Pulse-chase experiments and immunoprecipitations. For pulse-chase experiments, 2.5 A_{600} cells were taken from a logarithmically growing culture for each time point and were labelled with 62.5 μCi [^{35}S]-methionine. Growth, labelling, chase conditions and other experimental procedures, such as cell lysis, immunoprecipitation and SDS–PAGE, were performed as described[13].

Deglycosylation experiments and western analysis. Cells were grown at the indicated temperature in complete synthetic medium containing 2% glucose to an A_{600} of 3.0. Immunoprecipitation and deglycosylation of CPY* was performed as described[5]. Immunoprecipitated material was boiled in 50 μl UREA buffer before SDS–PAGE using a 8% gel and blotting. For western analysis, detection of the indicated proteins was performed using the respective antibodies.

Protease protection experiments. Spheroplasting and cell breakage were done as described[5]. For protease treatment of the pellet, trypsin was added to a final concentration of 0.5 mg ml^{-1} after resuspension of the pellet. The samples were incubated for 30 min on ice. If added, Triton X-100 was present at 1%. All treatments were stopped by TCA precipitation. After resuspending the pellet in 100 μl UREA buffer, CPY* was analysed by SDS–PAGE and immunoblotting.

β-Galactosidase activity test. After adding cycloheximide to a final concentration of 0.5 mg ml^{-1} at zero time ($t = 0$) to the logarithmically growing culture, 0.3 A_{600} of cells were taken for each time point, mixed with lysis buffer (0.6% Triton X-100, 0.75% ONPG, 2.25% β-ME, 0.15 M Tris-BCl, pH 7.5) and kept at −80 °C for 30 min. After incubation for 60–90 min at 37 °C, 75 μl of 1 M $NaHCO_3$ was added to the samples, debris was removed by centrifugation (20,000*g*, 3 min) and A_{405} was determined.

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Marking of active genes on mitotic chromosomes

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During development and differentiation, cellular phenotypes are stably propagated through numerous cell divisions[1]. This epigenetic 'cell memory' helps to maintain stable patterns of gene expression[2]. DNA methylation[3] and the propagation of specific chromatin structures may both contribute to cell memory[4]. There are two impediments during the cell cycle that can hinder the inheritance of specific chromatin configurations: first, the pertinent structures must endure the passage of DNA-replication forks in S phase[5]; second, the chromatin state must survive mitosis, when chromatin condenses, transcription is turned off, and almost all double-stranded DNA-binding proteins are displaced[6,7]. After mitosis, the previous pattern of expressed and silent genes must be restored. This restoration might be governed by mass action, determined by the binding affinities and concentrations of individual components. Alternatively, a subset of factors might remain bound to mitotic chromosomes, providing a molecular bookmark to direct proper chromatin reassembly. Here we analyse DNA at transcription start sites during mitosis *in vivo* and find that it is conformationally distorted in genes scheduled for reactivation but is undistorted in repressed genes. These protein-dependent conformational perturbations could help to re-establish transcription after mitosis by 'marking' genes for re-expression.

DNase I-hypersensitive sites in chromatin are useful indicators of gene activity and may persist during mitosis, despite cessation of transcription and dissociation of most transcription factors from mitotic chromosomes[6]. Many DNase I-hypersensitive sites are also sensitive to S1 nuclease and hence may have single-stranded features[8–10]. Melted regions of the human c-*myc* gene have been mapped in unsynchronized cells and shown to bind transcription factors *in vitro*[10,11]. During mitosis, the single-stranded properties of chromatin increase ~10-fold[12]. To test whether any mitosis-specific increase in single-stranded character might occur in previously characterized S1-sensitive regions of the human c-*myc* gene, bases reactive to potassium permanganate[10,11] were mapped by using ligation-mediated polymerase chain reaction (LM-PCR). Factors binding to such melted regions during mitosis may contribute to the inheritance of the c-*myc* gene chromatin structure through this

