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In Vivo Occupancy of Histone Gene Proximal Promoter Elements Reflects Gene Copy Number-Dependent Titratable Transactivation Factors and Cross-Species Compatibility of Regulatory Sequences

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Abstract To assess systematically the structural and functional aspects of histone gene transcription within a chromosomal context, we stably integrated an extensive set of human histone H4 gene constructs into mouse C127 cells. Levels of expression were determined by S_1 nuclease protection assays for multiple mouse monoclonal cell lines containing these human H4 genes. For each cell line, we quantitated the number of integrated human H4 genes by Southern blot analysis. The results indicate that the expression of the human H4 gene is in part copy number dependent at low gene dosages. However, the level of expression varies among different cell lines containing similar numbers of copies of the same H4 gene construct. This result suggests that position-dependent chromosomal integration effects contribute to H4 gene transcription, consistent with the roles of long-range gene organization and nuclear architecture in gene regulation. At high copy number, the level of human H4 gene expression per copy decreased, and endogenous mouse H4 mRNA levels were also reduced. Furthermore, in vivo occupancy at the human H4 gene immediate 5′ regulatory elements, as defined by genomic fingerprinting, showed copy number-dependent protein/DNA interactions. Hence, human and mouse H4 genes compete for titratable transcription factors in a cellular environment. Taken together, these results indicate cross-species compatibility and suggest limited representation in vivo of the factors involved in regulating histone H4 gene transcription.

Key words: histone gene transcription, chromosome, H4 gene, C127 cell, titratable transcription factors

Expression of mammalian histone genes, which encode the principal proteins responsible for packaging newly replicated DNA into chromatin, is regulated in a cell cycle-dependent manner. Multiple levels of control contribute to restriction of histone protein synthesis to S-phase and functional coupling of histone gene expression to DNA replication [Stein et al., 1984, 1994; Osley, 1991]. Stoichiometric synthesis of histone proteins is in part regulated by posttranscriptional mechanisms involving mRNA processing and mRNA stability, as well as translatability and subcellular distribution of histone gene transcripts. Cellular histone mRNA levels may also be regulated by an autonomous mechanism that determines histone mRNA stability through complexes of histone proteins with polysome-associated nascent histone polypeptides [Marzluff and Pandey, 1988; Morris et al., 1991; Peltz and Ross, 1987; Peltz et al., 1991; Stein and Stein, 1984; Zambetti et al., 1987, 1990]. There is also a growing appreciation for the complexities of transcriptional control operative in expression of histone genes [Bell et al., 1992; Bouterfa et al., 1993; Brignon and Chaubet, 1993; Fei and Childs, 1993; Khochbin and Wolffe, 1993; Ohtsubo et al., 1993; Palla et al., 1993; Stein et al., 1994; Sun et al., 1993; Wolfe and Grimes, 1993; Zahradka et al., 1993]. In proliferating mammalian cells, histone gene transcription continues throughout the cell cycle but is enhanced 2- to 5-fold at the G_1/S-phase transition point [Stein and Stein, 1984]. In contrast, histone gene transcription is downregu-

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lated at the onset of differentiation or during cell growth arrest in human and rodent cells [Bortell et al., 1992; Collart et al., 1988; Stein et al., 1989; van den Ent et al., 1993; Wright et al., 1992].

Both in vivo and in vitro experimental approaches have defined a modularly organized series of promoter regulatory sequences and cognate factors that determine transcriptional competency and cell cycle regulation of histone genes based on observations with a broad spectrum of cell culture systems [Stein et al., 1994; van *Wijnen et al., 1992b*]. Studies with transgenic mice have established that an H4 gene construct with 6.5 kB of 5′ regulatory sequences supports proliferation-dependent transcription during development [van Wijnen et al., 1991a]. In addition, we have established proliferation-dependent in vivo occupancy of key proximal cell cycle regulatory sequences in intact cells at single nucleotide resolution [Pauli et al., 1987; Ramsey-Ewing et al., 1994; Stein et al., 1989].

Stringent transcriptional control of histone gene expression in response to multiple cellular signalling mechanisms that mediate cell cycle and growth control require interaction of transcription factors with both proximal and distal regulatory elements. The spatial integration of these regulatory activities results in modulation of human histone gene transcription, and is paralleled by cell cycle related modifications in parameters of nuclear architecture [Chrysogelos et al., 1985, 1989; Dworetzky et al., 1992; Moreno et al., 1986, 1988; Pauli et al., 1989; Stein et al., 1994]. To account for the contribution of nuclear architecture, it is necessary to assess transcriptional regulation of histone gene expression in vivo within a chromosomal context, as fidelity of chromatin structure, nucleosome organization and higher order chromatin topology associated with gene-nuclear matrix interactions may be dependent on chromosomal integration.

In this study, we analyzed transcriptional control of histone genes by stable transfection of human H4 gene constructs containing intact or modified flanking sequences into murine C127 cell lines. Our data establish cross-species compatibility of rodent and human transcription factors and histone gene regulatory sequences, and reveal limited representation of distal and proximal transcription factors in vivo. Furthermore, cell line specific variations in H4 gene expression are consistent with contributions of chromosomal position effects and long-range spatial organization of the integrated gene copies.

MATERIALS AND METHODS

Construction of Stable Cell Lines

C127 mouse cells were cotransfected with 10 μg of each human histone H4 gene construct (Fig. 1), and 10 μg of pSV2neo via the $CaPO_4$ precipitation/glycerol shock method as described previously [Kroeger et al., 1987]. The cells were grown in Dulbecco's modified minimal essential medium containing 500 μg of G-418/ml for the selection of resistant cells. Resistant colonies were picked, expanded in 24 well Corning tissue culture plates, and subsequently passaged as clonal cell lines (monoclonals). The level of human H4 gene expression of each monoclonal cell line was determined by S1 nuclease analysis as previously documented [Kroeger et al., 1987]. The quantity of total cellular RNA in each sample was adjusted using values obtained by UV spectroscopy [Kroeger, 1988]. Transcriptional values were expressed as the ratio of the level of human H4 mRNA to the level of mouse H4 mRNA. The inclusion of an internal probe specific for the mouse H4 gene [Seiler-Tuyns and Paterson, 1987] permits normalization for the total amount of RNA in each S1 nuclease hybridization and provides a control for the amount of histone mRNA in each sample. The integrity and copy number of each gene construct was determined by Southern blot analysis of total genomic DNA from each cell line. Genomic DNA (10 μg) was cut to completion with EcoRI and XbaI, electrophoresed in a 1% agarose gel, and blotted onto a nylon membrane. The filter was hybridized at 68°C, for 20 h, with an EcoRI/XbaI fragment spanning the human H4 gene, which had been ^{32}P-labelled by nick-translation. The filter was washed three times, at room temperature, in 5 mM $NaPO_4$, pH 7.0, 1 mM EDTA, and 0.2% SDS, and then exposed to preflashed Kodak XAR-5 film at −70°C.

Characterization of In Vivo Protein/DNA Interactions by Dimethyl Sulfate Fingerprinting

In vivo protein/DNA interaction experiments were performed as described previously [Pauli et al., 1987]. In brief, the medium was removed from 10 cm culture plates of individual monoclonal cell lines containing the pFO003 construct and the cells were treated with 0.5% dimethylsulphate (DMS) in 2 to 3 ml of media

