Histone acetylation facilitates RNA polymerase II transcription of the *Drosophila hsp26* gene in chromatin

Karl P.Nightingale, Ralf E.Wellinger¹,
Jose M.Sogo¹ and Peter B.Becker²

Gene Expression Programme, European Molecular Biology
Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany and
¹Institut für Zellbiologie, ETH-Hönggerberg, CH-8093 Zürich,
Switzerland

²Corresponding author
e-mail: Becker@EMBL-Heidelberg.de

A number of activators are known to increase transcription by RNA polymerase (pol) II through protein acetylation. While the physiological substrates for those acetylases are poorly defined, possible targets include general transcription factors, activator proteins and histones. Using a cell-free system to reconstitute chromatin with increased histone acetylation levels, we directly tested for a causal role of histone acetylation in transcription by RNA pol II. Chromatin, containing either control or acetylated histones, was reconstituted to comparable nucleosome densities and characterized by electron microscopy after psoralen cross-linking as well as by *in vitro* transcription. While H1-containing control chromatin severely repressed transcription of our model *hsp26* gene, highly acetylated chromatin was significantly less repressive. Acetylation of histones, and particularly of histone H4, affected transcription at the level of initiation. Monitoring the ability of the transcription machinery to associate with the promoter in chromatin, we found that heat shock factor, a crucial regulator of heat shock gene transcription, profited most from histone acetylation. These experiments demonstrate that histone acetylation can modulate activator access to their target sites in chromatin, and provide a causal link between histone acetylation and enhanced transcription initiation of RNA pol II in chromatin.

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Introduction

In vivo, processes utilizing DNA as a template must contend with its packaging into chromatin, a complex array of nucleosomes, non-histone proteins and enzymatic activities which compact the DNA via a continuum of loosely defined higher order structures. Increasingly, it is being shown that this organization into chromatin adds an essential layer of transcriptional regulation (Felsenfeld, 1992, 1996). At the simplest level, chromatin appears as a regular array of nucleosomes. The structure of the nucleosome has been defined at high resolution (Luger *et al.*, 1997); 146 bp of DNA wind tightly around a central histone octamer core. From this compact structure the

flexible histone N-terminal tails reach out to contact sites, potentially situated on the nucleosome particle itself, as well as on the adjacent nucleosomes and in the intervening linker DNA. This leads to tightened histone–DNA contacts and to a stabilization of chromatin structure by promoting the folding of the chromatin fibre into higher order structures (Fletcher and Hansen, 1996; Luger *et al.*, 1997). As a functional consequence of these interactions the histone tails contribute to the transcriptional repression associated with nucleosome assembly. Individual nucleosomes impede the binding of factors to their cognate sites (Hayes and Wolffe, 1992) and act as a physical barrier to the elongating polymerase (Izban and Luse, 1991; Brown *et al.*, 1996). Deletion of the histone N-termini renders nucleosomal DNA considerably more accessible (Vettese-Dadey *et al.*, 1994). Transcription is further repressed by tail-dependent folding of the chromatin fibre *in vitro* (Hansen and Wolffe, 1992). Genetic experiments in yeast show that the histone tails contribute to both transcriptional regulation and the establishment of silencing at the yeast mating type loci via interactions with specific non-histone proteins (Grunstein *et al.*, 1995). It is likely that many of the observed functions of the histone tails are mediated by specific interactions with non-histone proteins.

The post-translational modification of the histone N-termini, most notably the acetylation of the ϵ -amino groups of conserved lysine residues, affects the properties of the histone tails (Turner and O'Neill, 1995; Grunstein, 1997). Studies on native chromatin and intact nuclei have documented a correlation between actively transcribed (and potentially active) regions and histone acetylation. A prominent example is the chicken β -globin domain which was shown to be both in a DNase I-sensitive configuration, and enriched in acetylated histones (Hebbes *et al.*, 1994). Histone acetylation has also been correlated with modified interactions of histone H1 (Perry and Annunziato, 1991). Conversely, inactive, silenced or heterochromatin domains are characterized by histone hypoacetylation (Braunstein *et al.*, 1993; Jeppesen and Turner, 1993). However, the correlation between transcriptional activity and histone acetylation does not hold when specific H4 isoforms are monitored, suggesting that acetylation of specific lysines, rather than bulk acetylation, may be important functionally (Turner, 1993; O'Neill and Turner, 1995; Turner and O'Neill, 1995).

At the level of the single nucleosome, bulk acetylation (Lee *et al.*, 1993) and, more specifically, acetylation of the H4 tails (Vettese-Dadey *et al.*, 1996), facilitates transcription factor binding to nucleosomal sites. The recent discovery of histone acetylase and deacetylase activities in both transcriptional regulators (Brownell and Allis, 1996; Ogryzko *et al.*, 1996) and a component of TFIID (Mizzen *et al.*, 1996) has fuelled proposals that the targeting of these activities to specific promoter-

proximal nucleosomes performs a central role in the control of transcription (Pazin and Kadonaga, 1997; Wolffe *et al.*, 1997). However, at this point it remains to be demonstrated that nucleosomes, rather than other factors involved in transcriptional control, are the physiological substrates for these activities. Both components of the general transcription machinery (Imhof *et al.*, 1997) and a transcription activator have been shown to be substrates for histone acetyltransferases (Gu and Roeder, 1997). In the latter study, acetylation of p53 by its co-activator p300 stimulates the binding of the activator to DNA. It therefore remains unclear whether the physiological target of the acetyltransferase p300 (Ogryzko *et al.*, 1996) is histones or transcription factors.

Clear correlations between acetylation and the increased transcription of specific genes by RNA polymerase (pol) II *in vivo* have been obtained by inducing global hyperacetylation in cells by treatment with the histone deacetylase inhibitor trichostatin A (TSA, Yoshida *et al.*, 1995; Bartsch *et al.*, 1996; Van Lint *et al.*, 1996a,b), but these studies are unable to distinguish whether the acetylation of histones, of other chromatin components or of the transcriptional machinery are causally linked to the increased transcription.