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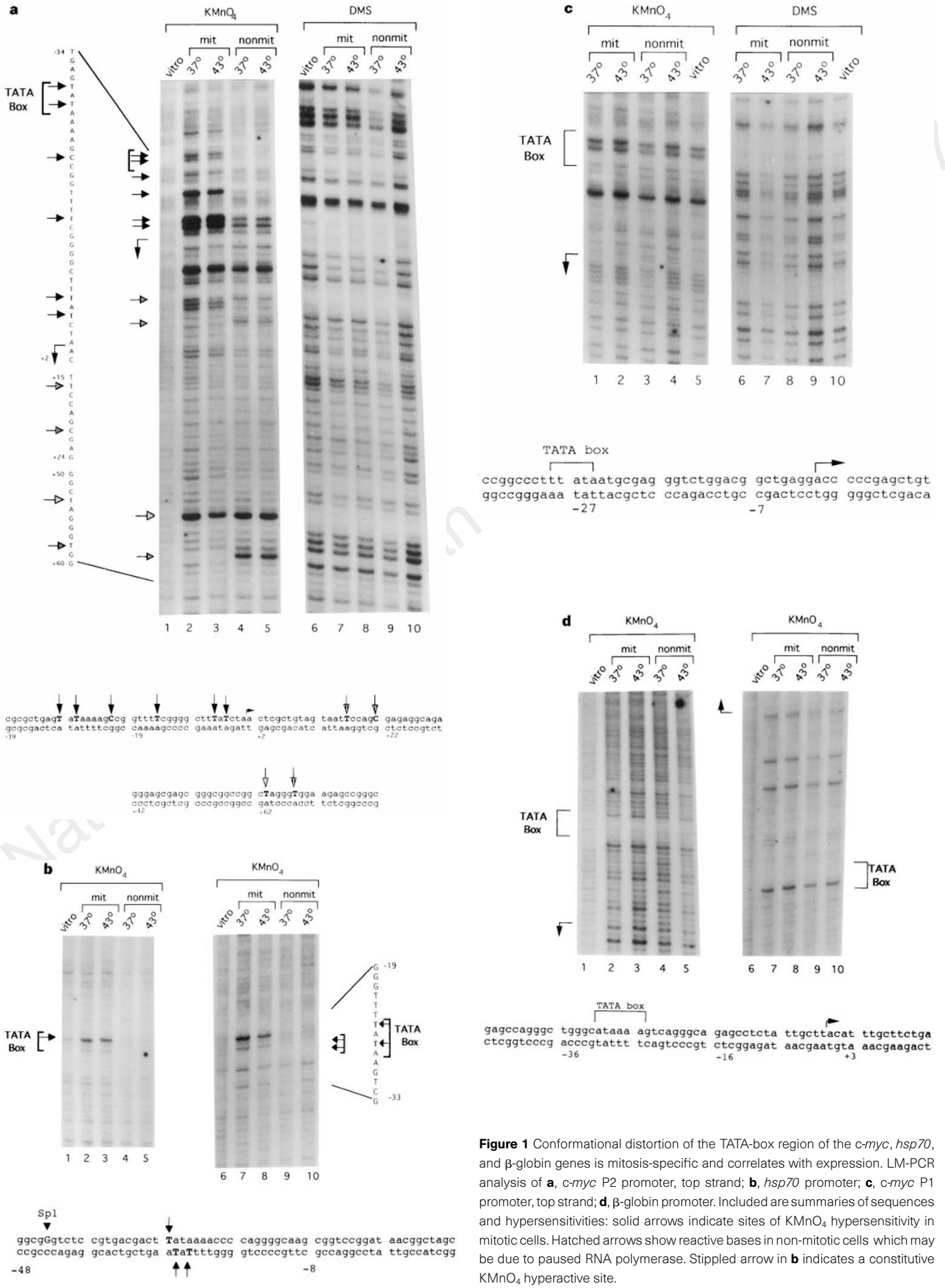

