice by using a set of fusion constructs containing a human H4 histone gene promoter linked to three different genes. Transgene expression in both fetal and adult mice paralleled endogenous mouse histone mRNA levels in most tissues, consistent with this promoter conferring developmental, cell growth-related transcriptional regulation. Our results suggest that HiNF-D is stringently regulated *in vivo* in relation to cell growth and support a primary role for HiNF-D in the proliferation-specific expression of H4 histone genes in the intact animal. Further, the data presented here provide an example in which apparent tissue specificity of gene expression reflects the proliferative state of various tissues and demonstrate that multiple levels of histone gene regulation are operative *in vivo*.**

Gene-regulatory programs that direct development from a unicellular, totipotent zygote to a multicellular, differentiated organism include those responsible for growth control of the cell division cycle. Histone gene expression is subject to stringent cell cycle regulation as evidenced by the temporal and functional coupling between histone protein synthesis and histone mRNA levels that parallel DNA synthesis (reviewed in refs. 1–5).

Cell cycle regulatory mechanisms involved in modulating DNA replication-dependent histone gene expression have been studied in numerous mammalian tissue culture systems by using cells that display a pluriform array of phenotypes, including those of normal diploid, transformed, and tumor-derived cells (6–31). These studies revealed that histone gene expression is regulated by multiple transcriptional and post-transcriptional mechanisms involving histone gene promoter sequences and cognate histone gene promoter factors (6–20), 3′ end mRNA processing activities (21–23), histone mRNA degradation (24–26), and subcellular localization of the mRNA (27, 28). Although selective processing and destabilization of histone mRNA contribute significantly to cellular histone mRNA levels in actively proliferating cells, expression is primarily down-regulated at the transcriptional level during the onset of differentiation, mediated by selective alterations in histone gene promoter protein–DNA interactions (29, 30). Similarly, when normal diploid cells in culture exit the cell cycle at quiescence, modifications in protein–DNA interactions parallel the transcriptional down-regulation of histone gene expression (31).

Regulation of the human H4 histone gene designated FO108 has been extensively characterized, and the transcriptional down-regulation of this gene at the cessation of proliferation at the onset of both differentiation and quiescence is associated with a selective loss of binding of the nuclear factor HiNF-D to a primary proximal promoter element designated "H4-Site II" (7, 17). However, to date our understanding of cell cycle and cell growth regulation of gene expression during the initiation and progression of differentiation is restricted to results obtained from cell culture models. Here, we address the regulation of histone gene expression *in vivo*.

Our experimental approach was as follows. First, we determined the levels of histone mRNA in mouse tissues as a measure of cell proliferative activity. Second, we assessed levels of HiNF-D binding activity in these tissues and the cross-species compatibility of mammalian histone gene transcription factors. Finally, we generated several transgenic mouse strains by introducing chimeric human histone promoter–reporter gene constructs into the mouse germ line to directly investigate the contribution of transcription in the regulation of histone gene expression in the mouse. The results are consistent with a role for HiNF-D as a primary mediator of transcriptional regulation of H4 histone gene expression in the whole animal.

MATERIALS AND METHODS

Plasmid Constructions. Plasmid FO003 contains a 7.5-kilobase (kb) human genomic DNA fragment (*Xba* I–*Xba* I) spanning the 5′ flanking region (6.5 kb) and mRNA coding region of the FO108 H4 histone gene (called H4-FO108) (19). Construct pF3cat (32) contains the 6.5-kb 5′ flanking sequence of pFO003, starting exactly at the histone mRNA cap site and extending to −6.5 kb (designated "F3" promoter), fused to a 1.6-kb *Hin*dIII–*Bam*HI fragment from pSV2cat (33). The pSV2cat fragment contains chloramphenicol acetyltransferase (CAT) mRNA coding sequences, the simian virus 40 (SV40) small tumor antigen intervening sequence (IVS) in the 3′ untranslated region, and the SV40 early polyadenylylation signal. Plasmid F3tat contains the F3 promoter fused to the first exon of the human immunodeficiency virus *tat* gene (HTLV-III isolate) and the SV40 IVS and polyadenylylation signal from pSV2cat (32).