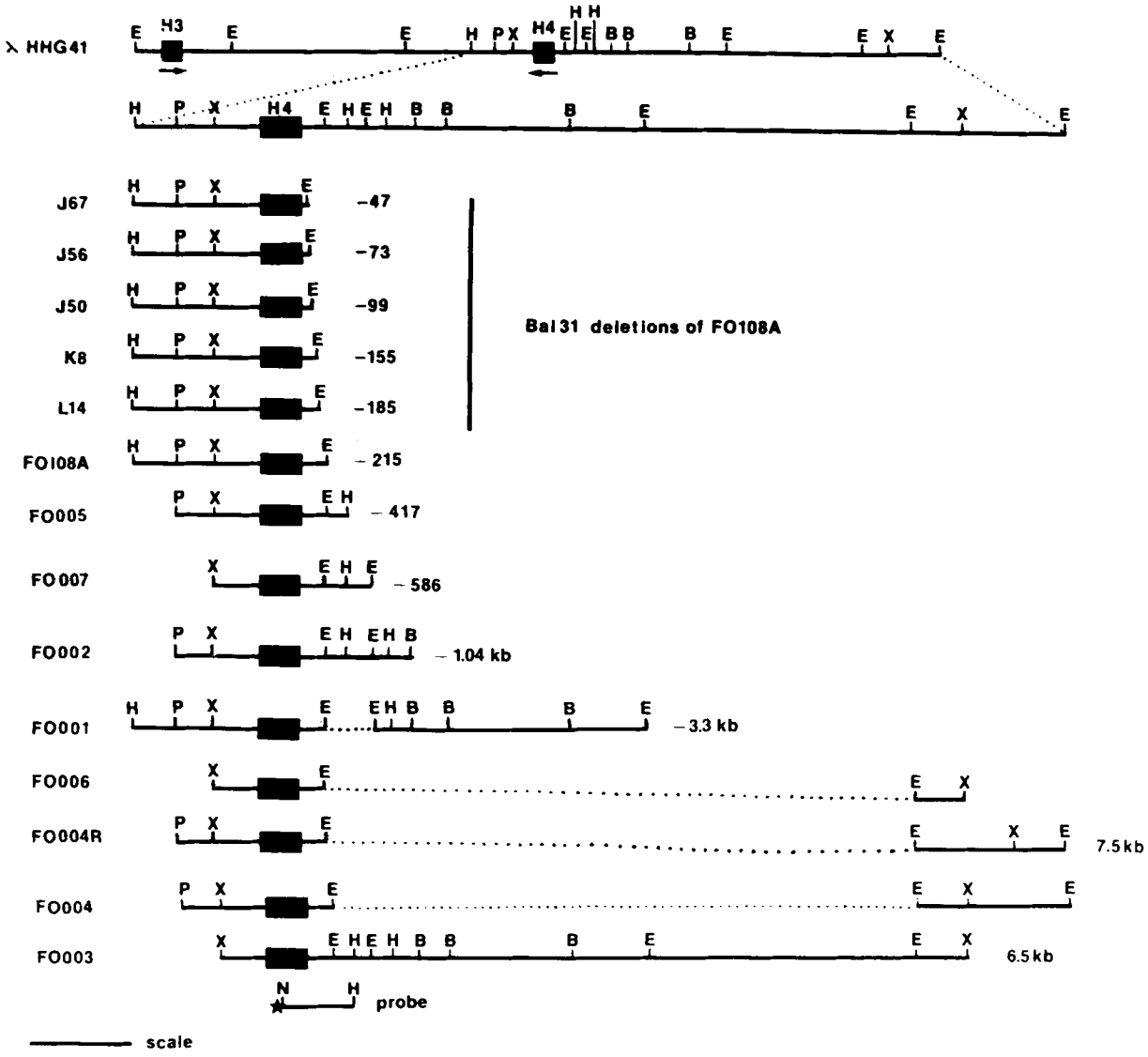


Fig. 1. Schematic diagram of human histone gene deletion constructs. The top of the drawing depicts the original lambda HHG41 clone used to generate the plasmid subclones listed below it. The distance from the end of the histone promoter sequence relative to the cap-site is indicated to the right of each construct. The scale at the bottom is 2 kB on the lambda HHG schematic and 1 kB on all others. Pertinent restriction sites are abbreviated as follows: EcoRI (E), BamHI (B), HindIII (H), XbaI (X), and NcoI (N). The most commonly used S1 nuclease probe, labelled at the NcoI site, is designated at the bottom of the figure.

for 1 or 2 min. After removal of DMS, the cells were washed twice in phosphate buffered saline with 60 mM Tris-HCl, pH 7.4, and scraped from the plate. The methylated DNA was purified, digested with Hinc II, and treated with piperidine. The chemical cleavage products were separated in 6% genomic sequencing gels, and electrotransferred to a nylon membrane. Hybridization was performed with the Hinc II 5′ upper strand probe [Pauli et al., 1987] at 65°C for 16 h, followed by eight washes at 65°C for 5 min in 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 1% SDS. The membrane was then exposed at −70°C to preflashed XAR-5 film.

RESULTS AND DISCUSSION

In Vitro Transcription and Protein/DNA Interactions in Histone Gene Regulatory Sequences

Promoter sequences and regulatory proteins that contribute to transcriptional control of a

representative human histone H4 gene (designated FO108) have been studied by a number of in vitro techniques [Sierra et al., 1983; van den Ent et al., 1993; van Wijnen et al., 1989, 1991b, 1992b; van der Houven van Oordt et al., 1992; Wright et al., 1992]. The interactions of proteins with distal and proximal H4 gene promoter elements have been analyzed at the molecular level using a number of different experimental approaches to determine sequence-specificity and functionality of regulatory binding events [Stein et al., 1994] (Fig. 2). As with many vertebrate transcription factors to date, histone gene promoter factors show evolutionary conservation and can be detected in proliferating cell lines derived from at least three distinct mammalian species (i.e., human, rat, and mouse). Furthermore, we have assessed correlations between the extent of H4 histone gene transcription and representation of cognate transcription factors in many distinct mammalian cell types [van Wijnen et al., 1989, 1991a, 1992b; van den Ent et al., 1993; Wright et al., 1992].

To assess the extent to which mouse C127 cells represent a suitable heterologous system for studying human H4 gene expression in vivo, we performed several experiments. Using cell free transcription analysis, we compared nuclear proteins from proliferating mouse C127 and human HeLa cells. In both cases, we observed that accurate cap site initiation of transcription occurs from human H4 histone gene templates and is sensitive to low levels of α-amanitin (1 μg/ml); furthermore, the relative efficiency of in vitro transcription, expressed per mg of protein, is similar between the mouse and human nuclear extracts (data not shown). These results establish that fidelity and efficiency of RNA polymerase II mediated H4 gene transcription in vitro are similar between mouse C127 and human HeLa cells. Also, the relative nuclear abundance of several transcription factors (i.e., SP-1, HiNF-D, HiNF-B, and HiNF-A) in mouse C127 cells is comparable to that of HeLa S3 cells [van Wijnen et al., 1988, 1992b]. These results are consistent with quantitative similarities in relative levels and transactivation potential of the heterologous mouse and human transcription factors in these two cell types. Taken together, in vitro transcription and protein/DNA interaction analysis of human HeLa S3 and mouse C127 cells suggests that mouse C127 cells represent a compatible heterologous system for functional studies on human H4 histone gene transcription in vivo.

In Vivo Transcriptional Analysis of Human H4 Gene Expression in Mouse C127 Monoclonal Cell Lines

To refine our understanding of the transcriptional regulation of histone genes, we have considered the involvement of both proximal and distal histone H4 promoter sequences. The in vivo properties of histone H4 gene (FO108) regulatory sequences have been established using transient expression assays, experiments with stably transfected episomal H4 genes and polyclonal cell lines containing chromosomally integrated H4 gene constructs, as well as transgenic mice experiments [Green et al., 1986; Helms et al., 1987; Kroeger et al., 1987; Ramsey-Ewing et al., 1994; van Wijnen et al., 1991a]. However, one of the key questions that has not been resolved in these studies is the extent to which gene copy number per cell and chromosomal integration influences the expression of both the test gene and the endogenous gene. One of our experimental approaches was to construct a systematic set of stable mouse cell lines that contain different numbers of gene-copies of a series of histone promoter deletion constructs (Fig. 1).

Figures 3 to 9 provide representative data from analysis of an extensive panel of monoclonal C127 mouse cell lines containing human H4 genes. Each cell line was characterized with respect to the level of expression of the integrated human H4 gene construct using S1 nuclease protection analysis and the gene copy number by Southern blot hybridization analysis (summarized in Table I). For example, we constructed a set of monoclonal cell lines containing the stably integrated human histone gene construct pFO003 (6.5 kB of 5′ flanking sequence) (Fig. 3). S1 nuclease protection analysis of total RNA from each cell line was performed by stringent solution hybridization simultaneously with two DNA probes of different lengths: one identical to the integrated human H4 histone gene, and the second specific for a representative cell cycle regulated mouse H4 gene [Seiler-Tuyns and Paterson, 1987]. The amounts of the two protected DNA probes represent a direct measure of human and mouse H4 mRNA levels that can be concurrently visualized on sequencing gels. The results for the pFO003 containing monoclonals show that each cell line expresses the integrated human H4 genes but that the