Figure 1 Conformational distortion of the TATA-box region of the c-*myc*, *hsp70*, and β-globin genes is mitosis-specific and correlates with expression. LM-PCR analysis of **a**, c-*myc* P2 promoter, top strand; **b**, *hsp70* promoter; **c**, c-*myc* P1 promoter, top strand; **d**, β-globin promoter. Included are summaries of sequences and hypersensitivities: solid arrows indicate sites of $KMnO_4$ hypersensitivity in mitotic cells. Hatched arrows show reactive bases in non-mitotic cells which may be due to paused RNA polymerase. Stippled arrow in **b** indicates a constitutive $KMnO_4$ hyperactive site.

phase of the cell cycle. This approach has identified and mapped melted DNA at promoters bearing paused RNA polymerases[13] and at other sites binding single-strand-specific factors[10,11]. The c-*myc* P2 promoter, a major region of S1 sensitivity in isolated nuclei, was analysed with $KMnO_4$ during mitosis in synchronized cells. In parallel, *in vivo* dimethylsulphate (DMS) footprinting of the *hsp70* promoter was used to monitor displacement of conventional double-stranded DNA binding proteins[6]. HeLa cells were treated with nocodazole for eight hours[6] (which synchronizes cells at metaphase), separated by shake-off into mitotic and non-mitotic cells, incubated for 30 min at either 37 °C or 43 °C (for analysis of the *hsp70* promoter), and treated *in vivo* with either DMS or $KMnO_4$ (ref. 9). Purified DNA was subjected to LM-PCR using primers that allowed visualization of the top strand of the human c-*myc* gene in the vicinity of the P2 transcription start site.

$KMnO_4$ reactions revealed unexpected mitosis-specific unwinding of the *myc* P2 TATA-box/start-site region (Fig. 1a; compare lanes 2, 3 with 4, 5). These changes were not apparent by DMS footprinting (Fig. 1a; compare lanes 7, 8 with 9, 10). Similar changes were noted in mitotic cells prepared without nocodazole and hence were not caused by blocking the cell cycle (data not shown). Controls confirmed that cells were properly synchronized (mitotic index of >95%), that dramatic mitotic displacement of the conventional double-stranded DNA-binding proteins Sp1 and HSF (heat-shock factor) from the *hsp70* promoter proceeded as described[6], and that upregulation of *in vivo* binding by HSF (and Sp1 to a lesser extent) occurred upon heat shock only in non-mitotic cells (data not shown).

The *hsp70* TATA-box region[6] was examined during mitosis. Like the c-*myc* P2 promoter, the *hsp70* TATA box experienced a conformational change that was specific for mitosis (Fig. 1b; compare lanes 2, 3 with 4, 5; and lanes 7, 8 with 9, 10). Again, these changes were not seen with DMS (data not shown). Not all TATA boxes were mitotically distorted, because no changes occurred at c-*myc* P1 (Fig. 1c; compare lanes 1, 2 with 3, 4), which also appeared to be vacant as judged by DMS footprinting. Perhaps the conformational changes at the *hsp70* and c-*myc* promoters marked these genes for transcriptional restart following mitosis. If so, the proximal promoter region of non-expressed genes should be unreactive with $KMnO_4$ during mitosis. As predicted, neither strand of the silent β-globin gene showed mitosis-specific changes (Fig. 1d, compare lanes 2, 3 with 4, 5, and lanes 7, 8 with 9, 10). Thus, stable gene activity correlated with perturbation of the TATA box during mitosis.