Generation of Transgenic Mice. The inserts of the above plasmids were isolated on agarose gels and purified on glass beads or on cesium chloride gradients. DNA concentrations were determined by using DNA fluorometry with Hoechst

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Abbreviation: CAT, chloramphenicol acetyltransferase.

33258 (Hoefer), and samples were diluted to 1 μg/ml before microinjection. Transgenic mice were generated by microinjecting 200–400 fertilized eggs derived from superovulating 21- to 24-day-old female FVB/N mice. Embryos surviving micro-injection were transferred into pseudo-pregnant foster mothers (15–20 zygotes per animal). Approximately 25% of the implanted embryos developed to term and were weaned when 3 weeks old. Three independent transgenic strains containing the FO003 construct, seven lines of F3cat mice, and one strain of F3tat mice were obtained.

RNA Blot-Hybridization (Northern) Analysis, S1 Nuclease Protection Analysis, CAT Assays, and Gel Retardation Assays. Isolation of cytosolic RNA from mouse tissues and subsequent Northern blot analysis and S1 nuclease protection analysis of H4 histone mRNA have been reported (19, 32, 34). The mouse H4 histone gene probe (designated mouse H4-AST in this study) was derived from pBR-mus-hi-I-H4-HinfI (35). The preparation of nuclear proteins, binding conditions for gel retardation assays, and competition assays have been described (17).

Protein for CAT assays was isolated by homogenization of tissue samples in 0.25 M Tris·HCl (pH 7.5) and incubation of crude extracts for 15 min at 60°C. Protein concentrations in the cleared supernatants were determined by Bradford assays (Bio-Rad). CAT assays were performed in a 200-μl volume containing 0.1 M Tris·HCl (pH 7.5), 100 μg of protein, 0.2 μCi of [^{14}C]chloramphenicol (1 μCi = 37 kBq), and 4 mM acetyl coenzyme A at 37°C for 4 hr. Reaction products were separated by TLC and subjected to autoradiography.

RESULTS

Tissue Distribution of DNA Replication-Dependent H4 Histone mRNA. Cell proliferative activity in the intact animal was initially studied by examining the tissue distribution of DNA replication-dependent H4 histone mRNAs in adult mice. Cytosolic mRNA isolated from several tissues was assayed by Northern blot analysis to establish overall H4 histone mRNA levels (Fig. 1). To discriminate between DNA replication-dependent and constitutively expressed or differentiation-specific H4 histone mRNA species (36–42), we performed S1 nuclease protection analysis (Fig. 1) using the cell cycle-regulated (16) mouse H4 histone gene we call H4-AST. The H4-AST probe is a representative mouse H4 histone gene cloned in pBR-mus-hi-I-H4-HinfI (35) that we have used previously to establish endogenous H4 histone mRNA levels in cultured mouse cell lines (19). Comparison of the results of both procedures indicates that levels of H4-AST histone mRNA have a similar tissue representation as the bulk of mouse H4 histone mRNA, suggesting that cell cycle-regulated H4 histone mRNAs comprise the majority of H4 histone gene transcripts. More importantly, these data show that levels of H4 histone mRNAs are highest in spleen, barely detectable in brain and kidney, and below the level of detection in adult liver. Because of the tight coupling between H4 histone gene expression and DNA replication, this indicates that the apparent tissue-related expression of H4 histone genes in spleen reflects the level of cell proliferation. In contrast, the absence of histone gene expression in tissues such as adult kidney and liver parallels the absence of histone gene expression observed previously in differentiated (29, 30) or quiescent cells (31) in culture.

