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Displacement of Sequence-Specific Transcription Factors from Mitotic Chromatin

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Summary

The general inhibition in transcriptional activity during mitosis abolishes the stress-inducible expression of the human *hsp70* gene. Among the four transcription factors that bind to the human *hsp70* promoter, the DNA-binding activities of three (C/EBP, GBF, and HSF1) were normal, while Sp1 showed reduced binding activity in mitotic cell extracts. In vivo footprinting and immunocytochemical analyses revealed that all of the sequence-specific transcription factors were displaced from promoter sequences as well as from bulk chromatin during mitosis. The correlation of transcription factor displacement with chromatin condensation suggests an involvement of chromatin structure in mitotic repression. However, retention of DNase I hypersensitivity suggests that the *hsp70* promoter was not organized in a canonical nucleosome structure in mitotic chromatin. Displacement of transcription factors from mitotic chromosomes could present another window in the cell cycle for resetting transcriptional programs.

Introduction

The global inhibition of transcriptional activity at the mitotic stage of the eukaryotic cell division cycle was established more than 30 years ago by autoradiographic studies that demonstrated a deficient incorporation of radiolabeled RNA precursors during mitosis (Taylor, 1960; Prescott and Bender, 1962; Littau et al., 1964; Johnson and Holland, 1965). Surprisingly, little is known of the mechanisms that govern transcriptional repression in this stage of the cell cycle. Current investigations in the mitotic repression of transcription have elucidated repressive mechanisms involving modification of components of the transcriptional machinery. Mitotic phosphorylation of the homeodomain of the Oct-1 transcription factor leads to inhibition of its DNA-binding activity (Roberts et al., 1991; Segil et al., 1991), and mitotic phosphorylation of a component of the RNA polymerase III transcription initiation factor TFIIIB suppresses transcription of the 5S and tRNA genes (Hartl et al., 1993; Gottesfeld et al., 1994). It is unclear whether inhibition by mitotic phosphorylation is a mechanism shared among the large set of basal and sequence-specific factors involved in the initiation of transcription. Mitotic repression in Drosophila has also been shown to involve the abortion of nascent transcripts, which precludes the expression of large regulatory genes in the rapid cell division cycles of the early embryo (Shermoen and O'Farrell, 1991; Rothe et al., 1992). The mechanism by which nascent RNAs are aborted in mitosis is unknown.

In our studies of the regulation of the heat shock response, we have confirmed that the transcriptional induction of the human *hsp70* gene is abolished in HeLa cells synchronized or arrested in mitosis. By analysis of the constitutive and inducible transcription factors that bind to the human *hsp70* promoter, we found that one factor, Sp1, showed an inhibition of specific DNA-binding activity in mitotic cell extracts. No decrease of DNA-binding activity could be found for the constitutively active CCAAT enhancer–binding protein (C/EBP) and G_n-binding factor (GBF). Also, the DNA-binding activity of the human heat shock transcription factor HSF1 could be induced upon heat shock of mitotic cells. Although these DNA-binding activities were retained during mitosis, genomic footprinting and immunocytochemical analyses revealed that the sequence-specific transcription factors were nonetheless displaced or excluded from the human *hsp70* promoter and from bulk chromatin in vivo. Our results suggest a possible mechanism for mitotic repression of the *hsp70* gene that involves the displacement of transcription factors from mitotic chromatin. The displacement mechanism appears to be distinct from competition between sequence-specific factors and a canonical nucleosome structure and may be related to the mechanisms that govern the condensation of chromatin during mitosis.

Results

Induction of Human *hsp70* mRNA Is Abolished in Mitotic Cells

In agreement with the general repression of transcription observed during mitosis, the induction of human *hsp70* mRNA was abolished in HeLa cells arrested at the onset of mitosis by treatment with nocodazole, an inhibitor of microtubule assembly (Figure 1A). By contrast, an approximately 7-fold induction of the steady-state level of *hsp70* mRNA was observed when asynchronous HeLa cells were heat shocked. The inducibility of *hsp70* mRNA was also abolished in mitotic HeLa cells synchronized by means of a double-thymidine block, but could be restored in the G1 phase, after cells were released from mitotic arrest (Figure 1A).

The inability to induce the expression of *hsp70* as part of the general mitotic repression leads to increased sensi-

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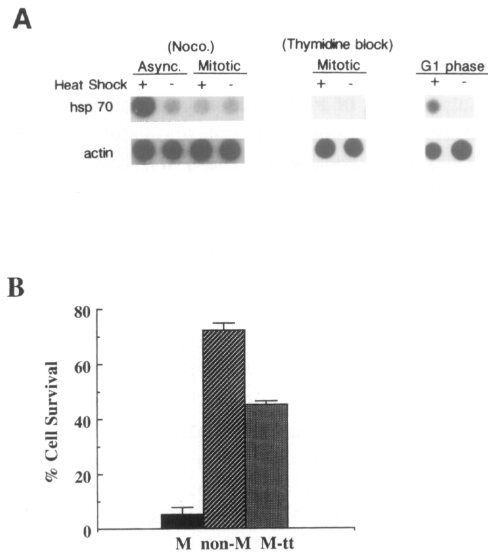


Figure 1. Induction of the Human *hsp70* Gene Is Abolished in Mitosis

(A) Dot blot analysis of *hsp70* RNA levels in asynchronous, mitotic, and G1 phase HeLa cells, before (minus) and after (plus) heat shock for 30 min at 43°C. HeLa cells were arrested in mitosis by treatment with nocodazole (Noco) or synchronized with a double-thymidine block; mitotic cells were collected by selective detachment. RNA from approximately 2.5 × 10^5 cells was loaded on each dot. The same filters were stripped and rehybridized with a β-actin cDNA clone as a control for the amount of RNA loaded.