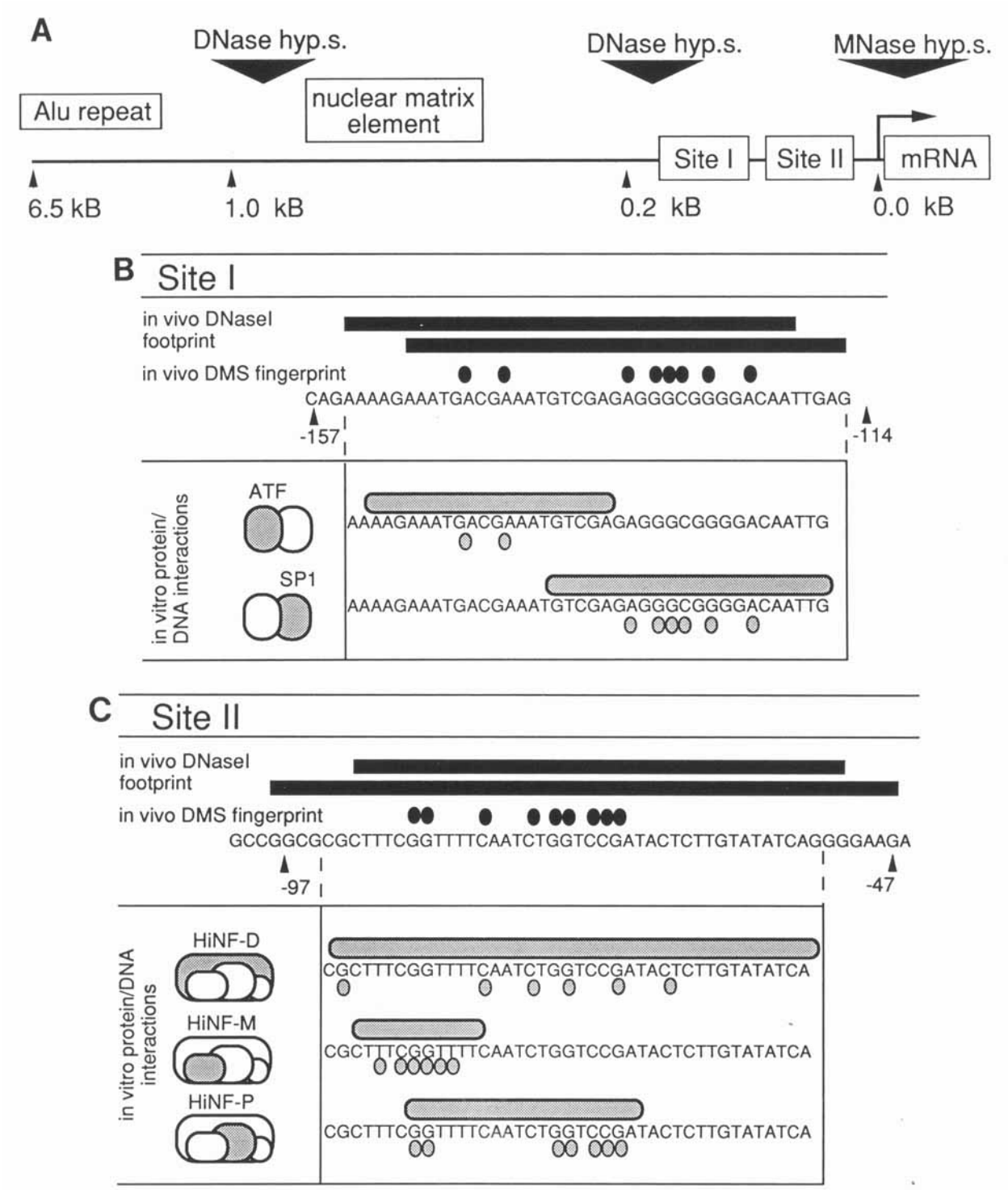


Fig. 2. Schematic representation of the human histone H4 gene designated FO108. **A:** Outline of the initial 6.5 kB of 5′ flanking sequence, which includes two in vivo protein/DNA interaction sites (Sites I and II) [Pauli et al., 1987], several DNase I and MNase I hypersensitive regions [Chrysogelos et al., 1985, 1989; Moreno et al., 1986, 1988], a putative nuclear matrix attachment region [Dworetzky et al., 1992; Pauli et al., 1989], as well as far upstream repetitive sequences (e.g., Alu repeat; [Collart et al., 1985]). **B** and **C** provide detailed descriptions of in vivo and in vitro protein/DNA interactions occurring at, respectively, Sites I and II. Indicated in B and C are in vivo DNase I footprints (thick black lines) and DMS fingerprints (black ovals), as well as the minimal binding domains (grey rounded boxes) and guanine contacts (grey ovals) of the cognate factors (i.e., ATF, SP1, HiNF-D, HiNF-M, and HiNF-P) that interact with these multi-partite binding domains in vitro [van Wijnen et al., 1992b; Stein et al., 1994].

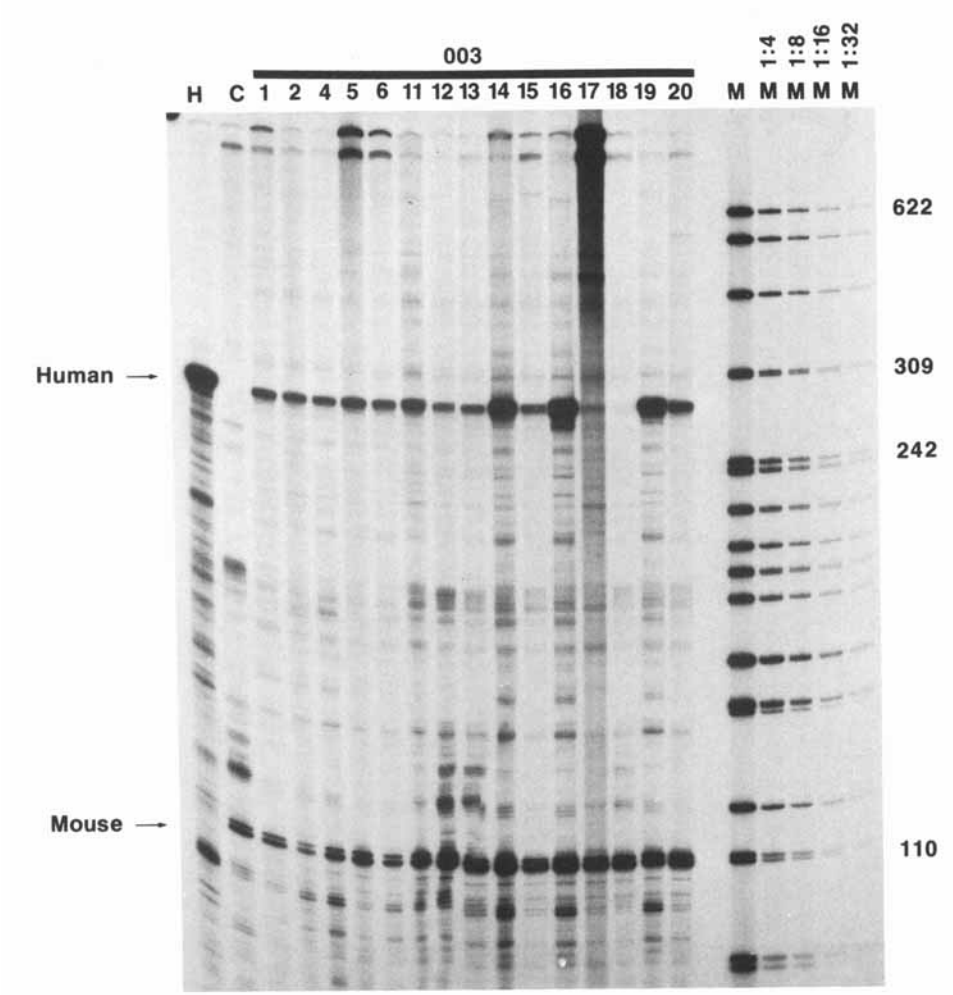


Fig. 3. Assessment of human H4 gene expression relative to the representation of mouse H4 gene transcripts in pFO003 monoclonal cell lines using S1 nuclease protection analysis. Shown is an autoradiogram of a sequencing gel analyzing the S1 nuclease protected products of both human and mouse H4 gene probes (indicated by arrows) in solution hybridization with RNA isolated from monoclonal cell lines. Each monoclonal was designated the term 003mX, with X representing the respective numbers indicated above the lanes. The correct locations of the protected human (280 nt) and mouse (110 nt) probes were established by size relative to a radio-labelled pBR322/HpaII marker (lane M), and by control reactions with human HeLa (**H**) and mouse C127 (**C**) RNA samples. Dilutions of the marker (last four lanes to the right) were loaded for densitometric purposes.

representation of these H4 mRNAs differs relative to that of the endogenous mouse H4 gene.

To correlate differences in human H4 gene expression levels relative to the integrity and number of gene copies integrated in each cell line, we performed Southern blot analysis. Chromosomal DNA from each cell line was digested by restriction enzymes, transferred to a solid support, and then hybridized with a probe that preferentially recognizes the human H4 gene (Fig. 4A). Quantitation of gene-copy number was calculated from densitometric comparisons of multiple autoradiographic signals representing both the integrated human H4 gene and standard quantities of a plasmid-derived restriction fragment spanning the H4 gene. In addition, the actual DNA quantities present on the Southern blot were normalized by subsequent hybridization with a probe for 18S ribosomal genes (Fig. 4B). The results for cell lines 003m17, 003m5, 003m15, and 003m1, for example, establish that they contain, respectively, 4, 13, 44, and 139 copies of the human H4 histone gene.