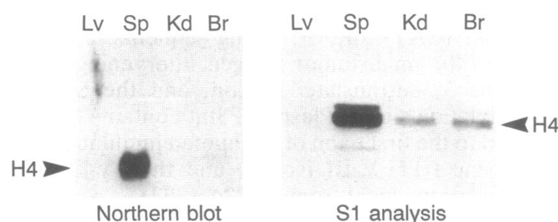

FIG. 1. Northern blot analysis (*Left*) and S1 nuclease protection analysis (*Right*) of cytosolic mRNA from adult mouse tissues with H4 histone gene-specific probes. Lanes: Lv, liver; Sp, spleen; Kd, kidney; Br, brain. The signal corresponding to cell cycle-regulated H4 histone mRNA is indicated by an arrowhead.

Tissue Distribution of the Proliferation-Specific, Cell Cycle-Regulated Human H4 Histone Gene Promoter Factor HiNF-D. The H4-Site II–HiNF-D DNA promoter–protein interaction has been implicated in control of FO108 human H4 histone gene expression during the cell cycle (6, 17), and loss of HiNF-D binding activity has been observed when proliferation is down-regulated at the onset of differentiation (29, 30) and quiescence (31). To test the relevance of these findings during development, nuclear extracts from different mouse tissues were assayed for the presence of HiNF-D binding activity (Fig. 2). Competition assays were performed to confirm that the sequence specificity of binding for the murine homologue was indistinguishable from that of human HiNF-D (Fig. 3). HiNF-D activity was detectable in adult spleen and thymus but was not observed in adult liver. The correlation between detection of HiNF-D activity and cell proliferative activity as reflected by mouse H4 histone mRNA levels is consistent with a functional relationship between the H4-Site II–HiNF-D DNA–protein interaction and the proliferation-specific transcription of H4 histone genes. Interestingly, high levels of HiNF-D activity were found in adult brain (Fig. 2). The physiological significance of high levels of HiNF-D activity in brain, with limited histone mRNA accumulation, remains to be established. However, the abundance of HiNF-D DNA binding activity is consistent with the persistence of histone gene transcription (as measured by CAT assays) in this tissue (see below).

Down-Regulation of H4-Site II–HiNF-D DNA–Protein Interaction During Mouse Liver Development. To explore the extent to which H4-Site II DNA–protein interactions are related to developmental regulation of the H4 histone gene promoter, we measured HiNF-D binding activity (Fig. 4) and histone mRNA levels (Fig. 5) during mouse development at the fetal and adult stages. HiNF-D binding activity was detectable in embryonic liver but was not observed in adult liver. However, relatively high levels of HiNF-D activity were found in both fetal and adult brain. These results indicate that HiNF-D is down-regulated by cell-growth mechanisms mediating the arrest of cell prolifera-

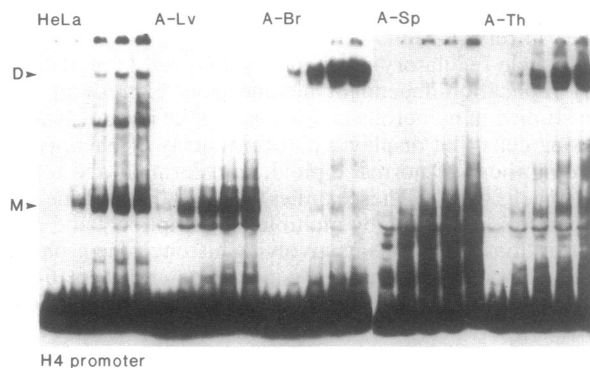

FIG. 2. Gel retardation assay using nuclear proteins from adult mouse tissues with a DNA fragment spanning the FO108 human H4 histone gene promoter region designated H4-Site II (nucleotides −70 to +1, relative to the H4 histone mRNA cap site). Lanes: A-Lv, adult liver; A-Br, adult brain; A-Sp, adult spleen; A-Th, adult thymus. Indicated by arrowheads are the protein–DNA complex mediated by HiNF-D (D) and the complex involving a 5′-dTCGGTT binding protein designated HiNF-M (M) (A.J.v.W., J.L.S., and G.S.S., unpublished data). Approximately 2, 4, 6, 8, and 10 μg of protein were added to each reaction mixture, respectively.