(B) Thermosensitivity of mitotic cells. HeLa cells arrested in mitosis by treatment with nocodazole and harvested by selective detachment (M), the residual nonmitotic population (non-M), and a similar mitotic population rendered thermotolerant by pretreatment for 0.5 hr at 42°C, 5 hr prior to mitosis (M-tt) were subjected to an acute heat shock at 43.5°C for 1 hr and analyzed by the colony formation assay. Note that the treatment with nocodazole was responsible for part of the thermosensitivity, as the survival for untreated, asynchronous cells was 93% (data not shown). The number of colonies formed after the acute heat shock was normalized to the number formed without heat stress.

tivity of mitotic cells to elevated temperatures. As shown by the colony formation assay, an acute heat shock (43.5°C) of mitotic HeLa cells arrested by nocodazole treatment resulted in 5% cell survival, while 72% survival was observed in a similarly treated, nonmitotic population (Figure 1B). When HeLa cells were rendered thermotolerant by pretreatment at a lower temperature (42°C) prior to mitosis, the mitotic cell survival increased from 5% to 45%. The enhanced thermosensitivity of mitotic cells appears to be due, in part, to a failure of induction of heat shock genes during mitosis.

Mitotic Disruption of Transcription Factor-DNA Contacts In Vivo

To investigate the mechanisms responsible for the mitotic repression of heat shock gene expression, we analyzed the in vivo interactions of sequence-specific transcription factors for the human *hsp70* promoter by genomic footprinting. HeLa cells were treated with the DNA alkylating reagent dimethyl sulfate (DMS), and the positions of DMS reactivity were determined using the ligation-mediated polymerase chain reaction (PCR) technique (Mueller and Wold, 1989; Garrity and Wold, 1992). In agreement with previous studies performed on asynchronous HeLa cells (Abravaya et al., 1991a, 1991b), we observed protection from or hypersensitivity to DMS methylation at G residues in comparison with the methylation pattern on free DNA. The changes in the methylation pattern occurred on the coding strand at the following locations: the distal GC box (−174 to −172, −170, −168, and −167), the proximal GC box (−48 to −43 and −41), and the CCAAT box (−64 and −63) (Figure 2A). We also detected a previously unrecognized methylation protection at three G's (−135 to −133) in a G array (G_n sequence). In unshocked HeLa cells, no genomic footprinting was detectable over the consensus nGAAn inverted repeats comprising both proximal and distal heat shock regulatory elements (HSEs). Following heat shock, changes in DMS reactivity revealed the induced binding of human HSF1 to the HSEs (Figure 2A).

In contrast with the in vivo footprints observed for asynchronous cells, genomic footprinting of HeLa cells arrested at mitosis showed the abolition of methylation protection or enhancement at the GC boxes, the CCAAT box, and the G_n sequence (Figure 2A). In addition, mitotic cells subjected to heat shock failed to show heat shock–inducible footprinting over the distal and proximal HSEs. Densitometer analysis of the autoradiograms quantitatively confirmed the loss of the in vivo footprints in mitotic cells (Figure 2C).

To confirm the results obtained with cells arrested in mitosis by treatment with nocodazole, we have also analyzed the genomic footprints in mitotic cells synchronized by means of the double-thymidine block. A significant loss of the constitutive and inducible in vivo footprints could be observed for these cells (Figure 2B). The combined results indicate that the human *hsp70* promoter is devoid of sequence-specific transcription factors during mitosis. The constitutive and inducible in vivo footprints on the *hsp70* promoter were restored when HeLa cells were released from mitotic arrest, along with the transcriptional inducibility (data not shown).

Among the sequence-specific factors in the cell capable of binding to the four different DNA elements on the *hsp70* promoter, there is direct evidence supporting in vivo interactions between HSF and the HSE, shown by immunofluorescent staining of Drosophila polytene chromosomes (Westwood et al., 1991). For the other elements, it is assumed that the most probable candidates are the following: Sp1 for the GC box (Jackson et al., 1990), the C/EBP family for the CCAAT box (Cao et al., 1991; reviewed by McKnight, 1992), and GBF for the G_n sequence (Hapgood and Patterton, 1994, and references therein).

DNA-Binding Activity in Mitotic Extracts

The loss of transcription factor binding in vivo could be due to an inhibition of their DNA-binding activities. Studies of the POU homeodomain transcription factor Oct-1 have shown that mitotic phosphorylation of the DNA-binding domain leads to inhibition of its DNA-binding activity (Roberts et al., 1991; Segil et al., 1991). To investigate the possibility of a similar inhibition for the constitutive and inducible transcription factors that bind to the human