Comparison of H4 mRNA expression level and gene copy-number for this representative set of monoclonals indicates that when fewer than 40 copies of the human H4 gene are present per cell (e.g., construct pFO003), there is a positive correlation between gene-copy number and level of human H4 gene expression (Figs. 3 and 4; Table I). However, at high gene dosage (139 copies per cell) overall expression level is no

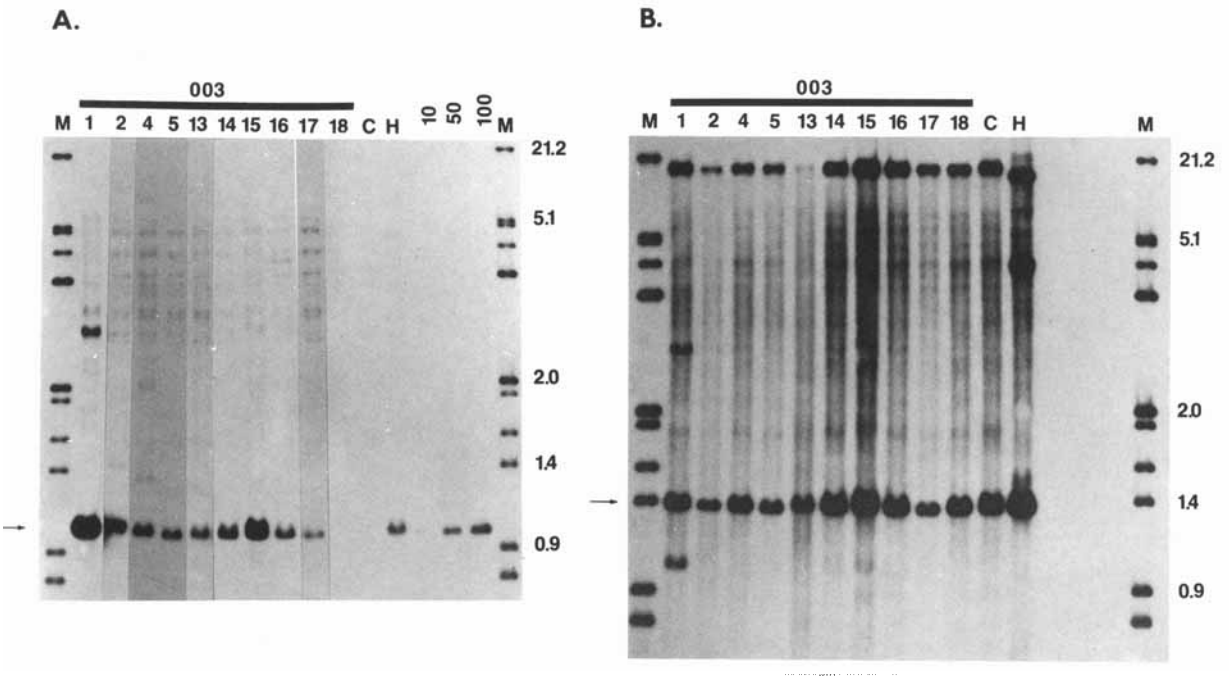


Fig. 4. Copy number determination of pFO003 monoclonal cell lines using Southern blot hybridization analysis. The autoradiogram in **A** shows the hybridization results of 10 μg chromosomal DNA (digested with EcoRI/XbaI) from each cell line with a nick-translated probe spanning the human H4 gene (1070 bp EcoRI/XbaI fragment of pFO002). Each monoclonal was designated the term 003mX, with X representing the respective numbers indicated above the lanes. Densitometric analysis of the genomic 1,070 bp band (indicated by the arrow to the left) was performed by comparison with signals obtained from exact quantities of H4 plasmid (quantities in pg depicted above the lanes) on several autoradiographic exposures. Genomic DNA from HeLa (lane H) and C127 (lane C) was run in parallel as a control for specific hybridization to the human H4 gene. Lane M represents the radio-labelled lambda HindIII/EcoRI marker. **B** shows the autoradiographic signals obtained after rehybridization with a probe homologous to mouse 18S ribosomal genes (indicated by arrow). This permits correction of densitometric values obtained in A for the amount of DNA present on the blot.

longer influenced by increasing copy-number. This result suggests there is a limit to the number of stably integrated copies of the human H4 gene that can be expressed.

The combined results obtained for a number of cell lines with different distal truncations of the human H4 promoter (Figs. 3 to 9, Table I, and data not shown), reveal two distinct effects that are independent of the length of promoter. First, cell lines containing the same construct and a similar number of intact human H4 gene copies show variations in the level of H4 expression (Table I). This indicates that chromosomal integration sites and long-range gene organization modulate H4 gene expression levels, similar to observations obtained with independent lines of transgenic mice [van Wijnen et al., 1991a]. Second, for constructs with at least 0.1 kB of 5′ flanking sequence (summarized in Table I, and Fig. 10), it appears that cell lines containing a low H4 gene copy number have a higher level of expression per integrated H4 gene than high copy number cell lines. Thus, these results suggest that stably integrated human H4 gene copies are in direct competition with each other for rate-limiting murine trans-activating factors.

The level of endogenous mouse H4 gene expression is typically higher than that of the integrated human H4 genes in low copy-number cell lines. However, in cell lines with high copy-numbers of the human H4 gene, we observed that mouse H4 gene expression was reduced to levels comparable to that of human H4 gene expression. Analysis of the relative amount of mouse H4 gene expression as a function of the number of human H4 gene copies (Fig. 11) shows that there is an inverse correlation between mouse H4 mRNA levels and human H4 gene copy-number. This copy-number dependent decline in endogenous mouse H4 gene expression suggests that the mouse H4 gene is in direct transcriptional competition with the human H4 gene. Our results are comparable to those obtained by Capasso and Heintz [1985] who also observed copy-number dependent effects on H4

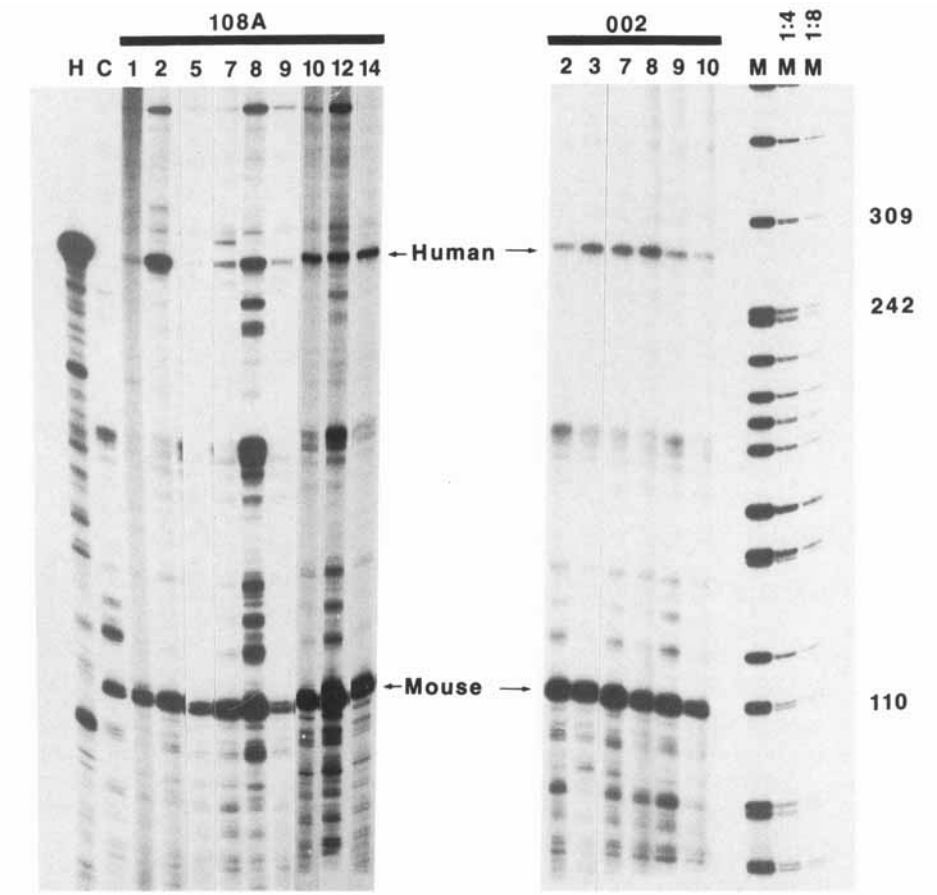


Fig. 5. S1 nuclease protection analysis monitoring expression of human and mouse H4 genes in monoclonal cell lines containing constructs pFO108A and pFO002. Indicated above the lanes are the abbreviated designations for each monoclonal cell line, as well as control S1 nuclease reactions (lanes H, C) and sizing markers (lane M) as outlined in the legend to Figure 3. The signals representing the level of human and mouse H4 mRNAs are indicated by arrows.

gene expression using a different human histone H4 gene transfected into mouse Ltk$^-$ cells.