If mitotic TATA-box marking were linked to the maintenance of gene expression, then no unwinding at the c-*myc* P2 TATA-box region should occur when c-*myc* transcription is repressed. In IMR32 neuroblastoma cells overexpressing N-*myc*, the c-*myc* locus is shut off and c-*myc* RNA is undetectable[10]. No change in DNA conformation of the c-*myc* P2 proximal promoter region was detected with $KMnO_4$ (Fig. 2a) or DMS (Fig. 2a, right), either in mitotic or non-mitotic IMR32 cells (Fig. 2a; compare lanes 6, 7 with 2, 3), indicating that mitosis-specific tagging of this start site was linked to gene expression. Likewise, no $KMnO_4$ hypersensitivity was induced on the complementary strand (Fig. 2a, right). DMS reactions in HeLa and IMR32 cells were indistinguishable (Fig. 2a, lanes 8–12). The lymphoma cell line CA46, which expresses c-*myc*, was also mitotically marked at P2 (Fig. 2a, lanes 4 and 5).

The lack of mitosis-specific marking of c-*myc* in IMR32 cells might simply have been due to a cell-line-specific defect in whatever machinery was required for unwinding. We therefore tested the mitotic $KMnO_4$ reactivity of the *hsp70* gene, which is expressed in both IMR32 and HeLa cells. The *hsp70* TATA-box region was similarly tagged in neuroblastoma cells (Fig. 2b, lanes 1 and 2) and HeLa cells (Fig. 2b, lanes 3 and 4), indicating that the mechanism for TATA-box unwinding is intact in IMR32 cells; therefore the