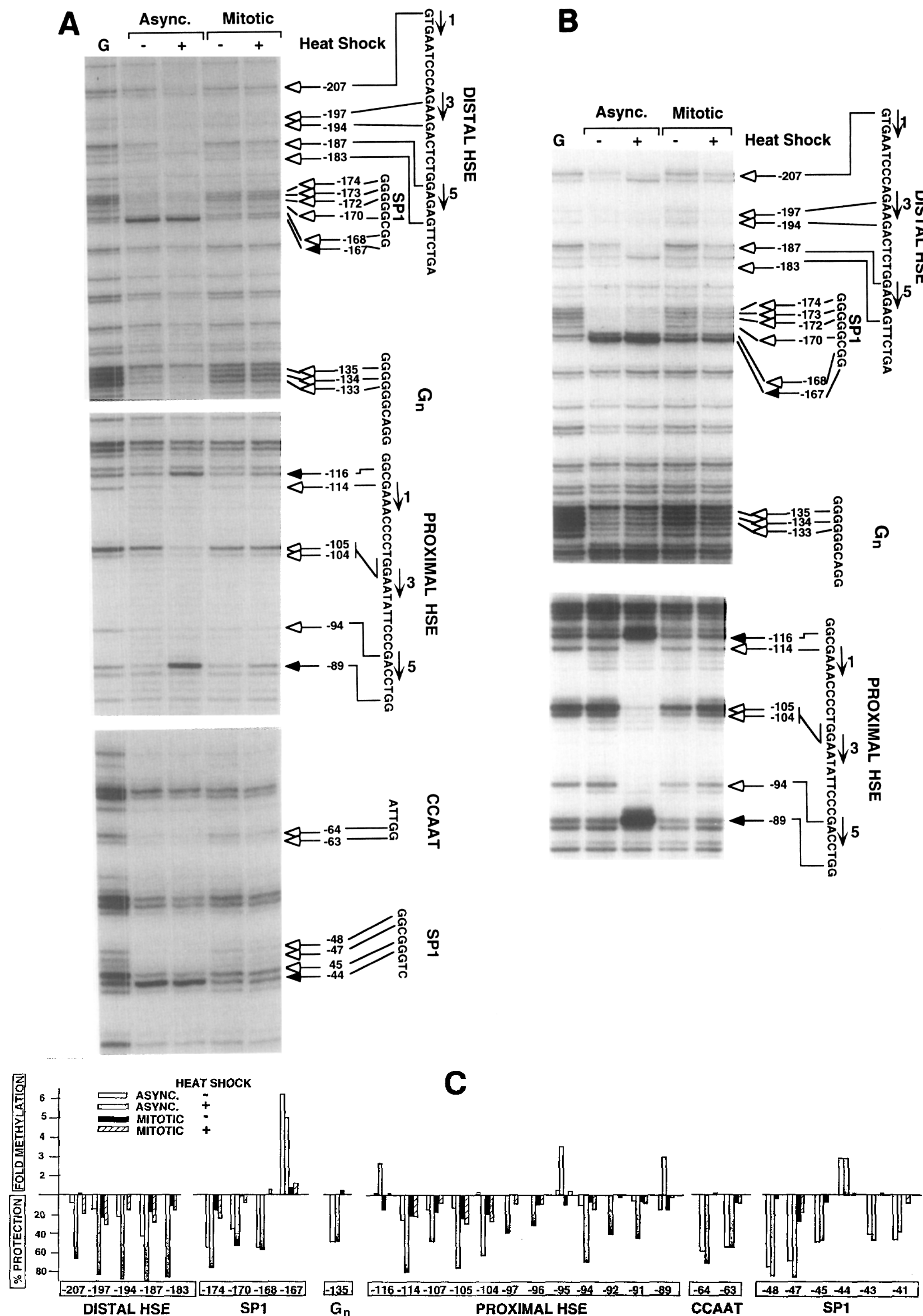


Figure 2. Genomic Footprinting of the Human *hsp70* Promoter from Asynchronous and Mitotic HeLa Cells

(A) Genomic footprint of the coding strand of the human *hsp70* promoter from HeLa cells arrested in mitosis by treatment with nocodazole for 8 hr. The genomic footprints of HeLa cells treated with nocodazole for 2 hr were identical to those from asynchronous cells.

(B) Genomic footprint of the coding strand of the human *hsp70* promoter from HeLa cells synchronized by a double-thymidine block. For (A) and (B), mitotic cells were subjected to a heat shock for 30 min at 43°C. As a control, the DMS reactivity at G residues for DNA purified from unshocked HeLa cells is shown (G). The changes in DMS reactivity found in asynchronous cells are noted at the corresponding G residues by open arrows (protection), and by closed arrows (hypersensitivity).

(C) The percent protection and fold hypermethylation calculated relative to the methylation at the corresponding G position from free DNA. Values were determined by densitometry.

hsp70 promoter, we determined the DNA-binding activities of the factors that interact in vitro with the GC box (Sp1), the CCAAT box (C/EBP family), the G_n sequence (GBF), and the HSE (HSF1). These binding activities were compared in extracts prepared from asynchronous HeLa cells and cells arrested in mitosis. As shown by electrophoretic mobility shift (Figure 3A) and Western blot assays (Figure 3B), Sp1 showed an approximately 5-fold reduction of specific DNA-binding activity in mitotic cell extracts. This decrease was correlated with a change in the electrophoretic position of Sp1 on SDS–polyacrylamide gels that was similar (but not necessarily identical) to results previously attributed to hyperphosphorylation by a DNA-dependent protein kinase (Jackson et al., 1990). As a positive control, we also confirmed the inhibition of Oct-1 DNA-binding activity in the same mitotic extracts (data not shown).

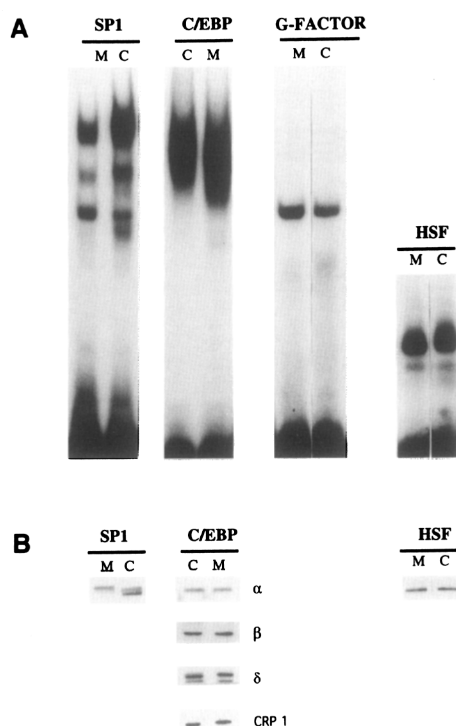


Figure 3. DNA-Binding Activity of Sp1, C/EBP, GBF, and HSF1 in Mitotic and Asynchronous HeLa Cell Extracts

(A) Electrophoretic mobility shift assays (EMSAs) were performed under conditions of DNA excess such that the amount of complex formed varied linearly with the amount of extract. The complexes observed are specific (the three top complexes for Sp1 and the major complexes for the other factors), as judged by competition with unlabeled binding sites.

(B) Western blot assays of whole-cell extracts prepared from asynchronous HeLa cells and cells arrested in mitosis. Western blots were reacted with antiserum specific for Sp1 (1:100 dilution); C/EBPα, C/EBPβ, C/EBPδ, CRP1 (1:100 dilution); human HSF1 (1:1000 dilution). Total extract protein (5 μg) was loaded per gel lane. The same amount of mitotic and asynchronous cell extracts used in the immunoblot analysis was subjected to EMSA. The amounts of factor:DNA complexes were quantified by densitometry. Both asynchronous and mitotic cell extracts were processed rapidly, in the presence of phosphatase inhibitors. In the absence of inhibitors, the reduction of Sp1-binding activity in mitotic extracts compared with asynchronous cell extracts was less than 2-fold, and the change in the SDS gel mobility was less pronounced, suggesting that these effects were due to endogenous phosphatase activity.