Interestingly, all mouse monoclonal cell lines tested remained capable of expressing the mouse H4 gene, even in mouse cell lines containing up to 250 copies of the human H4 gene. This persistence of mouse H4 gene expression at very high H4 gene copy number implies that transcriptional competition between mouse and human H4 genes is unequal. This differential competition may be due in part to position effects: the mouse H4 gene in its native location within the genome is invariably located in a chromosomal context (or chromatin domain) functionally evolved for optimal regulation, whereas multiple exogenous human H4 genes may integrate randomly in both transcriptionally favorable and unfavorable locations. In addition, differential competition may reflect heterogeneity between distinct copies of the H4 multigene family and possible differences in promoter-strength based on observed variations in the distribution of histone gene promoter regulatory elements within individual histone gene copies [van Wijnen et al., 1992b].

Although expression of the human H4 gene was observed in most cell lines with constructs containing at least 0.1 kB of 5′ flanking sequence, accurately initiated human H4 gene transcripts were not detected in cell lines with the pJ67 construct, which contains less than 0.1 kB of 5′ flanking sequence [Kroeger et al., 1987]. We extended this observation by analyzing additional cell lines with this construct containing different numbers of copies of this construct (Fig. 9). These data show that construct pJ67 does not express even when multiple intact copies are integrated, although active expression of the endogenous mouse H4 gene is observed. Apart from the absence of distal auxiliary regulatory elements that contribute to the level of H4 gene transcription [Stein et al., 1994], the

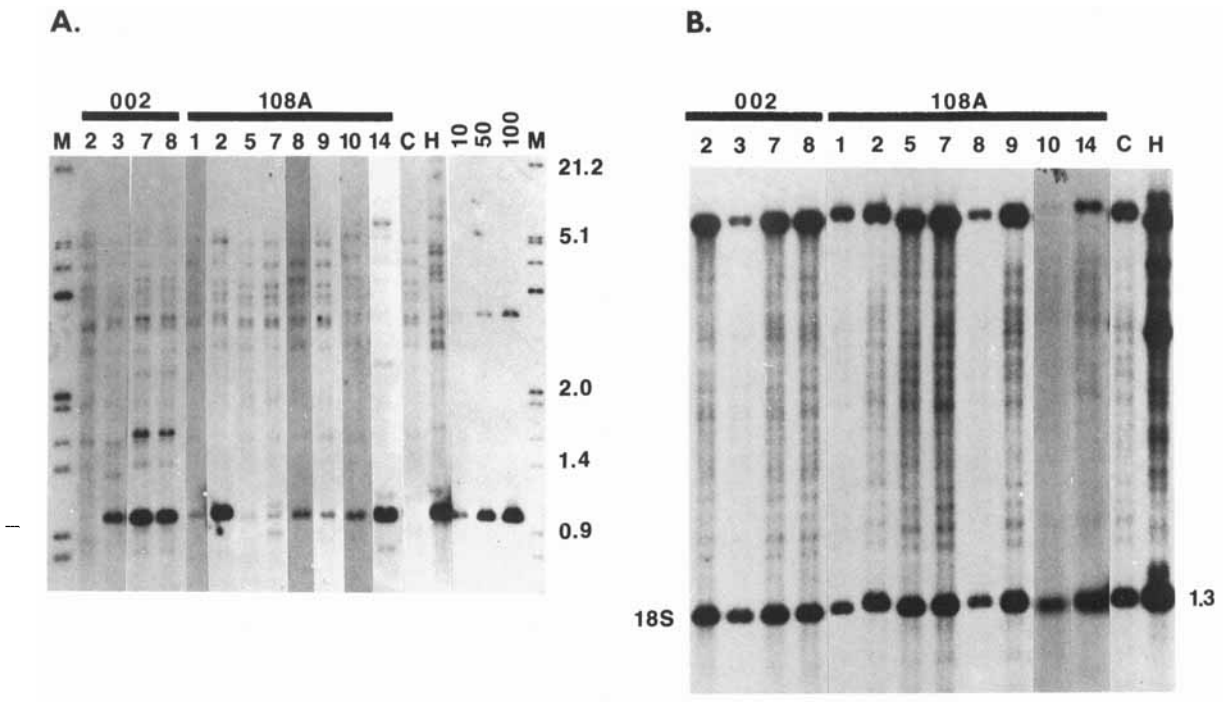


Fig. 6. Southern blot hybridization analysis establishing human H4 gene copy number in pFO108A and pFO002 cell lines. Indicated above the lanes are the abbreviated designations for each monoclonal cell line, as well as control hybridization reactions (lanes H, C) and sizing markers (lane M) as outlined in the legend to Figure 4. **A** shows the specific signal of the integrated human H4 gene obtained by hybridization with the human H4 gene probe (indicated by arrow) and **B** displays the signals obtained for the mouse 18S ribosomal gene (indicated by 18S).

truncated human H4 promoter present in pJ67 has a deletion of the cell cycle control region in the distal portion of site II that abolishes interactions of HiNF-M, HiNF-P, and HiNF-D with the H4 gene (Fig. 2). However, the pJ67 construct contains an intact TATA-box that has been shown to facilitate RNA polymerase II mediated transcription in cell free in vitro transcription systems [Sierra et al., 1983; van Wijnen et al., 1989]. Absence of H4 gene expression observed with the integrated human H4 gene pJ67 construct in vivo (Fig. 9) is consistent with the concept that histone gene specific DNA binding activities interacting with distal site II are absolutely required for cap-site specific initiation of transcription in vivo.

In Vivo Protein/DNA Interactions at the Proximal Human H4 Gene Promoter in Mouse C127 Monoclonal Cell Lines

Using in vivo genomic sequencing we have previously established occupancy of the H4 promoter in the intact cell at two protein/DNA interaction domains designated site I (nt −150 to −117) and site II (nt −91 to −50) [Pauli et al., 1987; see Fig. 2]. To directly examine occupancy at these sites in the human H4 gene promoter when integrated in the heterologous mouse C127 cell, we performed a series of genomic fingerprinting experiments with monoclonal cell lines that contain construct pFO003 (Fig. 12). Construct pFO003 has extensive 5′ flanking sequences (6.5 kB), is cell growth regulated in transgenic mice [van Wijnen et al., 1991a], and encompasses both proximal and distal DNase I hypersensitive sites observed for the native gene in human HeLa cells [Chrysogelos et al., 1985, 1989; Moreno et al., 1986, 1988]. We assessed whether the same protein/DNA contact points were present in mouse and human cells, as well as the extent to which factors were titratable by competition with increasing copy number of the introduced human H4 histone gene.

In vivo methylated DNA from several DMS treated cell lines was analyzed at single nucleotide resolution by genomic sequencing. Hybridization was carried out with the 5′ HincII upper strand probe (Fig. 12), which monitors the upper strand of the H4 promoter containing the majority of guanine protein-DNA contacts established in vivo. The results indicate that in a cell line with low copy number, pFO003m5 (13 cop-