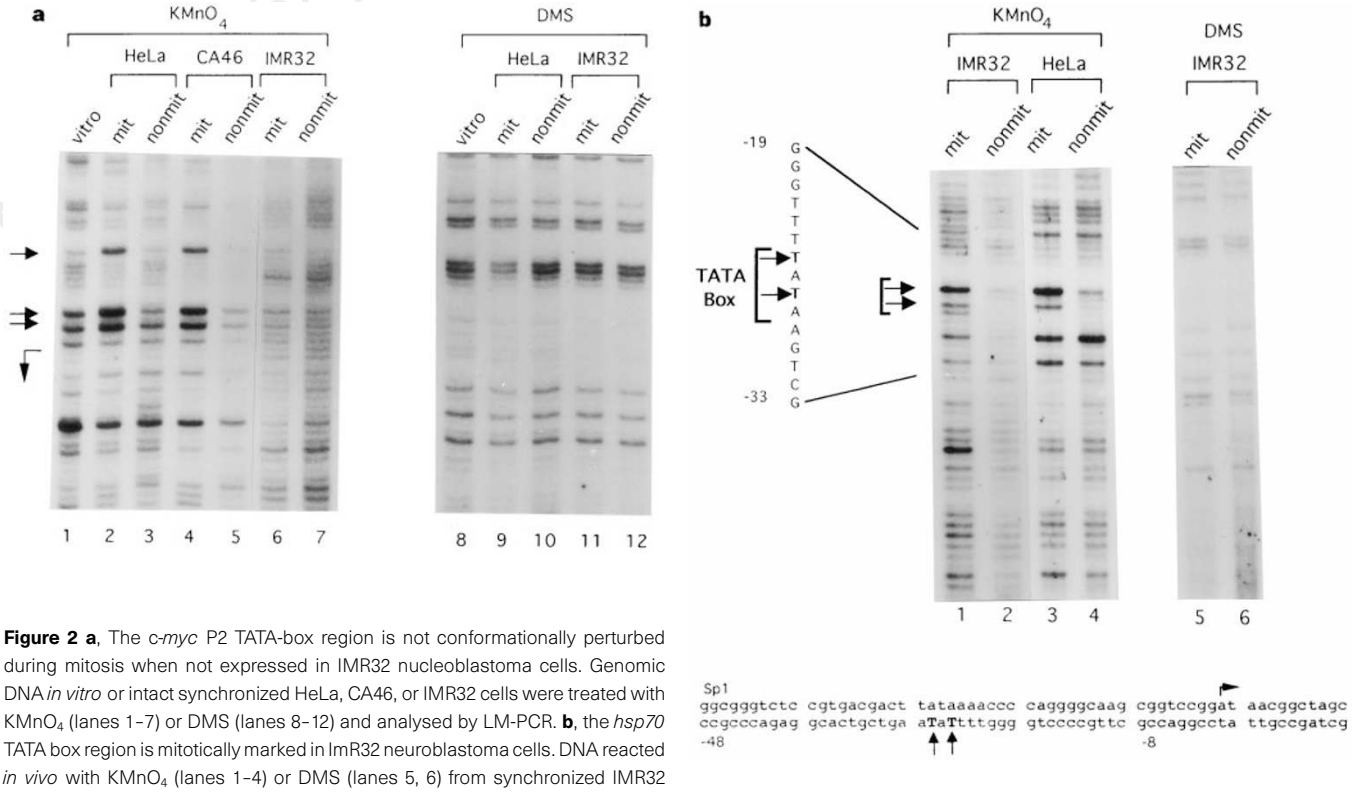

Figure 2 a, The c-*myc* P2 TATA-box region is not conformationally perturbed during mitosis when not expressed in IMR32 nucleoblastoma cells. Genomic DNA *in vitro* or intact synchronized HeLa, CA46, or IMR32 cells were treated with $KMnO_4$ (lanes 1–7) or DMS (lanes 8–12) and analysed by LM-PCR. **b**, the *hsp70* TATA box region is mitotically marked in ImR32 neuroblastoma cells. DNA reacted *in vivo* with $KMnO_4$ (lanes 1–4) or DMS (lanes 5, 6) from synchronized IMR32 neuroblastoma and HeLA cells was used to assess marking of the bottom strand of the *hsp70* promoter region via LM-PCR.

lack of unwinding at the c-*myc* P2 promoter in IMR32 was probably linked to the absence of c-*myc* transcription.

Genes lacking TATA boxes, including some 'housekeeping' genes, must also be restarted after mitosis. If mitotic marking contributes generally to re-expression, then expressed TATA-less genes such as the *ets*-2 protooncogene[14] should display mitotic perturbation near their initiation sites. Indeed, several nucleotides at the *ets*-2 start site and in the adjacent upstream sequence were mitotically reactive with $KMnO_4$ (Fig. 3, arrows), whereas downstream residues were reactive only in non-mitotic cells (Fig. 3, arrowheads), which is consistent with transcriptional melting. Furthermore, a TATA-less promoter that is silent in HeLa cells, that for the terminal deoxynucleotidyl transferase (TdT) gene[15,16] was devoid of mitosis-specific characteristics (data not shown).

What are the molecular requirements for placement of mitotic bookmarks? The diversity of affected promoters and the association with gene activity argues strongly against specific sequence recognition as the basis for mitotic marking. The correlation of mitotic tags with the