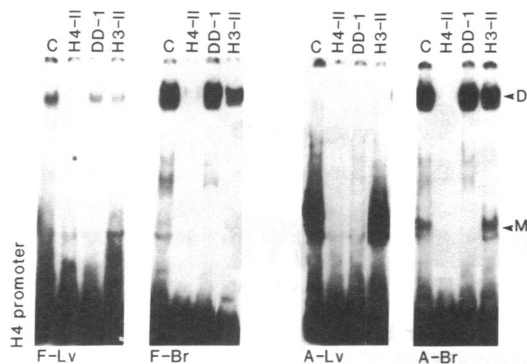

FIG. 3. Competition analysis of the murine homologue of HiNF-D using nuclear proteins from mouse fetal liver and brain (lanes F-Lv and F-Br) and adult liver and brain (lanes A-Lv and A-Br). Binding reactions were performed in the presence of a 500-fold molar excess of specific oligonucleotides. Control lane C contains no competitor DNA. Other lanes represent competition with the DS-II oligonucleotide spanning HiNF-D DNase I footprint sequences (lanes H4-II) (17), H3-Site II CCAAT-box sequences (lanes H3-II), and an oligonucleotide specific for HiNF-M (lanes DD-1) (A.J.v.W., J.L.S., and G.S.S., unpublished data).

tion during hepatic development, whereas other processes may be involved in constitutive control of HiNF-D binding activity in brain. The down-regulation of HiNF-D during liver development is temporally coupled to the cessation of H4 histone gene expression in this tissue (Fig. 5*D*). These results support a role for HiNF-D in the transcriptional regulation of H4 histone genes and suggest that the H4-Site II–HiNF-D DNA–protein interaction is developmentally regulated in liver and ultimately associated with control mechanisms mediating *in vivo* cell quiescence and cell differentiation in the intact animal.

Cell Growth-Related Transcriptional Regulation of Histone Gene Expression During Hepatic Development in Transgenic Mice. Cell cycle regulatory mechanisms involved in modulating levels of human H4-FO108 histone gene expression have previously been studied in a variety of normal and transformed mammalian tissue culture cells (5–7, 17, 19, 20). In this study, we examined the regulation of H4 histone gene expression by introducing the human H4-FO108 histone gene into the mouse germ line, where physiological mechanisms functionally related to cell cycle progression, cell growth control, and differentiation are operative (Fig. 5). The construct (designated FO003) used in these studies was a 7.5-kb human genomic fragment containing 6.5 kb of 5′ flanking sequence contiguous with the FO108 H4 histone gene mRNA coding region. Transgenic FO003 mice expressed the introduced human H4 histone gene in spleen, but expression was barely detectable in adult liver and brain (Fig. 5*A*). Similar results were obtained when we performed simultaneous S1 nuclease protection analyses of human and mouse H4 histone mRNA species (Fig. 5*B*). Transgene expression was reproducibly lower than that of the mouse H4-AST histone gene in three different transgenic strains. However, human H4-FO108 mRNA could only be detected in tissues that expressed high levels of the mouse H4-AST gene, indicating that the regulation of transgene expression parallels that of the endogenous mouse H4 histone gene.

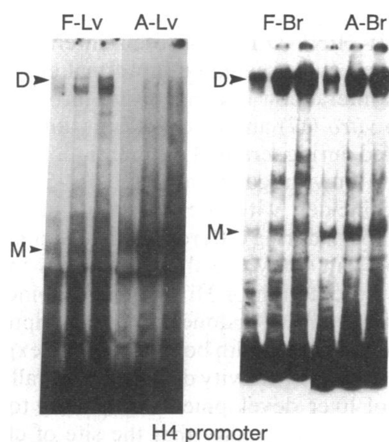

FIG. 4. Gel retardation assay using nuclear proteins from mouse liver and brain in the fetal (lanes F-Lv and F-Br) and adult (lanes A-Lv and A-Br) developmental stages. Indicated by arrowheads are the complexes mediated by HiNF-D (D) and HiNF-M (M). The doublet band observed with the HiNF-D complex is related to posttranslational modification (A.J.v.W., J.L.S., and G.S.S., unpublished data).