Recent *in vitro* studies utilizing a model dinucleosome, have demonstrated that histone acetylation has a direct effect on RNA pol III transcription (Ura *et al.*, 1997). In order to examine the consequences of histone acetylation on transcription by RNA pol II we adapted the cell-free chromatin assembly system derived from *Drosophila* embryos (Becker and Wu, 1992; Kamakaka *et al.*, 1993) to reconstitute chromatin from hyperacetylated histones purified from TSA-treated cells (Krajewski and Becker, 1998). Since histones are the only proteins with different acetylation status when acetylated chromatin is compared with control chromatin, any structural and functional differences can be causally linked to the acetylation status of the histones. Using this system we recently established a causal link between histone acetylation and an increased DNase I sensitivity of chromatin, as well as an increased flexibility of nucleosomal DNA (Krajewski and Becker, 1998). Here we extend these studies to assess the functional effects of histone acetylation on transcription by RNA pol II. The *hsp26* promoter serves as a useful model. We recently established highly efficient transcription of the *hsp26* promoter in *Drosophila* embryo extracts (Sandaltzopoulos *et al.*, 1995; Sandaltzopoulos and Becker, 1998). Chromatin assembly on this promoter represses transcription tightly. Heat shock factor (HSF)-specific transcription of a chromatin template lacking significant histone acetylation can be achieved only if transcription factors are allowed to bind to the promoter prior to chromatin assembly (Sandaltzopoulos and Becker, 1998). Using the *hsp26* gene as a model we now demonstrate that histone acetylation significantly affects the ability of RNA pol II to initiate transcription on a chromatin template by facilitating the access of key regulators to the promoter in chromatin. This finding establishes a causal relationship between histone acetylation and transcription by RNA pol II.

Fig. 1. Protein composition of the assembled chromatin. (Upper panels) input control (C) or hyperacetylated (A) histones (Hist.) and control (C) or acetylated (A) chromatin prior to (Chr.), or after incubation in transcription extract (Chr.+Ex) were separated on either a 15% SDS gel or on a TAU gel which can resolve the histone-acetylation isoforms. The protein size, core histones and acetylated histone H4 isoforms are indicated. (Lower panels) Western blot of the gel probed with tetra-acetylated histone H3 or tetra-acetylated histone H4 antibodies. Note that hyperacetylated H4 migrates slightly faster than control H4 on the SDS gel.

Results

Characterization of non-acetylated and acetylated chromatin templates

Chromatin was reconstituted using histones from either TSA-treated CV-1 cells, which accumulate hyperacetylated histone isoforms, or from untreated cells, in which the histones are primarily non-acetylated (Figure 1; Krajewski and Becker, 1998) onto a 7.75 kb plasmid containing a *hsp26* minigene (Sandaltzopoulos *et al.*, 1995). This process involves the prior depletion of the endogenous histones present in the chromatin assembly extract such that chromatin is assembled quantitatively from the input, exogenous histones (Krajewski and Becker, 1998). The chromatin assembly reaction generates complex chromatin containing many non-histone proteins and enzymatic activities.

An analysis of the histone isoform distribution of control and acetylated chromatin on Triton-acid-urea (TAU) gels revealed a significant deacetylation of histones during chromatin assembly (Figure 1). This deacetylation was not uniform; histone H4, a hallmark of acetylated chromatin, was not deacetylated significantly (Figure 1, lanes 8 and 10). Histone H2B is acetylated to an extraordinary degree in TSA-treated CV-1 cells, such that it runs anomalously on SDS gels (Figure 1, lane 2); however, significant deacetylation occurs during chromatin reconstitution and hence only moderately acetylated H2B iso-

forms (with a normal mobility on SDS gels) were found in reconstituted chromatin (Figure 1, compare lane 4 with lane 2 and lane 10 with lane 8). Histone H3 was also deacetylated during chromatin assembly such that only a modest degree of acetylation remained in the chromatin. As the reconstituted chromatin in this study was subsequently incubated in transcription extract (another potential source of histone modifying activities), it was important to document any further modifications that might occur during this time. We used chromatin coupled to paramagnetic beads to examine the protein composition of control and acetylated chromatin. Through Coomassie Blue (Figure 1, lanes 3 and 4) or silver (data not shown) staining, we found the protein composition of both chromatin samples to be very similar, both qualitatively and quantitatively. Furthermore, while many proteins from the transcription extract associate with chromatin, we failed to see major differences in the protein profiles (Figure 1, lanes 5 and 6). The histone isoform profile in the reconstituted chromatin was assessed by Western blotting and probing with antibodies raised against either (i) the tetra-acetylated isoform of histone H3 or (ii) the tetra-acetylated isoform of histone H4 (Figure 1, lower panels). This analysis confirmed the earlier assessment of the differential deacetylation of histone H3 and H4 during chromatin assembly (see TAU gel); while the hyperacetylation of H3 was reversed almost completely upon chromatin assembly (Figure 1, lanes 2 and 4), a high level of tetra-acetylated histone H4 was incorporated into the chromatin and was not diminished detectably upon incubation in transcription extract (Figure 1, compare lanes 2, 4 and 6). The main feature of chromatin reconstituted from hyperacetylated histones is therefore the high levels of histone H4 acetylation; however, some acetylation of H2B is also present (the gel systems do not permit the precise analysis of H2A acetylation). For convenience we refer to this chromatin as 'acetylated' and the corresponding control chromatin assembled with histones from untreated CV-1 cells (containing a low level of histone acetylation) as 'control' chromatin throughout the following analysis. The similarity of the protein profiles in the acetylated and control chromatin suggests that the structural and functional characteristics of acetylated chromatin are linked directly to the properties of nucleosomes rather than being an indirect consequence of a differential stoichiometric association of (a) non-histone protein(s).