activity of the c-*myc*, *hsp70*, *ets-2*, TdT or β-globin genes indicates that the conformational distortion is not due solely to chromosome condensation. However, the mitotic mark might represent buckling of naked promoter DNA, vacated during metaphase, under mechanical stress, but such a mechanism should have strained both c-*myc* promoters equally. Alternatively, melting might require a protein factor or cofactor. If this hypothetical protein"s half-life were sufficiently short, then the metaphase-specific $KMnO_4$ hyperactive sites at the c-*myc* P2 promoter should decay with cycloheximide treatment. Therefore, nocodazole-synchronized mitotic HeLa cells were divided into two aliquots and incubated for varying lengths of time, with or without 25 $\mu g\,ml^{-1}$ cycloheximide. Cycloheximide- and mock-treated cells were reacted *in vivo* in the presence of nocodazole with $KMnO_4$ for LM-PCR analysis. A cycloheximide-induced decrease in mitosis-specific $KMnO_4$ reactivity (relative to mock-treated cells) at the c-*myc* P2 promoter was first detected 3 h after cycloheximide treatment (Fig. 4a; compare lanes 5 and 6 with 3 and 4) and was clearly evident at 4 h (Fig. 4a, lanes 7 and 8). Also potentially turning over in this experiment, however, were proteins required for chromosome condensation; the resulting chromosome decompaction could compromise interpretation of *in vivo* $KMnO_4$ analysis. To exclude these complications, both mock- and cycloheximide-treated cells from the 4-h time point were fixed, stained[17] and microscopically examined; no changes in chromosome morphology induced by cycloheximide treatment were evident (data not shown). This experiment supports the existence of a protein required for the maintenance of the mitosis-specific mark at the c-*myc* P2 start site.