In contrast with the reduction of Sp1 DNA-binding activity, no significant changes of DNA-binding activity could be observed for GBF or the family of C/EBP proteins when asynchronous and mitotic cell extracts were compared (Figure 3). In addition, heat shock induction of the DNA-binding activity of HSF1 was normal in mitotic cell extracts. Hence, of the four sequence-specific factors potentially interacting with the human *hsp70* promoter, only Sp1 showed mitotic inhibition of its DNA-binding activity.

DNase I Hypersensitivity Is Retained in Mitotic Chromatin

A number of studies have documented the incompatibility of the binding of some transcription factors with the nucleosomal organization of DNA in chromatin (reviewed by Paranjape et al., 1994; Becker, 1994; Lewin, 1994). As both naked DNA and DNA wound over nucleosome core histones are freely accessible to reaction with small chemical probes such as DMS (Mirzabekov et al., 1977; McGhee and Felsenfeld, 1979), we investigated the chromatin structure of the human *hsp70* promoter in mitotic chromatin by digestion with DNase I. Like the region of highly accessible chromatin previously described for the Drosophila *hsp70* promoter (Wu, 1980), a series of DNase I hypersensitive cleavages mapping to the proximal region of the human *hsp70* promoter was observed in both mitotic and nonmitotic HeLa cell chromatin (Figure 4B). The DNase hypersensitive sites were not observed when naked HeLa cell DNA was used as a substrate (Figure 4A).

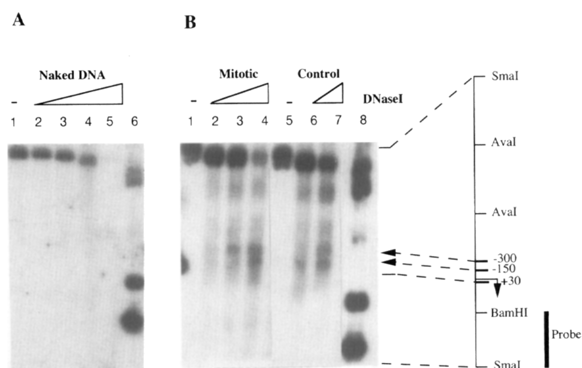


Figure 4. Analysis of Chromatin Structure at the Human *hsp70* Promoter

Autoradiograms showing the partial DNase I cuts on naked DNA (A) and on control (nonmitotic) and mitotic HeLa cell chromatin (B). (A) Naked HeLa cell DNA was digested for 3 min at 26°C with 0, 0.005, 0.0125, 0.025, and 0.05 U/μl of DNase I (lanes 1–5, respectively). (B) Control and mitotic cells were homogenized and digested for 3 min at 26°C with 0 (lanes 1 and 5), 0.2 (lane 2), 0.3 (lanes 3 and 6), and 0.5 U/μl (lanes 4 and 7) of DNase I. DNA was digested with SmaI, separated by electrophoresis, blotted, and hybridized with a 1.3 kb BamHI–SmaI fragment from the human *hsp70* gene. DNA digested with SmaI–PstI was used as a marker (lane 6 in [A] and lane 8 in [B]). Arrows indicate the hypersensitive sites generated over the *hsp70* promoter at position −300 and −150.

The retention of hypersensitivity to nuclease digestion suggests that the human *hsp70* promoter is not reassembled in a canonical nucleosome structure in mitotic chromatin.

General Dispersal of Transcription Factors from Mitotic Chromatin

The displacement of transcription factors from their site-specific targets could have a corresponding effect on the general association of these factors with mitotic chromatin. To investigate this possibility, we compared the bulk distribution of HSF1 in normal and heat-shocked HeLa cells at interphase and during mitosis. As shown by indirect immunofluoresence, both the inactive and activated forms of human HSF1 were localized to the cell nucleus at interphase (Figures 5A and 5B) (there is a subpopulation of HSF1 in the unshocked cell cytoplasm not revealed by the antibody; Wu et al., 1994). During mitosis, the unshocked and heat-shocked HSF1 were dispersed throughout the mitotic cytoplasm. In some cases, as shown in the figure, exclusion of HSF1 staining from the surface of the condensed metaphase chromosomes could even be observed. We have also observed a similar dispersal of Sp1 and C/EBP to the mitotic cytoplasm (data not shown). The exclusion of HSF1 from the mitotic chromosome does not appear to be due to the inaccessibility of the chromosome surface to reaction with antibody, as the metaphase chromosome could still be stained with antibodies against histone proteins (Figures 5C and 5D).

To assess whether such a dispersal of transcription factors was unique to factors that could bind to the *hsp70* promoter, we analyzed the bulk distribution of a number of other transcription factors by immunostaining. As shown in Figures 5E–5H, a similar dispersal of transcription factor staining from mitotic chromosomes was observed for Oct-1 (Fletcher et al., 1987), Oct-2 (Scheidereit et al., 1988), Ets-1 (Ghysdael et al., 1986), B-Myb (Gonda et al., 1985), c-Fos (Bohmann et al., 1987), E2F-1 (Kovesdi et al., 1986), and Bcl-6 (Ye et al., 1993). Interestingly, however, this dispersal was not observed for AP-2 (Williams et al., 1988).