As we intended to compare the transcriptional potential of the control with that of the acetylated chromatin templates, our initial concern was to establish conditions where the different templates were chromatinized equivalently. However, the interpretation of the standard assays of chromatin assembly by: (i) the generation of a nucleosomal ladder upon MNase digestion; or (ii) supercoiling analysis of deproteinized chromatin templates, is complicated by the structural differences between control and acetylated chromatin (Simpson, 1978; Bode *et al.*, 1980; Norton *et al.*, 1989). We therefore analysed the reconstituted chromatin using psoralen cross-linking and electron microscopy (Sogo *et al.*, 1984). This technique exploits the ability of tri-methyl psoralen to cross-link the two paired strands of DNA when irradiated with UV light. The agent is particularly useful for chromatin studies as it is not able to cross-link DNA wrapped around a nucleosome, and is

therefore restricted to the linker DNA, and does not disrupt chromatin structure (Conconi *et al.*, 1984). When the cross-linked DNA in chromatin is deproteinized, denatured and analysed by electron microscopy the plasmid appears as a series of single-stranded bubbles, which correspond to the previously nucleosomal DNA, interspersed by cross-linked linker DNA. Combining this technique with statistical analysis permits the quantification of several chromatin characteristics, including: (i) the absolute number of nucleosomes assembled onto specific templates; (ii) the length of protection from cross-linking (bubble size); and (iii) the overall degree of chromatinization.

A comparison of the structural characteristics of control and acetylated chromatin templates is presented in Figure 2. Chromatin was assembled from either control (C) or acetylated (A) histones, and aliquots of the samples were subjected to supercoiling analysis (Figure 2A), MNase digestion (Figure 2B) or psoralen cross-linking and electron microscopy (Figure 2C). The combination of these assays revealed that chromatin assembled from control and acetylated histones was remarkably similar. If assembled at the same histone:DNA ratio both types of chromatin constrained the same number of supercoils (Figure 2A), and showed the same nucleosome spacing upon MNase digestion; however, we consistently observed that the acetylated chromatin templates showed increased sensitivity to MNase (Figure 2B). Furthermore, representative electron micrographs of control and acetylated templates looked similar, even when the cross-linking was performed at different temperatures in an attempt to unveil any structural differences between the samples (Figure 2C; 0°C, upper or 37°C, lower). In both cases the DNA largely appeared as single-stranded bubbles, with very few extended double-stranded DNA regions, which would correlate to non-nucleosomal, naked DNA 'patches' in the assembled chromatin. No plasmids without bubbles were detected. The calculated *R*-values (indicating the extent of single-strandedness and therefore the degree of chromatinization) of the reconstituted chromatin (Table I), were very similar in control and acetylated chromatin and are close to those obtained from analyses of native chromatin in nuclei [0.71 and 0.81 (Sogo *et al.*, 1986; Widmer *et al.*, 1988; Gasser *et al.*, 1996)]. We conclude that the majority of the reconstituted chromatin is organized in arrays of regularly spaced nucleosomes and the density of the nucleosomes is comparable with that observed for native chromatin.

An examination of the total number of single-stranded bubbles in 100 randomly selected, control or acetylated 'minichromosomes' showed similar numbers irrespective of whether the cross-linking was performed at 0 or 37°C; control templates had an average of 29 ± 4 bubbles whereas acetylated templates had 31 ± 4 bubbles. Even though these results are not statistically significant, the analysis revealed a trend towards more nucleosomes in the acetylated chromatin in remarkable contrast to the sensitivity of this chromatin to MNase (Figure 2B) and to DNase I (Krajewski and Becker, 1998). Closer inspection showed that there were two populations of single-stranded bubbles; in addition to a majority of small bubbles, several larger bubbles (Figure 2C, arrows) were evident in all samples. We subjected 35 minichromosomes from each assembly or cross-linking condition to further examination and

Fig. 2. Characterization of control and acetylated chromatin. (A) Supercoiling analysis. Control (C) and acetylated (A) chromatin templates are compared with the natural level of supercoiling present in the naked template (N). Assuming a superhelical density in bacteria of -0.05 , the protein-free DNA contains -37.5 supercoils. The asterisk indicates nicked DNA templates. (B) Micrococcal nuclease analysis. Control (C) and acetylated (A) chromatin was digested with MNase for 30 s (lanes 1 and 3) or 3 min (lanes 2 and 4). The marker is a 123 bp ladder. (C) Electron micrographs of psoralen cross-linked material. Control (C, left panels) and acetylated chromatin (A, right panels) was cross-linked at 0°C (upper panels) or 37°C (lower panels). 'Dinucleosomal' single-stranded-bubbles are indicated by arrows. The bar corresponds to 500 nucleotides. (D) Distribution of single-stranded bubble sizes in control (C) or acetylated (A) chromatin cross-linked at 0°C (upper panels) or 37°C (lower panels). Bubble size is presented as nucleotides and directly corresponds to base-pairs in double-stranded DNA. The curves of best fit for the two bubble size populations are shown.

Table I. Number of single-stranded bubbles and detailed size measurement in control and acetylated chromatin cross-linked at different temperatures

Experiment	No. of molecules analysed	No. of ss-bubbles per molecule	No. of total ss-bubbles measured	Size of ss-bubbles	R-value
Non-acetylated (0°C)	35	26 ± 3	925	142 ± 27 250 ± 63	0.73 ± 0.06
Acetylated (0°C)	35	32 ± 4	1123	139 ± 27 251 ± 47	0.75 ± 0.07
Non-acetylated (37°C)	35	30 ± 3	1057	139 ± 30 261 ± 58	0.81 ± 0.05
Acetylated (37°C)	35	33 ± 3	1154	131 ± 29 273 ± 31	0.76 ± 0.07

measured the sizes of 1000 bubbles from each sample (Table I). The plots of the bubble size distribution on these minichromosomes (Figure 2D), and statistical analysis (Table I) reveal two populations of bubble size. A majority of small bubbles, ranging in size between 131 ± 29 bp (A, 37°C) and 142 ± 27 bp (C, 0°C), correspond to mononucleosomes. A smaller population of bubbles ranging in size between 250 ± 63 bp (C, 0°C) and 273 ± 31 bp (A, 37°C) may correspond to close-packed dinucleo-

somes. Interestingly, acetylated chromatin contained proportionally fewer 'dinucleosomes' (Figure 2D). In summary, this analysis confirmed that the control and acetylated templates were assembled to an equivalent degree, and at *in vivo* levels of chromatinization. Since the chromatin samples constrained equivalent numbers of supercoils, we used this as the diagnostic assay for equivalent chromatinization in the subsequent experiments.