These results provide *in vivo* evidence that a protein is required for mitosis-specific melting of the c-*myc* P2 start site. *In vitro* evidence for such a protein was sought by testing the salt-extractability of this factor from purified mitotic chromosomes. Nocodazole-arrested mitotic HeLa cells were lysed in hypotonic buffer, condensed chromosomes were centrifugally purified and then extensively washed with buffers of increasing ionic strength. Washed mitotic chromosomes were then treated with $KMnO_4$ and analysed by LM-PCR. The mitotic mark resisted extraction with 10 mM NaCl (Fig. 4b, lane 1), 200 mM NaCl (Fig. 4b, lane 2) and 300 mM NaCl (data not shown). Extraction with 400 mM NaCl yielded a pattern of $KMnO_4$ hypersensitive site very similar to that of non-mitotic cells (Fig. 4b; compare lanes 3 and 4). A mitotic marker was extracted, but 400 mM NaCl is also sufficient to mobilize nucleosomes and potentially alter other features peculiar to the mitotic chromatin of active genes.

Re-addition of the 0.4 M NaCl extract to the salt-washed mitotic chromosomes failed to restore the mitotic marker. It is unlikely that simple sequence recognition mediates direct formation of marker complexes at active promoters. If the marker itself instructs genes to

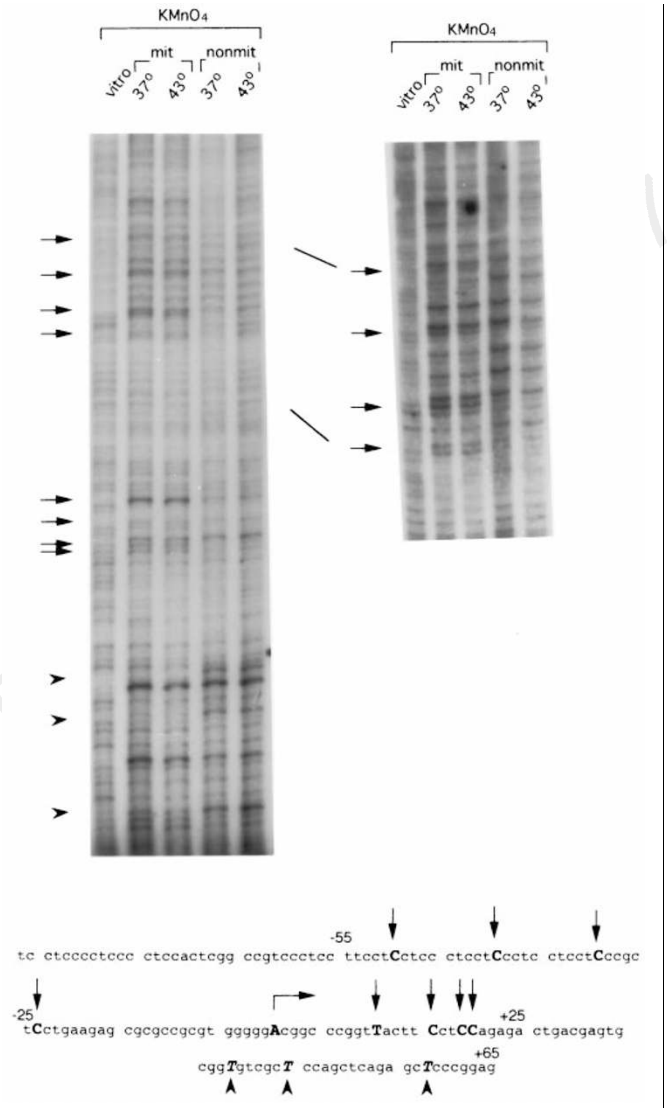

Figure 3 The *ets-2* start site is marked during mitosis. Left, bases mitotically reactive with $KMnO_4$ are indicated with arrows; arrowheads indicate bases selectively reactive in non-mitotic cells. Right, longer run of sequences upstream of –20. Bottom, summary of reactive bases; bent arrow indicates the start-site (+1).

be post-mitotically expressed, then removal of the marker should convert expressed genes to the default silent state. In the absence of transcriptional activity, reconstitution of the marker would not occur. Alternatively, the mitotic marker may act as an accessory factor to maintain a segment of DNA already configured for transcription, ready for reoccupancy by the transcription machinery. In this scheme, accurate reconstitution of the mitotic marker would be achievable unless the salt treatment disrupted native mitotic chromatin or removed cofactors.

The mitosis-specific $KMnO_4$ hypersensitivity indicates a nidus of single-stranded DNA at start sites, perhaps stabilized by bound protein. Two features of single-stranded DNA might facilitate transcription restart. First, nucleosomes, which might otherwise obstruct the promoter, have a reduced affinity for single-stranded DNA[18]. Second, the deformability of single-stranded DNA to flexural and torsional stress[19] facilitates the assembly of the transcription complex, especially if the returning TFIID or other basal components recognized the bookmarker[7]. *In vivo* footprinting indicates that other single-stranded DNA-binding proteins, hnRNP