The contribution of transcriptional regulation to histone gene expression was studied by using transgenic mice containing a chimeric construct (F3cat; Fig. 5*C*) composed of the H4 histone gene promoter (designated F3 and derived from construct FO003) fused to the bacterial CAT gene. Because posttranscriptional processes require unique histone mRNA sequences and secondary structures (21–26), substitution of the histone mRNA coding region and 3′ flanking sequences selectively eliminates the posttranscriptional component to histone gene expression facilitating direct assessment of transcriptional activity.

Transgenic mice expressing the F3cat construct from seven independent strains were examined for levels of CAT activity in a spectrum of tissues (Fig. 5*C*; also data not shown). Relative levels of CAT activity in three tissues were: (in ascending order) liver < kidney < spleen << thymus < brain. Hence, the tissue representation of reporter gene expression is qualitatively similar to both levels of H4 histone gene expression and HiNF-D activity in liver, kidney, and spleen, but not in brain (Fig. 5*C*). These results indicate that in these tissues, with the exception of brain, regulation of H4 histone gene expression is mediated primarily at the transcriptional level. Persistence of H4 histone gene transcription in brain, as directly reflected by histone promoter-driven CAT activity, is consistent with the constitutive presence of HiNF-D activity in both fetal and adult brain. The limited extent of histone mRNA accumulation indicates that histone gene expression in brain appears to be controlled at the level of mRNA stability or 3′ end processing, or both.

Based on the down-regulation of both H4 histone gene expression and HiNF-D activity during development, we investigated reporter gene expression in fetal mouse tissues to assess developmental regulation of histone gene promoter activity. Initially, we assayed CAT activity in four siblings of one strain of F3cat transgenic mice in fetal liver and brain from ≈16-day-old mouse embryos and other embryonic structures (Fig. 5*C*). CAT activity was readily detectable in fetal liver and brain and was comparable to that in other developing tissues. Interestingly, we observed that CAT activity in fetal liver was 2- to 8-fold higher than in fetal brain. In contrast, CAT activity in adult liver was 1/20th to 1/50th that in adult brain. The striking finding that the pattern of reporter gene expression differs in fetal and adult transgenic mice provided a first indication that the transcriptional regulation conferred by the H4-FO108 histone gene promoter is related to cell growth and development. This may in part reflect developmental modifications in the functions of these tissues—e.g., loss of haematopoietic activity in adult liver.

Since we observed that histone gene promoter activity as measured by CAT activity was modified during mouse liver development, we studied histone gene transcription more directly by measuring reporter gene mRNA levels rather than enzymatic activity. Transgenic mice were generated containing fusion constructs with the F3 human H4 histone gene promoter linked to the coding region of the human immu-