Histone acetylation enhances transcription of a chromatin template by RNA pol II

The transcriptional potential of the chromatin templates was assessed by *in vitro* transcription in a nuclear extract derived from heat-shocked *Drosophila* embryos. This extract transcribes the chromatin-free *hsp26* gene efficiently (Sandaltzopoulos *et al.*, 1995; Sandaltzopoulos and Becker, 1998). The assembly of control chromatin on the template repressed transcription profoundly [below and Sandaltzopoulos and Becker (1998)]; however, the level of repression is sensitive to the degree of chromatin assembly (data not shown), making it essential that each individual chromatin sample to be transcribed is characterized structurally by subjecting aliquots to MNase and supercoiling analyses. In all cases this confirmed that the two types of chromatin were similar qualitatively and quantitatively, both with respect to nucleosome spacing, as visualized by MNase digestion (Figures 3C and 4B), and in the amount of superhelicity constrained, which indicated that they were chromatinized equivalently (Figure 3B).

Transcription of chromatin templates was compared with templates subjected to a 'mock assembly' reaction, in which no exogenous histones were added. These templates were equivalent transcriptionally to naked DNA (Figure 4A; compare M with N), indicating that no other components of the crude chromatin assembly system, other than the histones, inhibit transcription. MNase digestion (Figure 3C; M) and supercoiling analysis (Figure 3B; M) demonstrate the extent of endogenous histone depletion. Assembly of control chromatin (C) repressed transcription of the *hsp26* promoter severely when compared with the robust transcription of the 'mock assembled' control (M). In contrast, acetylated chromatin (A) was significantly less repressive (Figure 3A). The magnitude of this effect was enhanced when a stoichiometric amount of histone H1 (i.e. 1 H1 per nucleosome) was incorporated into the chromatin. Histone H1 is incorporated with similar efficiencies into either type of chromatin (Krajewski and Becker, 1998). Note also the corresponding increase in nucleosomal repeat length of ~20 bp in both samples revealed by the MNase analysis in Figure 3C (Becker and Wu, 1992; Kamakaka *et al.*, 1993; Blank and Becker, 1995). Histone H1 repressed transcription from the control chromatin template much more than transcription from the acetylated template (Figure 3A) without inducing gross structural changes, as both control and acetylated templates continued to constrain equivalent numbers of supercoils (Figure 3B). The relative increase in transcriptional activity of acetylated chromatin was observed over a wide range of chromatin assembly conditions that generate chromatin with nucleosome repeat lengths between 153 and 185 bp (Blank and Becker, 1995; Figure 4).

The increased levels of *hsp26* RNA were revealed by extension of an oligonucleotide hybridized at position +71 relative to the transcription start site. Considering that TFIID still makes contacts down to position +35 on the *hsp26* promoter (Purnell *et al.*, 1994), it is likely that the positive effect of acetylation is not due to increased elongation through chromatin but rather is due to a promoter-proximal step, such as facilitated transcriptional initiation or promoter clearance. In keeping with an effect of histone acetylation on transcription initiation, we found

Fig. 3. Transcriptional analysis of *hsp26* gene in chromatin. (A) *In vitro* transcription of control (C) or acetylated (A) chromatin in the absence (–H1) or presence (+H1) of stoichiometric amounts of histone H1 is compared with transcription from a mock assembled template (M). Transcription from the chromatinized *hsp26* minigene template is indicated (Transcript), as is endogenous *hsp26* transcripts present in the extract (Endog. RNA) and a recovery control (Rec. Control). (B) Supercoiling analysis of the chromatin samples. Annotations as in (A). A naked DNA template (N) is included for comparison. The asterisk indicates nicked templates and relaxed plasmids that are not assembled into chromatin in a 'mock assembly' reaction (M), i.e. an assembly reaction in the absence of histones. (C) Micrococcal nuclease analysis of chromatin samples digested for 30 s or 3 min. Annotations as in (A). Marker is 123 bp ladder.

that the transcriptional difference between acetylated and control chromatin did not increase with the length of RNA transcript. The effect was identical when we measured RNA by annealing a primer at position +400, i.e. after transcription through 1–2 nucleosomes (Figure 5). The following observation of increased transcription factor access to acetylated chromatin also supports this interpretation.

Chromatin acetylation facilitates transcription factor access

Several studies have shown that histone acetylation can facilitate the binding of RNA pol II transcription factors to mononucleosomal DNA (Vettese-Dadey *et al.*, 1996; Ng *et al.*, 1997). We therefore examined whether increased factor access contributes to the increased transcription we

Fig. 4. Transcriptional analysis with chromatin of varied nucleosome densities. **(A)** *In vitro* transcription of control (C) or acetylated (A) chromatin templates assembled with increasing KCl concentrations (0, 6, 60, 90, 120 mM). Transcription of mock assembled (M) and naked DNA templates (N) are included for comparison. The gel is labelled as in Figure 3A. **(B)** Micrococcal nuclease digestion of chromatin assembled with increased KCl concentrations. Annotations as in (A). The nucleosome repeat length of the chromatin samples is indicated (NRL). Marker is a 123 bp ladder.

observed in acetylated chromatin, by making use of immobilized templates (Sandaltzopoulos and Becker, 1998). Linear *hsp26* templates were coupled to paramagnetic beads and chromatinized with the two types of histones. When the immobilized chromatin was purified on a magnet and assayed for transcription as before, the increased activity of acetylated chromatin was again apparent (Figure 6, lanes 1 and 2). To test whether transcription factor access was limiting, we pre-incubated the templates with transcription extract prior to chromatin assembly to allow the unhindered binding of transcription factors and components of the basal machinery. This procedure does not inhibit chromatin assembly as determined by the generation of a nucleosomal ladder upon MNase digestion (Sandaltzopoulos and Becker, 1998), but does generate a pattern of DNase I hypersensitive sites in the promoter similar to those seen *in vivo* (data not shown). Under these conditions chromatin assembly did not repress transcription and we observed similar amounts of transcription from control and acetylated templates (Figure 6, lanes 3 and 4). This suggests that the enhanced transcription in acetylated chromatin arises from the increased access of one or several components of the transcriptional machinery.