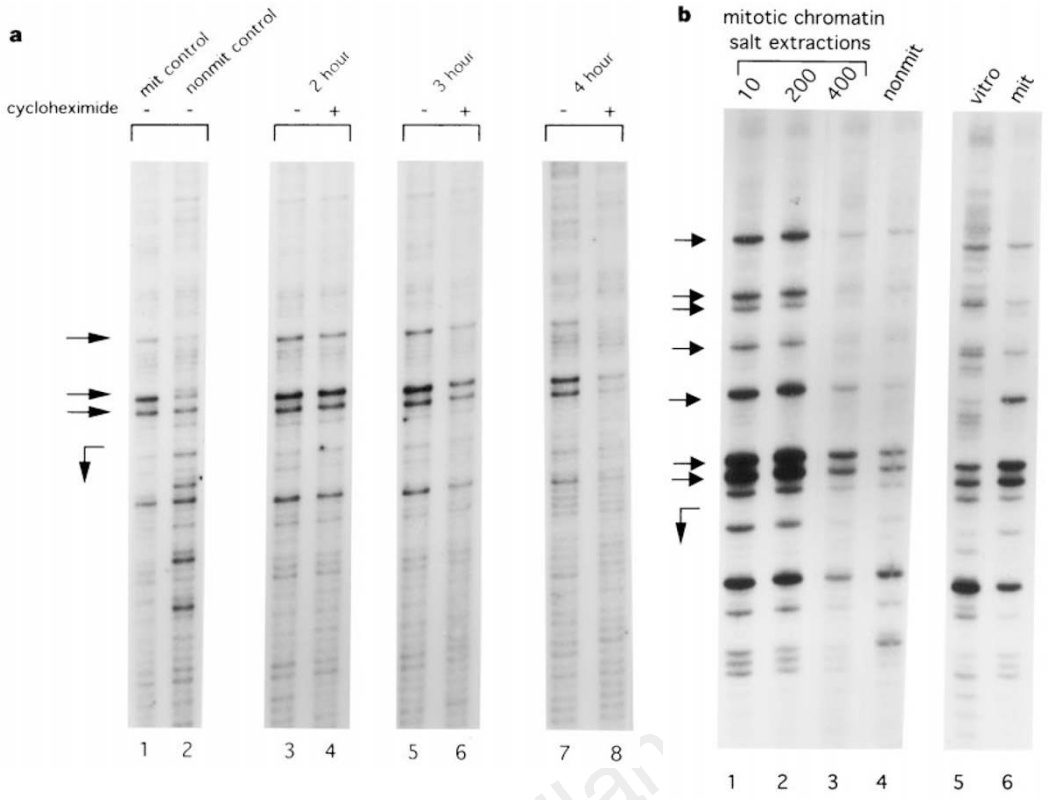

Figure 4 Protein requirement for the melting of the c-*myc* P2 TATA-box region during mitosis. **a**, Nocodazole-arrested mitotic HeLa cells were shaken off and separated into two nocodazole-containing flasks, one with cycloheximide, one without cycloheximide. At various times, samples were treated *in vivo* with $KMnO_4$, and purified DNA was analysed by LM-PCR using primers that visualized the top strand of the c-*myc* P2 promoter. **b**, Factor(s) required for mitosis-specific c-*myc* P2 promoter $KMnO_4$ hypersensitivity is salt-extractable. HeLa cells were treated with nocodazole for 8 h and mitotic cells were shaken off. Cells were then hypotonically lysed and mitotic chromosomes purified by centrifugation. Chromosomes were then extracted with increasing concentrations of NaCl, and treated with $KMnO_4$. The top strand of the c-*myc* P2 promoter was then analysed by LM-PCR.

K and FBP[9], remain bound to the human c-*myc* promoter during mitosis (data not shown) when the single-stranded character of bulk chromatin increases 10-fold[12]. Single-stranded DNA and associated factors may therefore be important for mitosis.

What is the source of single-stranded DNA during mitosis? Possibly transcription; melted DNA at promoter start sites in this scheme is stabilized by a factor, perhaps also interacting with the transcription machinery, just before M phase. Persistence of the marker through mitosis may require sustained torque. The requirement for topoisomerase I activity in chromosome condensation indicates that torsional strain is indeed present during compaction[20]. Recruitment of the cellular transcription machinery to genes marked by stressed DNA could be important to re-establish the correct range of expressed genes. □

Methods

Ligation-mediated PCR. *In vivo* footprinting was done as described[21]. Primers for LM-PCR analysis of the top strand of the c-*myc* P2 region were: TAGCCCCCTATTCGCTC, CCCCTATTCGCTCCGGATCTCCC and TTCGCTCCGGATCTCCCTTCCCAGGA. Primers for LM-PCR analysis of the top strand of c-*myc* P1 were: TCTCGAGGCAGGAGGGG, CGAGGCAGGAGGGGAGCCAGGGAC and GGGAGGAGGGGAGCCAGGGACGGCCGG. Primers for analysis of the bottom strand of the globin TATA-box region were: CACTTAGACCTCACCCTG. GACCTCACCCTGTCCAGCCACAC and CTGTGGAGCCACACCCTAGGGTTGGCC; primers for the top strand of globin were: GTCAGGTGCACCATGGTG, TGCACCATGGTGTCTGTTTGAGG and GTGTCTGTTTGAGGTTGCTAGTGAAC. Primers for the strand strand of the *ets-2* promoter were: CTCCGGGAGCTCTGAGC, CGGGAGCTCTGAGCTGGAGCGAC and GAGCTCTGAGCTGGAGCGACACCGCA.

LM-PCR analysis of mitotic chromosomes. Washed HeLa cells were Dounce-homogenized in 10 mM Tris, pH 8.0, 10 mM KCl. Mitotic chromosomes were pelleted by a 2-min spin in the microfuge and washed with a buffer containing 10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 0.5 mM EDTA, plus the indicated amounts of salt. LM-PCR analysis then proceeded as with intact cells. Fixing and staining of mitotic chromosomes was done as before[17].

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