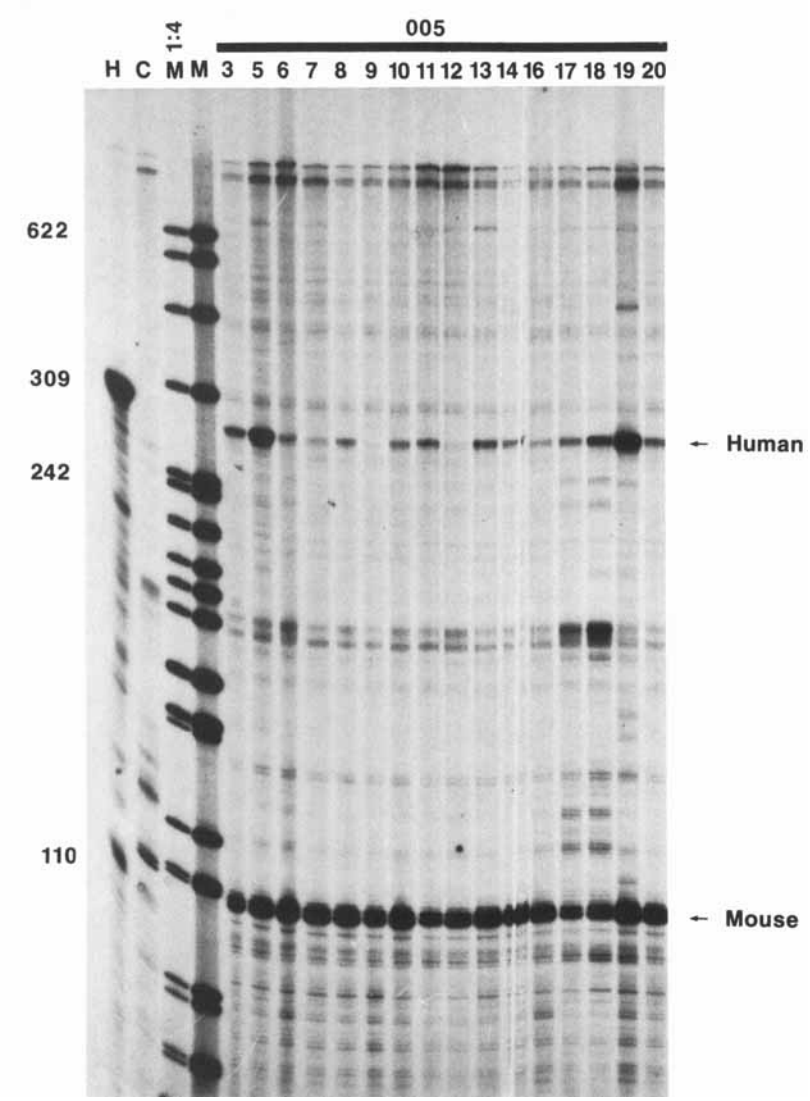


Fig. 7. S1 nuclease protection analysis monitoring expression of human and mouse H4 genes in monoclonal cell lines containing construct pFO005. The signals representing the level of human and mouse H4 mRNAs are indicated by arrows. Abbreviations above the lanes are outlined in the legend to Figure 3.

ies), a significant portion of the genes have protein bound to site I (G residues −123, −125, −126, and −127 are protected in vivo) (Fig. 12, lane 5). When the copy number of the cell line increases to 40, pFO003m6, there is still protein/DNA interaction detectable at site I (Fig. 12, lane 4). Finally, when 139 copies of the human histone gene are present, pFO003m1, there is no detectable protein interaction at site I (Fig. 12, lane 3).

The observation that in vivo protein occupancy is not detectable at high gene dosage is consistent with dispersion of factors between multiple identical copies within the same cell. The detection limit of genomic sequencing is subject to the following considerations. Simultaneous occupancy in the intact cell of at least 90% of a given binding site for all integrated H4 histone gene copies will result in a 10-fold reduction of guanine band intensity. In this case a clear protein/DNA contact is displayed in a genomic fingerprint. Lower levels of occupancy yield in vivo fingerprints with corresponding responsiveness (i.e., gained intensity of the G-residue signals). At a threshold level of occupancy (e.g., less than 50%) definitive interpretation of in vivo fingerprints is compromised. Additional factors that may contribute to minimal detectability of in vivo occupancy include site of integration and proximity to active or

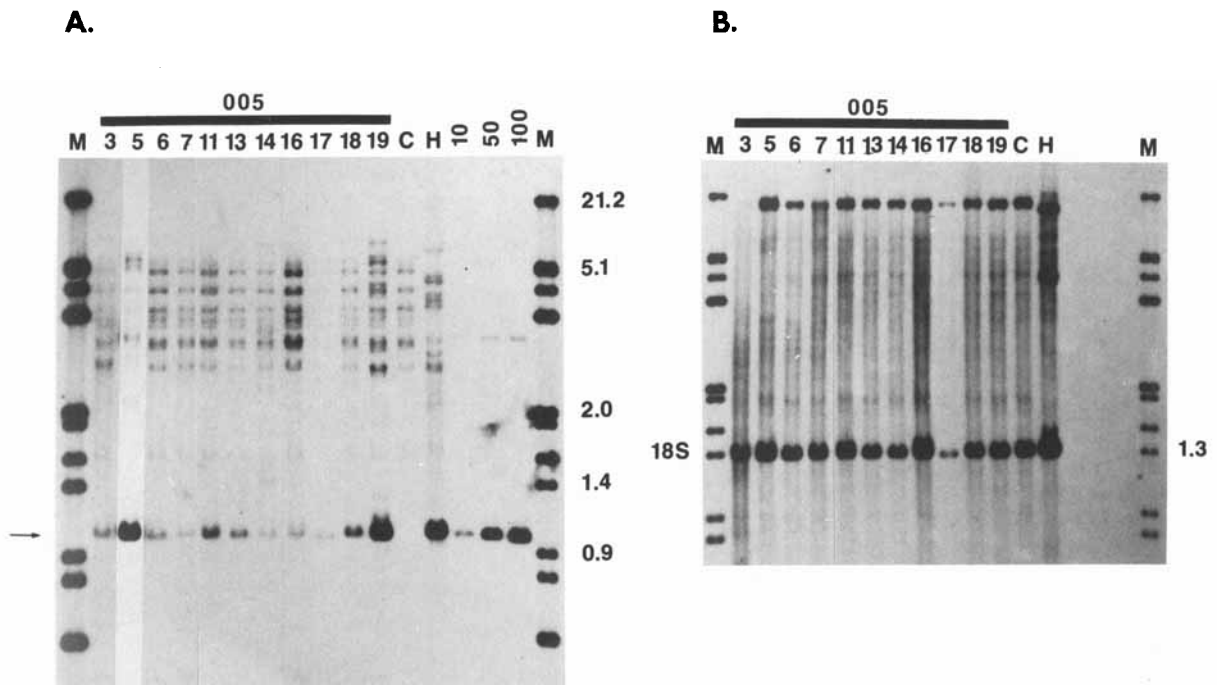


Fig. 8. Southern blot hybridization analysis establishing human H4 gene copy number in pFO005 cell lines. **A** shows the specific signal of the integrated human H4 gene (indicated by arrow) and **B** displays the signals obtained for the mouse 18S ribosomal gene (indicated by 18S). Abbreviations above the lanes are outlined in the legend to Figure 4.

inactive genes, as well as structural and spatial parameters imposed by genomic domains responsible for higher order chromatin structure. Furthermore, the dynamics of protein/DNA and protein/protein interactions may influence detection of in vivo contacts. Local concentrations of transcription factors within the nucleus or in the proximity of specific genomic domains may directly affect DNA binding affinities and/or cooperative protein/protein interactions. Signals that reflect cell cycle stage-specific interactions (e.g., during S phase) may be obscured because our studies were performed with exponentially growing (i.e., not synchronized) C127 cells.

It should be noted that site I encompasses functional binding sites for two ubiquitous transcription factors: SP-1 interacts with the proximal portion and members of the ATF family of transcription factors interact with the distal portion (Fig. 2). Thus, comparison of high and low copy number monoclonal cell lines (i.e., monoclonals pFO003m1 and pFO003m5) shows that addition of more than one hundred binding sites results in the titration of these general transcription factors to the extent that they are no longer detected by genomic DNaseI footprinting, with the number of unbound site I sequences apparently exceeding those that are bound in the high copy number cell line.

As a control for the location and occupancy of both sites I and II, we treated HeLa cells, early in S phase, with DMS in parallel with C127 cells and subjected both to the genomic fingerprinting protocol. Interestingly, there is no apparent occupancy of the human H4 histone gene at site II in C127 mouse cells, although the endogenous human H4 histone gene in human HeLa cells exhibits occupancy at sites I and II as previously reported [Pauli et al., 1987]. However, it is clear that site II performs a key regulatory function in C127 cells because deletion of the distal portion of Site II abolishes transcriptional initiation at the human H4 mRNA cap-site (Fig. 9). Thus, it is possible that differences in the detection of occupancy at sites I and II in C127 are quantitative. This raises the possibility that the histone gene specific site II binding proteins are an order of magnitude less abundant than the ubiquitous factors interacting with site I in C127 cells. Alternatively, accessibility of site II transcription factors to the exogenous human H4 gene in mouse C127 cells may be restricted due to the chromosomal location(s) where the gene is integrated.