In order to determine which transcription factors profited most from the histone acetylation we followed two comple-

Fig. 5. Studies on transcription elongation through chromatin. Transcription of control (C) or acetylated chromatin (A) compared with mock assembled (M) and naked DNA templates. *hsp26* transcripts of increasing length were detected by primer extension from primers annealed at positions +100, +200 or +400 downstream of the start site. The transcripts generated from the *hsp26* minigene are indicated by stars.

Fig. 6. Effect of pre-initiation complex on transcription in chromatin. **(A)** Experimental scheme. **(B)** *In vitro* transcription of immobilized control (C) or acetylated (A) chromatin templates without (-PIC), or with pre-initiation complex formation prior to chromatin assembly (+PIC). Transcription of mock assembled (M) and naked DNA templates (N) are included for comparison. The gel is labelled as in Figure 3A.

mentary approaches: (i) determination of the DNA sequences required for transcription in chromatin; and (ii) direct monitoring of the association of factors with the two types of chromatin.

The regulatory elements of the *hsp26* promoter are well-known from *in vivo* (Lu *et al.*, 1993) and *in vitro* studies (Sandaltzopoulos *et al.*, 1995). A proximal regulatory element includes the TATA box, proximal heat shock element (HSE) and an adjacent GAGA element, while a distal regulatory site corresponds to the distal

Fig. 7. *In vitro* transcription using *hsp26* promoter mutants. (A) Scheme of wild-type (wt) and mutant *hsp26* promoters used. The approximate positions of the sequence elements are indicated. (B) *In vitro* transcription of promoter mutant (M10, M8, M21, M4) and wild-type templates (wt) assembled into control (C) or acetylated (A) chromatin, or subjected to a mock assembly (M). Transcription from the wild-type and mutant *hsp26* minigene templates are indicated (Transcript). The gel is labelled as in Figure 3A.

HSE and GAGA binding sites (Figure 7A). Templates with mutated *hsp26* promoters were assembled into control and acetylated chromatin and analysed for their transcription potential as described previously (Figure 7B). A template bearing only the TATA box (M10) supported a very low level of transcription, even in the absence of chromatin (M) and there was no discernible transcription from chromatinized templates even after prolonged exposure (data not shown). Addition of the proximal HSEs however (M8), resulted in significantly increased transcription from both mock assembled and chromatinized templates, confirming the important role of the activator HSF. This minimal promoter, containing only proximal HSEs and the TATA box, clearly showed increased transcription from the acetylated template. The addition of GAGA elements (M21, M4) to the promoter enhanced transcription significantly, but to a similar degree on control and acetylated chromatin templates, such that the foldness of induction from acetylation remained the same. Interestingly, the GAGA elements did not increase transcription in the mock assembled control, confirming that GAGA factor is involved in overcoming chromatin-mediated transcriptional repression, but by a mechanism that does not profit from histone acetylation. These results suggest that the HSE and TATA box are the significant sequence elements for the increased transcription observed in acetylated chromatin.

This finding was confirmed independently by experi-

ments utilizing templates coupled to paramagnetic beads to examine the binding of specific transcription factors to control and acetylated chromatin. Chromatinized *hsp26* templates were incubated in transcription extract under standard transcription conditions (rNTPs), purified on a magnet, and the bound proteins were analysed by Western blotting using relevant antibodies (Figure 8). In the presence of non-specific competitor DNA (which is also present in the *in vitro* transcription reactions) all the proteins examined bound specifically to the *hsp26* templates when compared with the reduced, non-specific binding to the vector (pBluescript) control; however, only HSF showed a clear increased binding to the acetylated chromatin templates (Figure 8; compare lane 2 with lane 3). In contrast, the C-terminal domain of RNA pol II (CTD), GAGA factor (GAGA) and several components of the basal machinery, including TBP, TFIIA and TAFII150 (data not shown), showed no detectable difference in binding to control or acetylated chromatin. In summary, we present evidence that histone acetylation facilitates transcription by RNA pol II at an early step by increasing the access of HSF to its target elements in the *hsp26* promoter.

Discussion

The assembly of chromatin from purified acetylated histones under physiological conditions allowed the first

Fig. 8. Western blot analysis of protein composition of chromatin templates. Control (C) or acetylated (A) chromatinized *hsp26* templates were incubated in transcription extract in the presence of ATP. The samples are compared with a control chromatin pBluescript template (pBS). The protein composition of the chromatin samples was separated on SDS gels, Western-blotted and subsequently probed with antibodies specific for the RNA pol II (CTD), HSF, GAGA or TBP. A silver stained gel of the histones in the transferred gel is presented to confirm equal loading (histones).

direct assessment of the effect of histone acetylation on transcription by RNA pol II. The analysis revealed a clear causal relationship between the acetylation of histones, particularly of histone H4, and the efficiency of transcription by RNA pol II. The effect was not due to increased efficiency of elongation but arose from the increased association of HSF with the acetylated chromatin templates. To reach this conclusion we characterized the structure of control chromatin extensively and then compared the structure with that of acetylated chromatin. The combination of psoralen cross-linking and electron microscopy allowed visualization of the high quality of the chromatin reconstitution and allowed us to address several open questions concerning the structure of acetylated chromatin.

A causal relationship between histone acetylation and transcription by RNA pol II

Chromatin containing elevated levels of histone acetylation, particularly of histone H4, was transcribed $\sim 5\times$ better than control chromatin containing low levels of histone acetylation. This moderate stimulation is comparable in magnitude with that seen for transcription by RNA pol III *in vitro* on dinucleosome templates (Ura *et al.*, 1997), but is two orders of magnitude lower than the effects observed for specific promoters upon TSA treatment of tissue culture cells (Girardot *et al.*, 1994; Schlake *et al.*, 1994; Van Lint *et al.*, 1996a,b) or in *in vitro* reconstituted chromatin (Sheridan *et al.*, 1998). However, these large TSA inductions observed may well reflect the cumulative effect of this agent on several targets; TSA treatment is known to induce histone hyperacetylation, but may also result in the acetylation of components of the basal machinery (Mizzen *et al.*, 1996; Imhof *et al.*, 1997) or critical transcriptional activators (Gu and Roeder, 1997; Gu *et al.*, 1997). This suggests that the transcriptional consequence of histone acetylation may be relatively modest. In *Drosophila* males, elevated levels of H4 Lys16

acetylation on the X chromosome correlate with a 2-fold increase in transcription from X-linked genes (Bone *et al.*, 1994). This is a small but vital effect, as failure to up-regulate X-linked genes is lethal for males (for review see Kelley and Kuroda, 1995). We suggest that histone H4 acetylation is causally involved in this small but precise increase of transcription.