The general dispersal of HSF1 and the other sequence-specific factors to the mitotic cytoplasm could be due to a simple dilution effect caused by breakdown of the nuclear envelope at the end of mitotic prophase. We investigated this question by permeabilizing interphase HeLa cells with a nonionic detergent, Nonidet P-40 (NP-40), which damages nuclear and cytoplasmic membranes by extracting membrane lipids. As might be expected, HeLa cells treated with NP-40 showed a near complete loss of nuclear staining for the inactive form of HSF1, while the activated form of HSF1 retained substantial nuclear staining. Nuclear staining was also retained for Sp1 and C/EBP in permeabilized interphase cells (Figure 6A). This retention was confirmed biochemically by Western blot analysis, which showed that approximately 50% of the active form of HSF1, 95% of C/EBP, and 40% of Sp1 remained in the nuclear preparation after NP-40 treatment (Figure 6B). The results indicate that these sequence-specific factors possess a general affinity for interphase chromatin and suggest that their dispersal in mitosis (at least for HSF1 and C/EBP) is unlikely to be due to a simple increase in the volume of the nuclear compartment.

Correlation with Chromatin Condensation and Decondensation

To gain further information on the timing of transcription factor dispersal from mitotic chromatin, we visualized the subcellular distribution of the activated human HSF1 in relation to the stages of mitosis (Figure 7). While HSF1 remained localized in the nucleus during early mitotic prophase, the first signs of dispersal to the cytoplasm were evident by late prophase, roughly coincident with the condensation of chromatin and breakdown of the nuclear envelope. The dispersal of HSF1 was maintained throughout metaphase, anaphase, and early telophase, but by late telophase, when the chromosomes have decondensed, staining was restored within the daughter nuclei. The general dispersal and subsequent reassociation of HSF1 on chromatin appears to be roughly coincident with the process of chromatin condensation and decondensation in the cell cycle. Similar results were obtained with C/EBP and Sp1 (data not shown).

Discussion

Mechanisms for General and Site-Specific Displacement of Transcription Factors during Mitosis

We have found that the site-specific interactions of several transcription factors with the human *hsp70* promoter in vivo are abolished during the progression of HeLa cells through mitosis. In addition, the interactions of these factors with bulk chromatin are abolished during mitosis. While Sp1 showed a significant decrease of in vitro DNA-binding activity in mitotic cell extracts, no change in binding activity was measurable for the C/EBP family, GBF, or the active form of HSF1. These results suggest that the loss of in vivo protein–DNA interactions is not a consequence of an overall inhibition of the DNA-binding activities. How could transcription factors be displaced or excluded from high affinity sites at specific promoters as well as from nonspecific sites on bulk chromatin? The correlation between the dispersal of transcription factors and the condensation of chromatin during mitosis suggests that the two events may be linked.

The compaction of chromatin necessarily involves neutralization of the negative charges of DNA within the chromatin that are insufficiently counterbalanced by the basic residues of the core and linker histones (Widom, 1986; Clark and Kimura, 1990; Schwarz and Hansen, 1994). A partial neutralization of the residual negative charge in chromatin could occur by the enzymatic removal of acetyl groups from modified lysine residues of the core histones, and there is substantial biochemical and immunocytochemical evidence indicating increased histone deacetylation in mitotic chromatin (Chahal et al., 1980; D'Anna et al., 1983; Turner, 1989; Turner and Fellows, 1989; Jeppesen et al., 1992).

The residual negative charge could also be neutralized

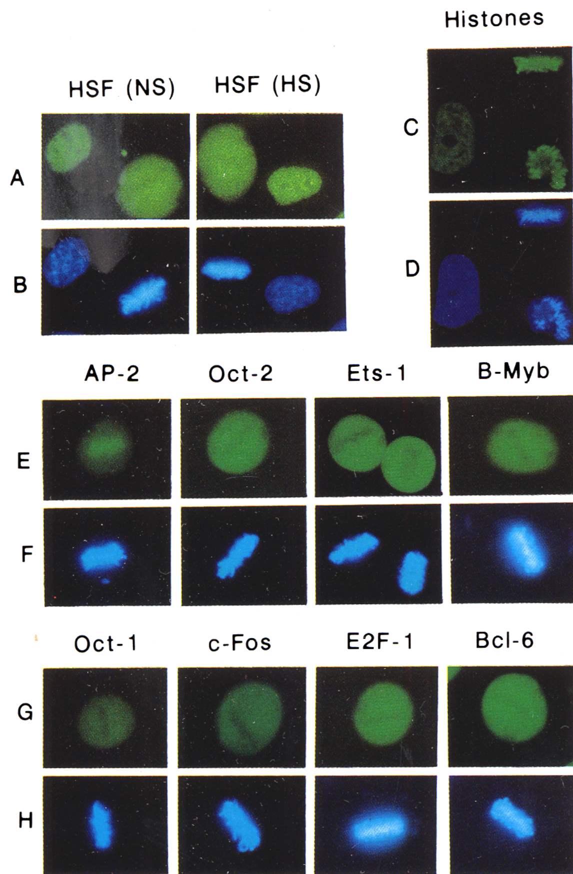


Figure 5. Indirect Immunofluorescent Staining of Transcription Factors and Histones in HeLa Cells in Mitosis

NS, nonshock; HS, heat shock (30 min, 43°C). (A), (C), (E), and (G) show distribution of transcription factors. (B), (D), (F), and (H) show Hoechst 33352 staining for DNA.