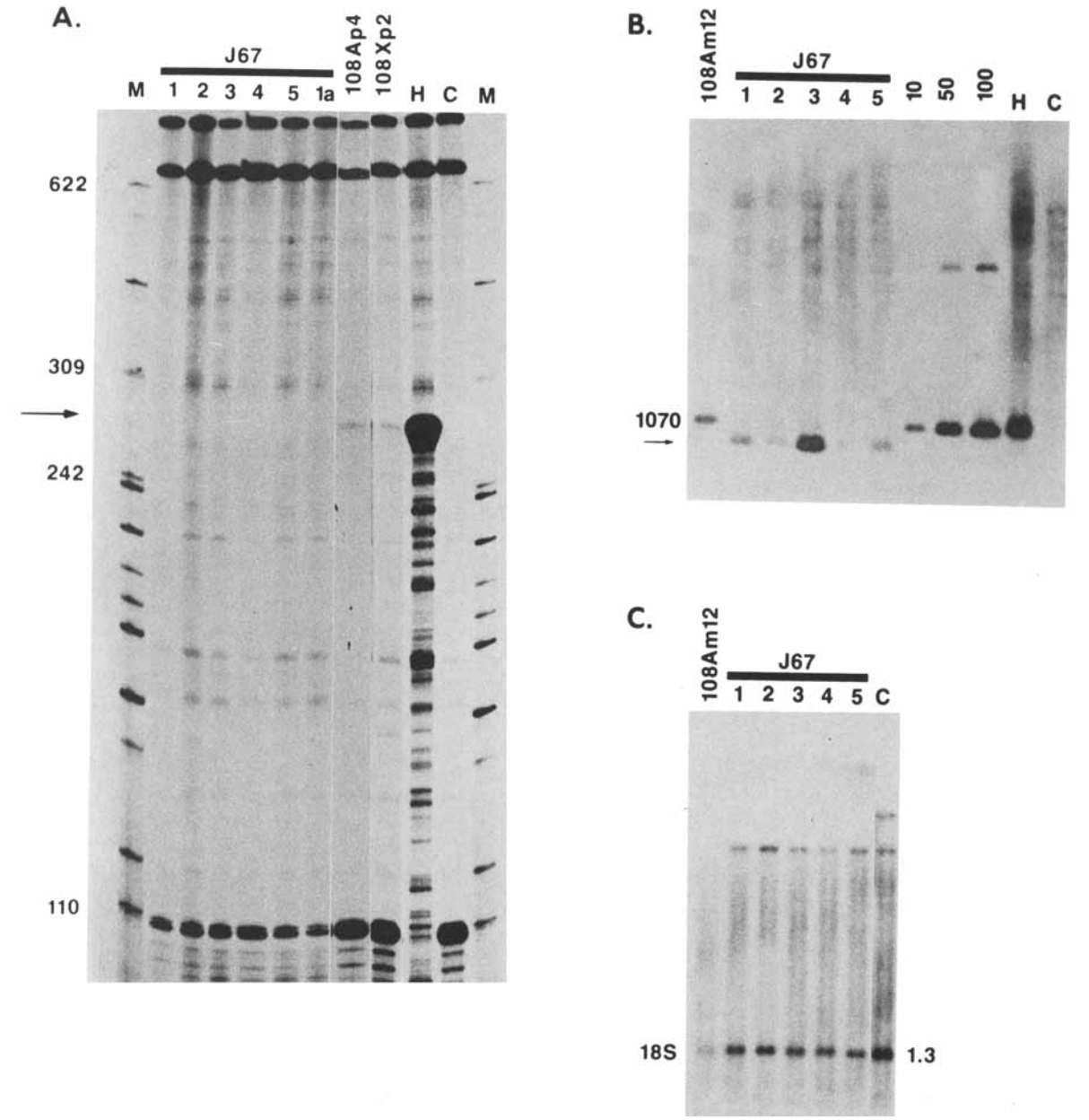


Fig. 9. S1 nuclease protection analysis of pJ67 polyclonal cell lines showing absence of correct cap-site initiation of human H4 mRNAs in vivo with construct pJ67. **A** shows S1 nuclease analysis of 25 μg total cellular RNA from six pJ67 polyclonal cell lines (lanes 1–5 and 1a). As positive controls, results are shown for the polyclonal cell lines pFO108Ap4 and pFO108Xp2, as well as untransfected HeLa (In H) and mouse C127 (In C) cells. Lane M represents the radio-labelled pBR322/HpaII molecular weight marker. The location of the protected fragment of the human H4 probe (observed only in lanes 108Ap4, 108Xp2 and H) is indicated by the arrow. **B** and **C** show Southern blot analysis for copy-number determination of the integrated pJ67 construct (B) relative to the mouse 18S ribosomal gene (C) as outlined in Figure 4. B shows the copy-number for J67 polyclones 1 to 5 and polyclone FO108Am12, as indicated above the lanes. The position of the truncated EcoRI/XbaI fragment of pJ67 is indicated by the arrow. Consistent with the size of the promoter deletion in pJ67, the pJ67 derived fragment runs faster than the full length 1,070 bp EcoRI/XbaI fragment which is observed in lanes 108m12 and H. No specific EcoRI/XbaI fragment is observed for genomic DNA from untransfected mouse C127 cells (lane C). Different amounts of the corresponding restriction fragment spanning the human H4 gene derived from plasmid DNA (lanes 10, 50, and 100; representing pg DNA) was included for quantitation purposes. C shows the blot of B reprobed for the 18S mouse ribosomal fragment (1.3 kB) to normalize copy-number for the amount of DNA transferred onto the blot.

TABLE I. Quantitation of Monoclonal Cell Line Expression*

Clone	Exp	CN	Exp/CN	Clone	Exp	CN	Exp/CN
K8m13	9.1	1	9.1	005m14	45.0	1	45.0
K8m17	18.0	1	18.0	005m16	31.0	1	31.0
K8m14	17.0	1	17.0	005m7	12.0	1	12.0
K8m18	3.0	5	0.6	005m11	38.0	2	19.0
K8m8	3.2	13	0.2	005m6	16.0	2	8.0
K8m9	18.0	28	0.2	005m3	78.0	2	39.0
Avg	11.4	8	7.5	005m13	97.0	3	32.3
Std	6.6		7.7	005m18	81.0	5	16.2
				005m17	43.0	5	8.6
108Am7	12.3	1	12.3	005m19	63.0	22	2.9
108Am1	1.3	1	1.3	005m5	128.0	31	4.1
108Am5	5.6	1	5.6	Avg	54.6	6	20.1
108Am9	21.0	1	21.0	Std	35.4		14.0
108Am10	11.0	1	11.0				
108Am12	14.3	4	3.6	108Xm2	8.6	1	8.6
108Am8	41.0	4	10.3	108Xm3	2.1	1	2.1
108Am2	94.0	19	4.9	108Xm9	2.1	1	2.1
108Am14	24.0	30	0.8	108Xm5	22.6	2	11.3
Avg	24.9	7	7.9	108Xm6	20.8	30	0.6
Std	26.8		6.1	Avg	11.2	7	4.9
				Std	8.9		4.2
002m9	3.6	ND	—				
002m10	13.5	ND	—	004m6	7.5	1	7.5
002m2	13.5	1	13.5	004m14	25.0	1	25.0
002m3	17.5	7	2.5	004m2	5.0	11	0.5
002m8	17.9	14	1.3	004m8	238.0	38	6.3
002m7	8.4	16	0.5	004m11	12.5	90	0.1
Avg	12.4	10	4.5	004m10	17.5	154	0.1
Std	5.0		5.3	004m19	540.0	188	2.9
				004m1	127.0	252	0.5
003m17	10.0	4	2.5	Avg	122.0	92	5.4
003m13	30.0	8	3.8	Std	176.0		7.9
003m4	180.0	10	18.0				
003m5	50.0	13	3.8	007m1	23.4	ND	—
003m14	177.0	21	8.4	007m2	20.8	ND	—
003m16	678.0	23	29.1	007m4	14.2	ND	—
003m2	650.0	41	15.9	007m8	3.4	ND	—
003m15	40.0	44	0.9	007m9	13.5	ND	—
003m1	250.0	139	1.8	007m10	12.3	ND	—
Avg	228.6	34	9.4	007m12	95.7	ND	—
Std	243.3		9.1	Avg	26.1		
				Std	31.3		

*The data displayed here summarize the relationship between levels of expression and copy-number for each monoclonal cell line. Autoradiograms of S1 nuclease protection and Southern blot hybridization analyses were scanned by laser densitometry as described by Kroeger [1988]. The left columns refer to the construct used for each cell line, and the specific clone designation (Clone). The second column from the left in each case shows the relative expression of human H4 mRNA (Exp). Human H4 gene expression was normalized relative to the amount of mouse H4 mRNA, and represents the ratio of human and mouse S1 signals (arbitrarily multiplied by 100). The second column from the right in each case provides the copy-number for each cell line (CN). The copy number was quantitated by densitometry of several autoradiographs of Southern blots as described in Figure 4. Included in the copy-number determinations were plasmid controls of precise amounts that served as standards for each calculation. The right columns show the expression per copy for each cell line, which was calculated by dividing relative expression by gene copy-number (EXP/CN). For each construct, the average relative expression and average expression per copy (Avg), as well as the corresponding standard deviations (Std), are indicated below the corresponding cell line data-sets.