Our analysis clearly establishes a role for histone acetylation in transcription but it may underestimate the actual effect. First of all, the treatment of cells with the deacetylase inhibitor TSA presumably generates unphysiological acetylation levels. Work from Turner's laboratory suggests that it is not the absolute level of histone H4 acetylation that matters, but rather the precise acetylation site on the N-terminus of H4. Isoform-specific antibodies revealed that H4 acetylated at Lys16 correlated with euchromatic chromatin, and hence potentially active sites, whereas H4 acetylated at Lys12 was enriched in transcriptionally inactive heterochromatin (Turner *et al.*, 1992). It is therefore possible that, for example, acetylation of Lys12 in tetra-acetylated H4 counteracts the activating effect of Lys16. Similarly, it is also possible that acetylation of histone H3 affects transcription. Recently Allis and co-workers (Kuo *et al.*, 1998) observed that activated transcription correlated with the targeting of H3 and H4 acetylation by the Gcn5 acetyltransferase to the vicinity of a yeast promoter. The deacetylation of histone H3 during chromatin assembly prevented us from determining the effect of H3 acetylation on transcription.

Finally, although our analysis does not rule out an effect of histone acetylation on RNA pol II elongation, especially through long arrays of nucleosomes, the stimulation of transcription we observed appeared to arise solely from the increased ability of HSF to interact with acetylated chromatin and was therefore due to an early event, related to transcription initiation or promoter clearance.

The association of a transcription activator, not of the basal RNA pol II machinery, is limiting to transcription in chromatin

In a recent study, Jones and colleagues (Sheridan *et al.*, 1998) reported a striking stimulation of transcription after TSA treatment of chromatin reconstituted on the HIV-1 enhancer. An important difference between those experiments and ours is that the effect of TSA on the HIV-1 enhancer in chromatin required the binding of transcription factors to the enhancer prior to chromatin assembly, whereas we have assayed the opening of chromatin by transcription factors added subsequently to tightly assembled chromatin. While it is possible that the acetylation of non-histone proteins contributed to the large induction observed by Sheridan *et al.* (1998), it is also possible that acetylation stimulates chromatin opening at various, promoter-specific, steps. While we assayed an initial chromatin opening event, Sheridan *et al.* (1998) focused on events subsequent to the binding of key regulators.

Our data appear to be in contrast with recent experiments (Mizuguchi *et al.*, 1998), which found that transcription of *Drosophila* chromatin did not require histone acetylation, but only the action of NURF, a nucleosome-remodelling factor which is also abundant in our transcription extracts (Tsukiyama and Wu, 1995). However, a

closer comparison between our experiments and those of Mizuguchi *et al.* (1998) revealed a fundamental difference. While our experiments involved the binding of native HSF to a native promoter, these authors analysed the ability of a synthetic activator, comprising the activation domain of HSF fused to the yeast Gal4 DNA binding domain, to interact with chromatin. The DNA binding domains of HSF and Gal4, however, differ markedly in their ability to contact nucleosomal DNA binding sites; Gal4-derivatives bind nucleosomal DNA with reasonable efficiency while HSF is unable to do so (Taylor *et al.*, 1991). This is because it is impossible to arrange an HSE on the surface of a nucleosome such that all three HSF monomers can interact with their penta-nucleotide consensus element. This suggests that NURF and histone acetylation act at different points in the transcriptional process; NURF induces transcription by remodelling nucleosomes to which factor(s) (Gal4-HSF) are already bound, whereas histone acetylation facilitates an earlier step; the actual binding of a transcription factor (HSF) to its site in chromatin. HSF can therefore be added to the list of factors in which histone acetylation can stimulate binding to chromatin. In nucleosome binding experiments, Workman and colleagues showed that acetylation of the N-terminus of H4, rather than that of H3, facilitated the binding of transcription factors (Vettese-Dadey *et al.*, 1996), a finding that is consistent with the fact that our acetylated chromatin is characterized by high levels of acetylated H4.

A further important new conclusion from our analysis is that the interaction of the activator (HSF) was the only limitation for transcription; equivalent amounts of TFIID, TFIIA, GAGA factor and RNA pol II were found in both acetylated and control chromatin (Figure 8). Transcription levels from acetylated chromatin were as high as if the entire transcription machinery had been allowed to associate with the promoter prior to chromatin assembly. Also, under those circumstances HSF remains a crucial activator for heat shock genes in chromatin (Becker *et al.*, 1991; Sandaltzopoulos and Becker, 1998). The presence of GAGA elements in the promoter enhanced transcription of control and acetylated chromatin significantly, consistent with an interaction of GAGA factor with HSF stabilizing HSF binding to DNA (Mason and Lis, 1997). Furthermore, the facilitated binding of HSF did not result in a corresponding increase in the association of TFIID or the other general transcription factors examined. This is consistent with our recent observation that HSF stimulates transcription by facilitating re-initiation from potentiated promoters in chromatin, rather than by recruiting TFIID (Sandaltzopoulos and Becker, 1998). It is possible that the presence of GAGA factor facilitated the recruitment of the basal transcription machinery to the promoter, which remained poised to be activated by HSF (Lu *et al.*, 1993). HSF action involves the release of a paused polymerase (reviewed in Lis and Wu, 1994) perhaps by recruitment of factors that relieve chromatin-mediated repression downstream of the transcription start site (Brown *et al.*, 1996; Brown and Kingston, 1997).

Possible mechanisms

In principle, histone acetylation can affect the binding of transcription factors to chromatin at two levels; either at

the level of the single nucleosome that occludes the binding of a factor to a site and/or at the level of the folding of the nucleosomal fibre into higher order structures. There is some evidence that histone acetylation may facilitate the binding of transcription factors to isolated mononucleosomes. While it is controversial whether the binding of the pol III transcription factor TFIIA to mononucleosomes can profit from the effect of histone acetylation (Lee *et al.*, 1993; Howe and Ausio, 1998) a number of pol II activators bind acetylated nucleosomes preferentially (Vettese-Dadey *et al.*, 1996). This must reflect a significant structural difference between individual acetylated and non-acetylated nucleosomes, possibly arising from the reduced constraints on the DNA in hyperacetylated nucleosomes (Norton *et al.*, 1989, 1990). A recent study suggests that acetylated nucleosomes are inherently unstable, such that transcription factor binding induces the complete disruption of the particle (Ng *et al.*, 1997).