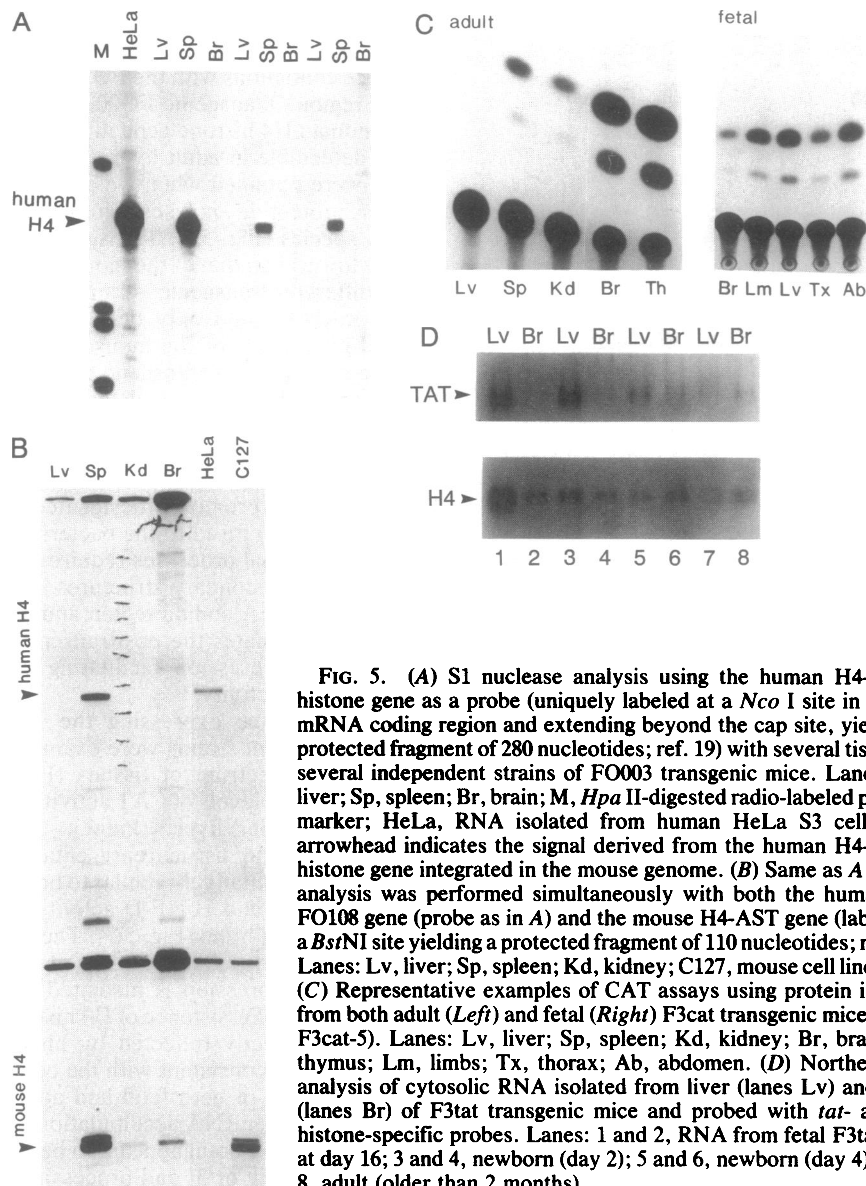

FIG. 5. (*A*) S1 nuclease analysis using the human H4-FO108 histone gene as a probe (uniquely labeled at a *Nco* I site in the H4 mRNA coding region and extending beyond the cap site, yielding a protected fragment of 280 nucleotides; ref. 19) with several tissues of several independent strains of FO003 transgenic mice. Lanes: Lv, liver; Sp, spleen; Br, brain; M, *Hpa* II-digested radio-labeled pBR322 marker; HeLa, RNA isolated from human HeLa S3 cells. The arrowhead indicates the signal derived from the human H4-FO108 histone gene integrated in the mouse genome. (*B*) Same as *A* except analysis was performed simultaneously with both the human H4-FO108 gene (probe as in *A*) and the mouse H4-AST gene (labeled at a *Bst*NI site yielding a protected fragment of 110 nucleotides; ref. 19). Lanes: Lv, liver; Sp, spleen; Kd, kidney; C127, mouse cell line C127. (*C*) Representative examples of CAT assays using protein isolated from both adult (*Left*) and fetal (*Right*) F3cat transgenic mice (strain F3cat-5). Lanes: Lv, liver; Sp, spleen; Kd, kidney; Br, brain; Th, thymus; Lm, limbs; Tx, thorax; Ab, abdomen. (*D*) Northern blot analysis of cytosolic RNA isolated from liver (lanes Lv) and brain (lanes Br) of F3tat transgenic mice and probed with *tat*- and H4 histone-specific probes. Lanes: 1 and 2, RNA from fetal F3tat mice at day 16; 3 and 4, newborn (day 2); 5 and 6, newborn (day 4); 7 and 8, adult (older than 2 months).

nodeficiency virus-specific viral tat protein (Fig. 5*D*). Use of this reporter facilitated simultaneous examination of both tat mRNA and endogenous H4 histone mRNA levels by Northern blot analysis.