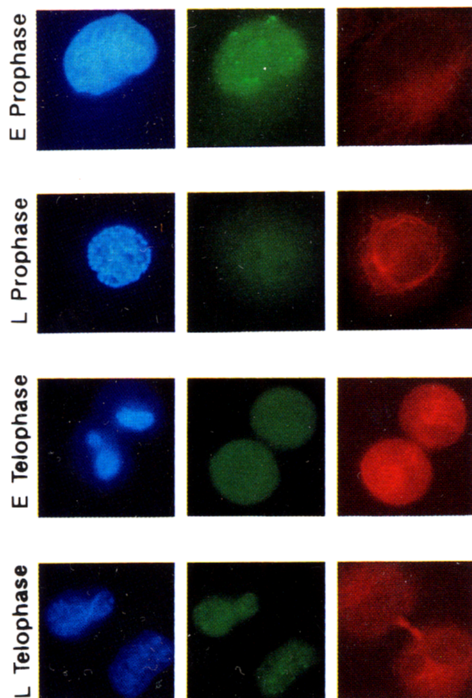
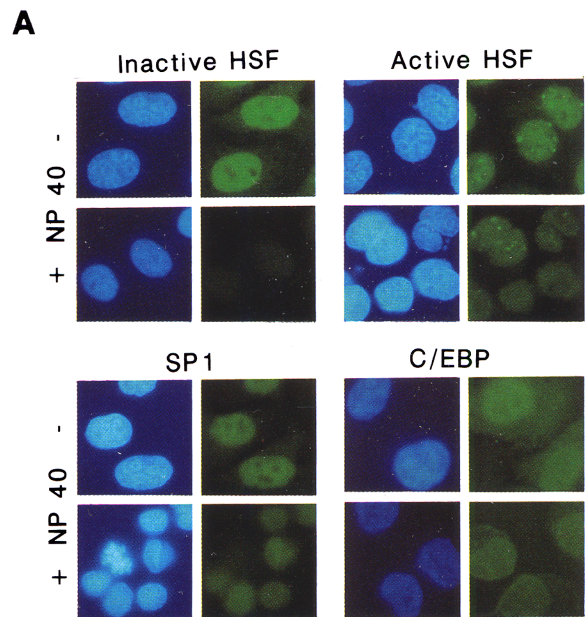


Figure 7. Cytological Localization of Activated HSF1 in Prophase and Telophase

HeLa cells were heat shocked at 43°C for 30 min, fixed and stained with antiserum to human HSF1 (central panel, in green), anti-tubulin antibody (right panel, in red), and Hoescht 33342 (left panel, in blue). At early prophase, HSF1 is associated with chromatin, but at late prophase, displacement of HSF1 occurs and is maintained to early telophase. At the end of mitosis in late telophase, the association of HSF1 with chromatin is restored. Between early and late telophase, when the chromosomes are still partially condensed, HSF1 can sometimes be localized within the daughter nuclei, but this relocalization appears to be due primarily to nuclear import without significant association with chromatin, as the nuclear staining was substantially reduced when cells at this stage were permeabilized with NP-40. Similar observations not shown were obtained with Sp1 and C/EBP.



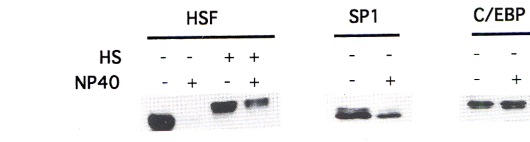


Figure 6. Distribution of Transcription Factors in Interphase HeLa Cells After Permeabilization with NP-40

(A) Indirect immunofluorescent staining for human HSF1 in HeLa cells heat shocked for 30 min at 43°C and for C/EBPα and Sp1, and HSF1 in unshocked HeLa cells; the cells were permeabilized with 0.2% NP-40 prior to immunostaining. The upper two panels correspond to untreated (minus NP-40) cells stained for DNA and the active and inactive forms of human HSF1, for Sp1, and for C/EBPα. Lower two panels correspond to NP-40-treated (plus NP-40) HeLa cells stained for DNA and the active and the inactive forms of human HSF1, for Sp1, and for C/EBPα.

(B) Western blot analyses of whole-cell extracts prepared from asynchronous HeLa cells treated with 0.2% NP-40 for 3 min. Blots were incubated with antiserum specific for Sp1 (1:100 dilution), C/EBPα (1:100 dilution), and human HSF1 (1/1000 dilution).

by the binding of endogenous spermine and spermidine, ubiquitous polycations that are present in millimolar concentrations in mammalian cells (McCormick, 1978). An immunocytochemical study has shown association of polyamines with highly condensed chromatin in metaphase and anaphase chromosomes (Hougaard et al., 1987). Like the modification of histones, the net positive charge (and consequently the DNA binding affinities) of spermine and spermidine can also be modulated by ace-

tylation and deacetylation (Seiler, 1987; Casero and Pegg, 1993; Matthews, 1993), thus providing a facile means of regulating the binding of polyamines to chromatin.

Besides facilitating the condensation of chromatin, the deacetylation of histone tails and the binding of spermine and spermidine to mitotic chromatin would have the additional effect of competing with the DNA-binding domains of transcription factors for electrostatic interactions with DNA. It is possible that such competitive interactions account for the observed displacement of transcription factors from mitotic chromatin. Indeed, the presence of nonacetylated core histones in the nucleosome impede transcription factor–DNA interactions (Lee et al., 1993), and the inclusion of submillimolar concentrations of polyamines in a nuclear isolation buffer was sufficient to erase the genomic footprints at a number of transcription factor–binding sites on the human *PGK-1* promoter (Pfeifer and Riggs, 1991).

Both histone deacetylation and polyamine binding could equally operate to displace transcription factors from bulk chromatin as well as from specific sites such as the human *hsp70* promoter. However, the extent of histone association and modification is unclear within DNase I hypersensitive promoter sequences in chromatin (reviewed by Lewin, 1994). The nonlocalized binding of polyamines to DNA (Wemmer et al., 1985; Schmid and Behr, 1991) could make a more significant contribution to transcription factor displacement at hypersensitive promoter sequences, while maintaining a nonnucleosomal structure sensitive to DNase I digestion. It should be noted that other histone modifications such as the phosphorylation of histone H1 and H3 are associated with the mitotic condensation of chromatin and could also play a role, albeit indirectly, in transcription factor displacement from chromatin (Bradbury, 1992; Roth and Allis, 1992). The functional significance of other agents such as factors involved in higher order chromatin folding (reviewed by Peterson, 1994) or in changes of DNA structure or topology also cannot be excluded.

Apart from the above mechanisms, the reduced DNA-binding activity of Sp1 by a change in its phosphorylation status may also contribute to its displacement and that of neighboring transcription factors at specific promoters by a loss of cooperative interactions, or by alterations in the local chromatin configuration that disfavor factor occupancy. The leading role of single transcription factors in a hierarchical process of factor loading in vivo has been observed, for example, for the vertebrate *RARβ2* gene (Dey et al., 1994) and for Drosophila heat shock genes (reviewed by Lis and Wu, 1993).