Similarly, histone acetylation has clear consequences for higher order chromatin structure; hyperacetylated chromatin adopts a more 'open', extended structure (Garcia-Ramirez *et al.*, 1995), and has been shown to be globally sensitive to DNase I *in vivo* (Hebbes *et al.*, 1994). We have recently shown, using thermal untwisting assays, that the DNA in reconstituted acetylated chromatin is less restrained than in control chromatin (Krajewski and Becker, 1998). This may be reflected in the trend towards reduced *R*-values observed in acetylated, but not control, chromatin when psoralen cross-linking is performed at elevated temperatures (Table I). However, the structural basis of these changes and their functional consequences remain unexplored. Similarly, the effect of histone acetylation on nucleosome–nucleosome interactions is undefined, although inter-nucleosomal contacts involving the N-terminal tails are likely to have a significant impact on higher order chromatin structure. This may reflect experimental difficulties; the histone tails are highly flexible and contacts are presumably transitory; indeed, tail contacts are known to differ when interactions are examined in an array of nucleosomes or in an isolated mononucleosome (Usachenko *et al.*, 1994; Fletcher and Hansen, 1995). It is also unclear whether the inter-nucleosomal contact observed in the recent high resolution crystal structure of the nucleosome, in which one of the H4 tails interacts with the H2A–H2B dimer on a neighbouring particle (Luger *et al.*, 1997), is relevant physiologically or is due simply to electrostatic interactions promoted by packing forces. It is interesting to speculate that the close-packed dinucleosomes detected by psoralen cross-linking in our reconstituted chromatin (Figure 2D) and which appear to be modulated by histone acetylation (Table I), correspond to nucleosome–nucleosome interactions.

In summary therefore, chromatin higher order structure is clearly modified by histone acetylation, but whether this has a functional effect is unclear. The observation that histone acetylation is a domain-wide phenomenon (Turner *et al.*, 1992), combined with recent evidence that histone acetylation can be targeted to a defined area surrounding promoters (Kuo *et al.*, 1998), suggests that acetylation has functional consequences at the level of both the individual nucleosome and higher order structure.

Nucleosomes containing high levels of H4 acetylation constrain equivalent superhelicity when compared with control nucleosomes

Bradbury and colleagues have shown that hyperacetylation of H3 and H4 results in a reduced nucleosome linking number change, such that acetylated nucleosomes only constrain 0.8 superhelical turns, 20% less than the one superhelical turn constrained by unmodified nucleosomes (Norton *et al.*, 1989, 1990). However Lutter *et al.* (1992) were unable to detect an equivalent effect of butyrate-induced histone acetylation *in vivo*. The reconstitution of chromatin with regular, physiological nucleosome spacing and with *R*-values (degrees of chromatinization) close to *in vivo* levels enabled us to re-evaluate the controversy; according to Bradbury's determination we would expect that our 7.75 kb acetylated chromatin template should contain up to eight more nucleosomes than control chromatin templates at identical levels of supercoiling. The direct determination of nucleosome numbers by psoralen cross-linking and electron microscopy analysis of the denatured plasmids clearly showed that this was not the case. Titration of equivalent histone amounts into the chromatin assembly reaction led to chromatin with equivalent levels of supercoiling, *R*-values and bubble numbers. A slight increase in the number of mononucleosome-derived bubbles in acetylated chromatin reflects the reduced number of dinucleosome-derived bubbles in this chromatin. However, these deviations can only be evaluated as trends since the high standard deviation in these experiments does not permit further conclusions; within the limits of the assay, acetylated and control chromatin are remarkably similar. Our analysis differs in several aspects from those of Bradbury and colleagues (Norton *et al.*, 1989, 1990) which might explain the different results. (i) The degree of histone acetylation was influenced by deacetylases in the chromatin assembly system and hence our final levels of acetylation, notably of histone H3, might have been lower than that of Norton *et al.* (1989, 1990). Since the degree of acetylation of histone H4 was clearly comparable, if not higher in our experiments, it is possible that, for example, the acetylation of histone H3 is the main determinant of the reduced linking number change observed. (ii) The reconstitution procedures differed fundamentally. Norton *et al.* (1989, 1990) used a salt gradient dialysis reconstitution procedure in which the octamer core, consisting of the globular histone domains is known to bind DNA considerably before the tails (Walker, 1984), while our chromatin was reconstituted under physiological conditions. This affects the precise interactions of the tails with DNA and adjacent nucleosomes which in turn may influence the linking number change. (iii) Norton *et al.* (1989, 1990) reconstituted sub-saturating levels of nucleosomes whereas we assembled chromatin with nucleosome densities similar to *in vivo* levels. It is known that the nucleosome density in chromatin affects its propensity to fold into higher order structures (Hansen and Lohr, 1993), perhaps by affecting tail-mediated nucleosome–nucleosome interactions which, in turn, may influence the linking number change. Finally, it cannot be excluded that, as yet undefined, non-histone proteins associated with chromatin reconstituted in the *Drosophila* embryo extracts also affect the levels of superhelicity.

Materials and methods

Plasmids and probes

The *Drosophila hsp26* minigene (*phsp26M*), and the mutant *hsp26* template derivatives (M10, M8, M21, M4), have been described previously (Sandaltzopoulos *et al.*, 1995). To immobilize the templates these plasmids were cleaved in the polylinker using *NotI* and *SpeI* to generate one short (13 bp) and two long fragments. The fragment containing the *hsp26* gene was selectively biotinylated by incorporation of biotin-21-dUTP and biotin-14-dATP. The short biotinylated fragment and free dNTPs were removed by gel filtration and the DNA quantitated photometrically. Immobilization to Dynabeads M280 (Dynal, Oslo) was as described previously (Sandaltzopoulos *et al.*, 1994). Agarose gel analysis of the DNA fragments remaining in the supernatant after the coupling reaction allowed precise quantification of the immobilized template DNA. The oligonucleotides used in the primer extension assays corresponded to the upper strand as follows; '100': +71/+101; '200': +208/+238; '400': +408/+438. Primer '100' was used in all experiments except when stated otherwise.