Analysis of cytosolic mRNA from liver and brain at the fetal, newborn, and adult developmental stages revealed that in liver, levels of tat mRNA and mouse H4 histone gene mRNA decline in parallel, whereas in brain, levels of both mRNAs are at comparable, constitutive levels during development. Hence, we observe a coupling between alterations in proliferation-related, tissue-specific patterns of reporter gene expression and similar modifications in H4 histone gene expression, suggesting that the human H4 histone gene promoter mediates developmental, cell-proliferation-related transcriptional regulation in the intact animal.

DISCUSSION

The proximal promoter of the FO108 human H4 histone gene has been shown to confer cell cycle-regulated expression to reporter genes in tissue culture cells (A. Ramsey-Ewing, G.S.S., and J.L.S., unpublished observations). The region responsible for cell cycle regulation coincides with the *in vivo* DNA–protein interaction domain H4-Site II (7). The distal part of this binding domain contains a DNA sequence element that can be found in analogous regions of other mammalian, cell cycle-regulated H4 histone gene promoters (17). The H4-Site II–HiNF-D DNA–protein interaction has been shown to be cell cycle-regulated in normal diploid cells (6), although this interaction is constitutive throughout the cell cycle both *in vitro* (17) and *in vivo* (7) in tumor cells and is down-regulated during terminal differentiation *in vitro* and *in vivo* (29). The down-regulation of the H4-Site II–HiNF-D interaction coincides with the cessation of histone gene transcription, both during differentiation (29) and quiescence (31). In the present study, we show that in the intact animal, the tissue representation of HiNF-D DNA-binding activity correlates with levels of histone gene transcription (as measured by reporter genes) with both trans-gene expression and HiNF-D DNA-binding activity declining in parallel during the progression of liver development from fetus to adult. One cannot dismiss the possibility that the site of chromosomal integration of the reporter gene may in part influence the level of trans-gene expression. However, together with our previous findings, these data strongly indicate that HiNF-D may be a rate-limiting factor in the transcription of cell cycle-dependent H4 histone genes, and that transcriptional control is a primary mode of regulation when cells cease to divide during quiescence or differentiation.

Interestingly, a constitutive level of both HiNF-D activity and histone gene promoter activity was found in mouse brain throughout development. The abundance of HiNF-D activity in brain, when accumulation of cell cycle-dependent histone mRNAs does not occur, may suggest a novel trans-activating function for this factor in nondividing specialized cells. Alternatively, this finding could reflect a loss in stringent regulation of HiNF-D DNA-binding activity in brain, perhaps because of the presence of mitogenic factors in this tissue. Active histone gene transcription may be neutralized by the functional redundancy of the multiple regulatory levels controlling histone mRNA accumulation. In particular, posttranscriptional mechanisms, including the possible absence of histone mRNA 3′-end processing activities (21–23) or the presence of exonuclease activity rapidly degrading histone mRNA (24, 25), may contribute dramatically to the shutdown of histone gene expression, while the competency of H4 histone genes to be transcribed is not abrogated in brain. The finding that the FO108 H4 histone gene promoter, F3, is active in brain underscores the multilevel regulation of histone genes *in vivo*.

In conclusion, using transgenic mice containing human H4 histone promoter–reporter gene fusion constructs, we find that transcriptional regulation of H4 histone gene expression during mouse liver development may reflect variations in proliferative activity in this tissue. Moreover, the regulation of the H4 histone gene promoter appears to involve developmental control of cell cycle-dependent H4-Site II–HiNF-D DNA–protein interactions. Our findings are consistent with the hypothesis that multiple transcriptional and posttranscriptional mechanisms are involved in histone gene regulation during development in the intact animal, but that the H4-Site II–HiNF-D DNA–protein interaction represents a predominant regulatory on/off switch which may determine the competency of the histone gene 5′ flanking region to promote transcription.

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