Reassembly in Interphase

How do transcription factors reassemble on specific promoters during the transition from mitosis to G1? The retention of DNase I hypersensitivity in condensed mitotic chromatin for the human *hsp70* promoter and for glyceraldehyde 3-phosphate dehydrogenase (Kuo et al., 1982) suggests the maintenance of a noncanonical chromatin configuration at promoter sequences during mitosis. This structure could serve as a landmark for the reassembly of active transcription factors on the decondensing chromatin. It will be of interest to investigate the roles of histone modification, polyamine binding, and hierarchical transcription factor interactions by means of in vitro reconstitution using interphase and mitotic extracts.

Perspectives

The present study represents a comprehensive analysis of the interactions of sequence-specific transcription factors with DNA on a specific promoter and on bulk chromatin during mitosis. It is unclear whether the site-specific displacement of transcription factors from mitotic chromatin we have observed is a common phenomenon, or if it is restricted to a few promoter elements and transcription factors in the genomes of higher eukaryotes. The bulk dispersal from mitotic chromosomes as revealed by immunostaining appears to be characteristic of many transcription factors, but it is not completely general. In addition to AP-2, the bulk interactions of at least one sequence-specific transcription factor, the serum response factor $p67^{SRF}$, are apparently immune to mitotic displacement; immunofluorescent studies also show association of this factor with the condensed chromatin of metaphase chromosomes (Gauthier-Rouviere et al., 1991).

It will be of interest to analyze the protein–DNA interactions of promoter and enhancer elements of other genes during mitosis, particularly those involved in developmental pathways. These studies would explore perhaps the most intriguing implication of our findings, that the mitotic displacement of transcription factors might present a hitherto unrecognized window besides the transient disruption in S phase to be exploited for the resetting of some transcriptional programs.

Apart from the general mechanisms discussed above, it is conceivable that the mitotic repression of heat shock genes could also involve an inhibition of an aspect of the heat shock signaling pathway involved in the transcriptional competence of HSF. Finally, the thermosensitivity of HeLa cells arrested in mitosis by nocodazole and by the chemotherapeutic agent taxol (M. A. M.-B., unpublished data) confirms and extends earlier studies showing increased thermosensitivity of cultured mammalian cells in mitosis (Westra and Dewey, 1971). Our results suggest a molecular mechanism for the enhanced thermosensitivity of mitotic cells that has been the basis of combined thermal therapy and chemotherapy with antimitotic agents (Hahn et al., 1993). The general abrogation of transcription during mitosis also implies the absence of a transcriptional response to any type of external stress and underscores the vulnerability of this phase of the cell cycle to environmental damage.

Experimental Procedures

Cell Culture and Synchronization

HeLa S3 cells were grown in minimal essential medium (S-Medium, GIBCO), supplemented with 10% fetal calf serum and 30 mM $CaCl_2$. Cells were arrested in mitosis by treatment with 50 ng/ml nocodazole for 8 hr. The double-thymidine block was performed as described

(Stein and Borun, 1972). Mitotic cells were collected by selective detachment by manual shaking of the tissue culture flasks. The mitotic index was monitored by chromosomal staining with the fluorescent dye Hoechst 33342 (1 μg/ml) and was found to be 95%–100% for nocodazole-arrested cells and 90% for cells synchronized by the double-thymidine block. After release from nocodazole arrest, the cells were found to recover and progressed through the cell cycle, as measured by incorporation of radiolabeled thymidine. Heat shock was performed at 43°C for 30 min except where indicated. For the experiment analyzing DNase I hypersensitivity, cells were synchronized with a double-thymidine block and nocodazole was introduced 1 hr before detachment of mitotic cells to ensure arrest of cells in mitotic synchrony. The control (nonmitotic) population was harvested from the cells remaining after mitotic detachment.

RNA Dot Blots

Whole-cell lysates for RNA dot blot analysis were prepared by heparin–DNase I treatment as previously described (Krawczyk and Wu, 1987). Cell pellets were lysed by freeze–thaw in 10 mM Tris–HCl [pH 7.4], 2.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM vanadyl ribonucleoside complex (VRC) (Sigma) and 1.5 mg/ml heparin (Sigma), treated with DNase I, and mixed with denaturation solution. After centrifugation, supernatant equivalent to 2.5×10^6 cells was loaded on a dot blot apparatus. Nitrocellulose filters were hybridized for 16 hr at 65°C using a human *hsp70* probe (a linearized human *hsp70* plasmid [pH 2.3]; Wu et al., 1985) or a β-actin probe. After hybridization, the filters were washed with 1× SSC, 0.5% SDS followed by three washes with 0.1× SSC, 0.1% SDS, each wash for 20–30 min at 65°C.

Thermosensitivity Assay

HeLa cells were arrested in mitosis after 8 hr of treatment with nocodazole and harvested by selective detachment. As nonmitotic controls, we used the cells remaining after the detachment procedure. Thermotolerant cells were generated by a mild heat shock at 42°C for 30 min, 5 hr before mitosis. HeLa cells were subjected to an acute heat shock at 43°C for 1 hr, after which they were counted and plated at a density of 2×10^3–5×10^3 cells/dish. Colonies were visualized by crystal violet staining after 6 days of incubation at 37°C.

Genomic Footprinting

HeLa cells were arrested in mitosis after 8 hr of nocodazole treatment or synchronized by a double-thymidine block. Mitotic cells were harvested by selective detachment. As asynchronous controls, cells were untreated or exposed to nocodazole for only 2 hr. Cells to be heat shocked were incubated at 43°C for 30 min (25 min for cells synchronized by the double-thymidine block) and were treated with 0.1% DMS for 2 min. DNA was isolated and cleaved with piperidine. Genomic footprinting was performed by using LM-PCR (Dey et al., 1992; Garrity and Wold, 1992). For footprinting of the coding strand, primer 1 was 5′-CCTGGGCTTTTATAAGTC-3′ (18-mer, −14 to −31), primer 2 was 5′-ACGGAGACCCGCCTTTTCCCTTCTG-3′ (25-mer, −36 to −62), and primer 3 was 5′-CGGAGACCCGCCTTTTCCCTTCTGAGCC-3′ (29-mer, −36 to −66). For footprinting of the coding strand near the proximal GC and CCAAT boxes region, primer 1 was 5′-AGCCGCACAGGTTCGCTCT-3′ (19-mer, +98 to +80), primer 2 was 5′-AGCCTTGGGACAACGGGAGTCACTC-3′ (25-mer, +75 to +51), and primer 3 was 5′-GCCTTGGGACAACGGGAGTCACTCTCG-3′ (27-mer, +74 to +54). Labeled PCR products were resolved on a 6% sequencing gel.