Histone isolation and chromatin assembly

Control and hyperacetylated histones were prepared from untreated or TSA (Wako) treated Green Monkey kidney CV-1 cells as described previously (Krajewski and Becker, 1998). Chromatin assembly extracts were derived from 3- to 6-h-old *Drosophila* embryos (Becker and Wu, 1992; Blank *et al.*, 1997), and endogenous histone depletion and chromatin assembly was performed as described previously (Blank *et al.*, 1997; Krajewski and Becker, 1998). Typically, a chromatin assembly reaction contained 600 ng plasmid DNA, 1.8 µg purified histones, 20 µl depleted chromatin assembly extract, 14 µl 10× reaction buffer and EX-80 in a total volume of 140 µl (Blank *et al.*, 1997). Assembly was for 6 h at 26°C. Chromatin assembly was monitored by micrococcal nuclease digestion and supercoiling analysis (Krajewski and Becker, 1998).

Psoralen cross-linking

Psoralen cross-linking of *in vitro* assembled chromatin was performed essentially as described by Gasser and Laemmli (1986). Typically, two standard chromatin assemblies (600 ng) were pooled, and half the chromatin examined by psoralen cross-linking while the remainder was subjected to MNase digestion or supercoiling analysis (Krajewski and Becker, 1998). To cross-link the sample, 140 µl chromatin assembly (600 ng DNA) was placed in a inverted Eppendorf cap, such that the sample forms a shallow volume, and the cap placed on a heating block maintained at the required cross-linking temperature. The sample was mixed with 5 µl 4, 5', 8-trimethylpsoralen (200 µg/ml, Sigma) and incubated for 5 min in the dark to permit sample equilibration. This was subsequently irradiated under four UV lights (366 nm, G15T8 Sylva) for 5 min at 25 J/m² (~4.5 cm). This cycle of psoralen addition, equilibration and subsequent sample irradiation was repeated a further three times to ensure cross-linking was to completion. After transfer of the sample to a tube and the addition of 50 µl Stop mix (2.5% sarkosyl, 100 mM EDTA pH 8.0) and 4 µl RNase A (10 mg/ml) the sample was incubated at 37°C for 30 min. The samples were subsequently deproteinized by the addition of 4 µl 20% SDS and 10 µl Proteinase K (10 mg/ml), and incubated at 37°C overnight, prior to extraction with phenol/chloroform and chloroform and final ethanol precipitation. The plasmid DNA was subsequently converted to an open circular form, by resuspension of ~300 ng DNA in 100 µl buffer containing ethidium bromide (100 mM NaCl, 15 mM MgCl₂, 20 mM Tris pH 8.0, 0.2 mg/µl EtBr), and the addition of 0.5 µl DNase I (2 U). The sample was incubated at 30°C for 10 min prior to extraction with phenol/chloroform, chloroform and final ethanol precipitation. The DNA was dissolved in water to a concentration of 50 ng/µl. The efficiency of plasmid relaxation was analysed on an agarose gel.

Electron microscopy and quantitative analysis

Purified and nicked plasmid DNA was spread for electron microscopy under denaturing conditions as described previously (Sogo *et al.*, 1984), except that the psoralen-cross-linked DNA was incubated for 15 min at 42°C in a buffer containing 50% formamide and 0.5 M glyoxal. The denatured DNA was placed directly into redistilled water. The size of the single-stranded bubbles was calculated from the contour length of the plasmid DNA (7.75 kb) as described previously (Gasser *et al.*, 1996).

Transcription and primer extension

The preparation of transcription extract from heat-shocked *Drosophila* embryos and the standard reaction were as described by Sandaltzopoulos and Becker (1995). Either 30 ng of plasmid (6 fmoles) or 30 ng of immobilized DNA (9.5 fmoles) were transcribed with an excess of extract protein (12.5 µl). Non-specific DNA binding inhibitors were titrated with 1 µg of pBluescript for 5 min prior to the addition of the template. Preparation of the recovery control RNA, the isolation of the RNA templates and subsequent primer extension were as described by Sandaltzopoulos *et al.* (1995).

Experiments with immobilized templates

Chromatin assembly on immobilized templates was as described above, but was performed in 250 µl tubes (Bio-Rad) and with the addition of 0.025% NP-40 to the assembly mixture, which prevents aggregation of the paramagnetic beads. Chromatin assembly reactions were rotated continuously to avoid settling of the beads. Pre-initiation complex (PIC) formation on immobilized templates, washing and subsequent chromatin assembly was performed as described previously (Sandaltzopoulos and Becker, 1998). Briefly, 600 ng immobilized templates were incubated for 40 min in 200 µl standard transcription reaction lacking rNTPs at 26°C. The PIC-containing templates were purified magnetically, washed with a mixture of 25 µl HEMG-100 and 25 µl transcription premix, repurified and incubated directly in the chromatin assembly reaction. After 6 h of incubation the assembled templates were washed twice as before, purified, and an aliquot (30 ng per template) was resuspended in a standard transcription reaction. For analysis of chromatin-associated proteins, standard chromatin assembly reactions were pooled to permit 1.5 µg DNA template per sample, and the chromatin was incubated in a scaled-up transcription reaction (total volume 200 µl) in the absence of additional rNTPs but with 2.5 mM ATP. Following incubation for 30 min at 26°C, the beads were washed twice with 25 µl HEMG-100/25 µl transcription premix prior to final resuspension in 10 µl SDS loading buffer. To reveal the acetylation status of the histones, Western blots were probed with antibodies directed against tetra-acetylated H3 or H4 (Upstate Biotechnology, New York). HSF and GAGA factor were detected using polyclonal antisera raised in rabbits against the full-length purified proteins expressed as histidine-tagged fusions in bacteria. Antibodies against the polymerase CTD, TBP and TAFs were kind gifts of Y.Nakatani (NIH, Bethesda, MD).

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