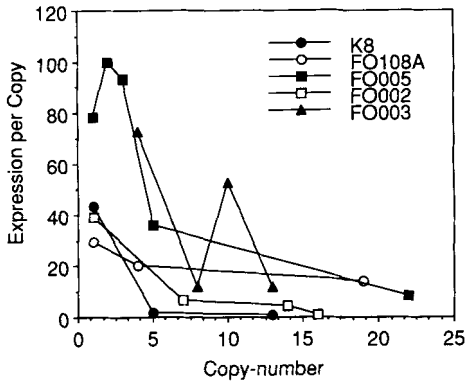


Fig. 10. Copy-number dependent expression of the human H4 gene stably integrated into mouse C127 cells. The effect of human H4 gene copy-number in cell lines on the expression of the human H4 gene was analyzed by plotting the average expression per H4 copy versus the cell line H4 gene copy-number using data from Table 1. The average expression per copy for monoclonals with the same copy-number was pooled and represented as a single point, whereas the remainder of points represent values for single monoclones. The results for each construct (i.e., K8, FO108A, FO005, FO002, and FO003) are indicated by separate connecting lines with corresponding symbols (see internal legend).

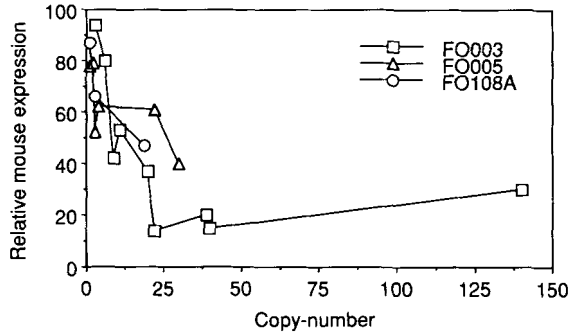
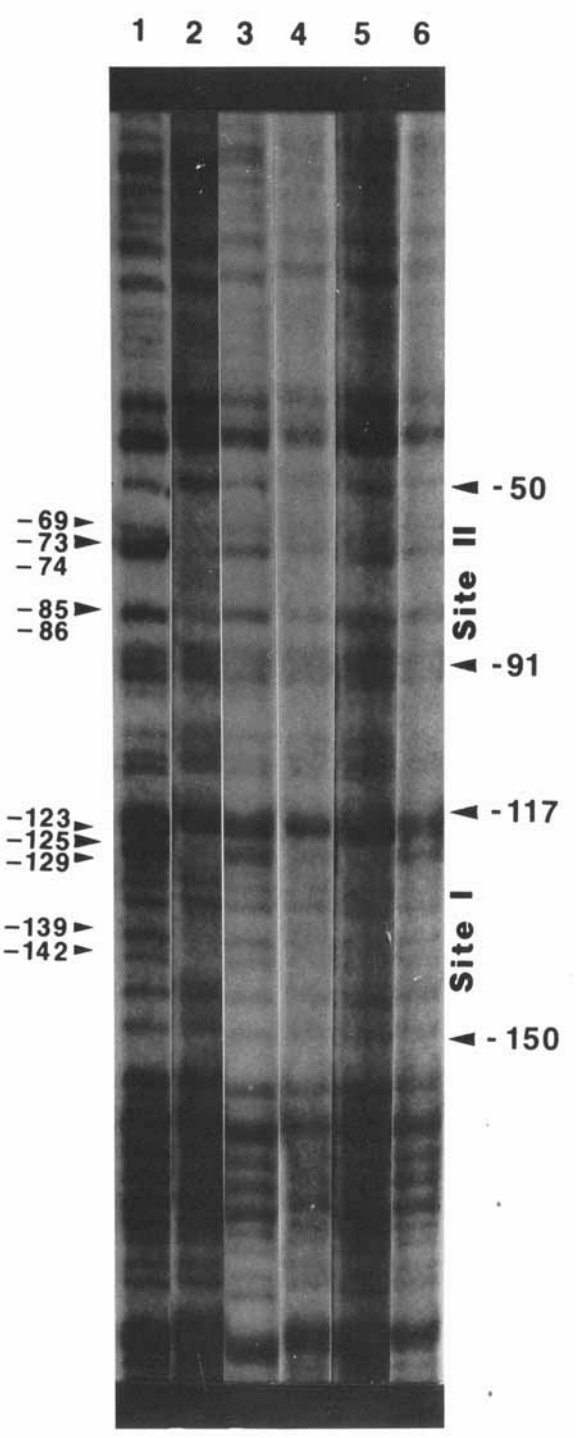


Fig. 11. The level of expression of the endogenous mouse H4 gene is dependent on the copy-number of the integrated human H4 gene. S1 nuclease protection data for several cell lines were analyzed by densitometry. The relative expression of the mouse H4 gene was expressed as the percentage of the densitometric values for mouse H4 mRNA relative to the total value of mouse and human H4 mRNA. Relative mouse H4 gene expression was plotted as a function of human H4 gene copy-number for each construct (i.e., FO003, FO005, and FO108A) (see internal legend).

CONCLUSIONS

The results presented in this study have several implications. First, we have analyzed regulation of histone gene transcription with both heterologous mouse [this work; Kroeger et al., 1987; van Wijnen et al., 1991a] and homologous human [Helms et al., 1987; Sierra et al., 1983; Ramsey-Ewing et al., 1994; van Wijnen et al., 1989; Wright et al., 1992] experimental cell systems by the combined application of both in vitro and in vivo transcriptional analyses, as well as in vitro and in vivo protein/DNA interaction assays. Results from promoter binding studies demonstrate that all histone gene transcription factors characterized to date are detectable in cells from at least three distinct mammalian



species (mouse, rat, and human) [van Wijnen et al., 1988, 1989, 1992a,b; van den Ent et al., 1993]. Furthermore, irrespective of the species in which studies were performed, both in vitro and in vivo functional assays show identity in cap-site utilization for transcriptional initiation, and show strong similarities in the importance of histone gene regulatory elements [Kroeger et al., 1987; van Wijnen et al., 1989; Wright et al., 1992; Ramsey-Ewing et al., 1994]. We conclude that there is extensive functional cross-species compatibility between histone gene regulatory sequences and transcription factors in mammalian species.

Second, we have carefully determined the levels of human H4 gene expression in over 60 distinct mouse C127 cell lines, and correlated this with intactness and gene-copy number of the integrated gene. We observed in vivo differences in histone gene transcription in monoclonal cell lines with a similar number of human H4 gene copies, suggesting that the chromosomal integration site, and, by extension, the long-range spatial organization of histone genes, influences the level of histone gene expression. Furthermore, there appears to be a relationship between the number of copies of the human H4 gene present and the level of expression per copy of the gene. In vivo genomic fingerprinting studies show that human H4 gene promoter occupancy is decreased with increasing gene-copy number. Taken together, these results support the concept that there are a maximum number of H4 genes that can be appropriately regulated for expression.

Copy-number dependent levels of H4 gene transcription reflect in vivo competition for key transcriptional components. Competition may directly influence availability of rate-limiting transcription factors, which mediate specific protein/DNA and/or protein/protein interactions. The synergism and antagonism of activities interacting at spatially arranged arrays of distinct multipartite promoter regulatory elements, as well as the multisubunit composition of cognate transcription factors, may affect cooperativity and the distribution of activities among multiple transcription units as copy number of the histone gene varies. However, further considerations are the effects of in vivo competition on parameters of nuclear architecture, including nucleosome organization, chromatin structure, and gene/nuclear matrix interactions, as well as on catalytic components of transcriptional control. Thus, although in vivo competition may impinge on availability of histone gene transcription factors, we propose that multifactorial interdependent mechanisms ultimately determine the copy-number related competency to transcribe human H4 genes in mouse C127 cells.

Fig. 12. Copy-number dependent in vivo occupancy of promoter elements of the human H4 gene in mouse C127 cells. DNA from several cell lines was analyzed by genomic sequencing with the 5′ HincII upper strand probe [Pauli et al., 1987] which monitors the sense-strand of the H4 promoter. **Lane 2** is a control lane showing the location of Site I and Site II in HeLa cells (derived from synchronized cells progressing through early S phase). All G residues exhibiting protection in HeLa cells are noted on the side of the figure (large arrows indicate that several G residues are protected; small arrows indicate protection of a single G residue) (see Fig. 2 for diagram). Genomic DNA from HeLa cells was processed in parallel with genomic DNA from the pFO003 cell lines (**lanes 3–5**). Lanes 3 to 5 contain genomic DNA from, respectively, pFO003ml (139 copies), pFO003m6 (40 copies), and pFO003m5 (13 copies). **Lanes 1** and **6** contain, respectively, pFO003 plasmid DNA and deproteinized Hela DNA, which were DMS treated as a control for the expected sequencing pattern of the G residues.

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