For quantitation, the autoradiograms were scanned with a laser densitometer (Molecular Dynamics). Scans were taken twice, and an average for each G residue was used for quantitation. The integrated area obtained for each G residue from the footprinted region was normalized relative to the value of an unaffected G nucleotide outside the footprint. The percent protection or fold hypermethylation in vivo was determined relative to the corresponding reactivity of the G residue in the free DNA lane.

Protein Extraction, Western Blotting, and Gel Mobility Shift Assay

Whole-cell protein was extracted from asynchronous and mitotic HeLa cell pellets by free–thaw in 0.4 M NaCl buffer containing 10 mM HEPES (pH 7.9), 0.4 M KCl, 0.1 mM EGTA, 5% glycerol, 0.5 mM DTT, 0.1 mM AEBSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 25 mM NaF, 5 mM Na orthovanadate, 1 mM Na pyrophosphate, and 5 μM microcystin (Dulić et al., 1994). Western blotting was performed using the Amersham ECL system according to the instructions of the manufacturer. Total cellular protein (5–10 μg) was separated by SDS–PAGE (7% or 12% polyacrylamide) and electrophoretically transferred to nitrocellulose. The primary antibodies were α-HSF1 (Rabindran et al., 1993), α-Sp1, α-C/EBPα, α-CRP1 (Santa Cruz Biotechnology), α-C/EBPβ, α-C/EBPδ (gift of S. L. McKnight) used at 1:500 (Sp1) and 1:100 (all others) dilutions. The secondary antibody was goat anti-rabbit IgG conjugated to peroxidase at 1:20,000 dilution.

For EMSA, each DNA binding reaction mixture (25 μl) contained between 5–10 μg of protein extract, 1 ng of labeled duplex oligonucleotide (Sp1, ATT CGA TCG GGG CGG GGC GAGC; C/EBP: CCT TTG GCA TGC TGC CAA TAT G; HSE: GGG CAG AAT TTC TAG AAT CAG C; GBF, TGT CGA GGG GGG CAG GGG TAG AA), 0.01% NP-40, 1.5 μg of BSA, and 1–4 μg of Escherichia coli DNA (HSF1 and GBF) or 0.5 μg of poly(dI–dC) (Sp1 and C/EBP). The protein–DNA complexes were subjected to native electrophoresis on 0.8% agarose, 0.5× TBE gels (HSF1), or on 4% polyacrylamide, 0.5× TBE gels for the other transcription factors.

Analysis of Chromatin Structure

Cells were homogenized in nuclear buffer (60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris-HCl [pH 7.4], 0.5 mM DTT, 0.1 mM AEBSF, and 300 mM sucrose; Wu, 1989) and digested for 3 min at 26°C with DNase I (Worthington). The reaction was terminated by the addition of SDS and EDTA to a final concentration of 0.5% (w/w) and 0.01 M, respectively. DNA was purified as described (Wu, 1980), digested with SmaI endonuclease, separated by electrophoresis on a 1.4% agarose gel in 1× TBE, blotted, and hybridized with probe labeled by random priming to a specific activity of 1×10^9–3×10^9.

Indirect Immunofluorescence

HeLa S3 cells were cultured in 1 ml slide flasks. Cells were fixed in 4% formaldehyde in PBS for 20 min at room temperature, followed by methanol for 10 min. After blocking with 3% BSA in PBS, 0.1% Tween 20 for 30 min at room temperature, slides were incubated with a 1:500 α-HSF1 antiserum, 1:350 α-Sp1, 1:100 α-C/EBP, 1:1000 α-tubulin, 1 μg/ml α-histone (Boehringer), 1:250 α-AP-2, α-Oct-1, α-Oct-2, α-Ets-1, α-B-Myb, α-cFos, α-E2F, α-Bcl-6 (Santa Cruz Biotechnology) in PBS, 3% BSA for 2 hr, followed by incubation for 1 hr with rhodamine-conjugated goat anti-mouse (for α-tubulin) and FITC-conjugated goat anti-rabbit IgG (for the other proteins), used at 1:250 dilution in PBS, 3% BSA. After each incubation with antibody, slides were extensively washed with PBS, 0.05% Tween 20 twice for 10 min each at room temperature.

Permeabilization of Cells with NP-40

HeLa cells were treated with nuclear buffer containing 0.2% NP-40 for 3 min. Cells were washed, centrifuged briefly on the microscope slide, fixed, and stained as described above for immunostaining. Alternatively, after NP-40 treatment, total nuclear protein was extracted. For this purpose, asynchronous cells were scraped from the culture flask and pelleted. Cells were resuspended in nuclear buffer, or in nuclear buffer containing 0.2% NP-40, and incubated for 3 min at room temperature. The treated cells were pelleted and total protein was extracted by heating in SDS–PAGE sample buffer (95°C, 5 min). Proteins were separated in SDS–PAGE, and Western blot analyses were performed as described above.

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Note Added in Proof

Mitotic displacement of transcription factors has been observed for the human phosphoglycerate kinase 1 promoter (Hershkovitz, M. and Riggs, A. D. [1995]. Proc. Natl. Acad. Sci. USA *92*, 2379–2383); the GAGA transcription factor is immune to mitotic displacement at GA/CT-rich chromosome regions of the preblastoderm Drosophila embryo (Raff, J. W., Kellum, R. and Alberts, B. [1994]. EMBO. J. *13*, 5977–5983).