The EMBO Journal vol.13 no.2 pp.373–379, 1994

Transcriptional repression by nucleosomes but not H1 in reconstituted preblastoderm *Drosophila* chromatin

Raphael Sandaltzopoulos, Thiemo Blank and Peter B.Becker[1]

European Molecular Biology Laboratory, Gene Expression Programme, 69012 Heidelberg, Germany

[1]Corresponding author

Communicated by G.Schütz

Chromatin reconstituted in an extract from preblastoderm *Drosophila* embryos represses transcription by RNA polymerase II. We have assembled regularly spaced nucleosomes on DNA attached to paramagnetic beads enabling the efficient purification of chromatin templates for transcription studies. We have used diagnostic salt extractions to establish that transcriptional repression of immobilized chromatin was largely due to nucleosome cores. When purified H1 was incorporated into chromatin, resulting in increased repeat lengths to 200–220 bp, the contribution of H1 to transcriptional repression was negligible. If more H1 was added no regularly spaced chromatin was obtained and only under these conditions was transcriptional inhibition by H1 apparent. We conclude that efficient repression of transcription by polymerase II in this system does not require the presence of histone H1.

Key words: chromatin structure/histone H1/nucleosome/ preblastoderm embryos/repression of transcription

Introduction

The context for regulated transcription *in vivo* is set by chromatin, the association of DNA with histones and a wealth of nonhistone proteins of largely unknown function (summarized by van Holde, 1988). A number of genes have been described in recent years whose transcriptional repression and activation involves interaction of transcription factors with nucleosomes, the ubiquitous basic unit of chromatin (reviewed by Grunstein, 1990; Wolffe, 1990; Felsenfeld, 1992; Kornberg and Lorch, 1992; Croston and Kadonaga, 1993; Workman and Buchman, 1993). We are interested in the role of chromatin components in the regulation of transcription by RNA polymerase II (pol II) and the specific features of transcription factors that allow them to function in concert with chromatin.

In *Drosophila* open chromatin structures at a variety of promoters are presumably established very early during embryonic development (Lowenhaupt *et al.*, 1983). Nevertheless, transcription of mRNA is not detected until the beginning of cellularization in the blastoderm stage (Anderson and Lengyel, 1979, 1981; Edgar and Schubinger, 1986). To study the establishment of active promoter structures in *Drosophila* we have described an efficient chromatin assembly system from extracts of preblastoderm fly embryos (Becker and Wu, 1992) which resembles the one derived from *Xenopus* oocytes or eggs (Almouzni and Mechali, 1988; Shimamura *et al.*, 1988) in many respects. The extremely rapid replications in preblastoderm *Drosophila* embryos are accompanied by an equally efficient chromatin assembly which relies entirely on maternal pools of chromatin precursors, such as histones and their carriers. Extracts of early embryos use these endogenous components to assemble plasmid DNA into nucleosomes with a regular repeat length of ~180 bp. A linker histone has not been identified in very early fly embryos yet, but exogenously added histone H1 is incorporated, increasing the repeat length to 200–220 bp (Becker and Wu, 1992). Thus the crude chromatin assembly extract from preblastoderm *Drosophila* embryos offers the opportunity to reconstitute and study chromatin with physiological spacing in the presence of nonhistone chromatin proteins and presumed but as yet unknown histone modifications and may be useful to reconstruct the events that lead to the formation of active promoter structures during early development.

Nucleosome assembly in this extract is paralleled by inhibition of transcription on chromatin templates. The kinetics and degree of inhibition are not changed upon incorporation of H1 (Becker and Wu, 1992). The interpretation of results from coupled assembly/transcription assays is compromised by the crudeness of the assembly system which may contain nonspecific soluble inhibitors and thus does not allow the identification of the transcriptional repressors. Until biochemical fractionation of the extract provides a reconstitution system of much reduced complexity, the reconstituted template must be purified from the assembly reaction prior to *in vitro* transcription. This is generally done by sucrose gradient sedimentation (Shimamura *et al.*, 1988; Becker and Wu, 1992; Laybourn and Kadonaga, 1992) which is time consuming and may change the state of chromatin. In order to be able to purify reconstituted chromatin for analysis of its composition and to identify the transcriptional repressors we have developed a procedure to reconstitute and analyse chromatin on long linear DNA molecules immobilized on paramagnetic beads. Nucleosomes are assembled on immobilized DNA with regular spacing. Reconstituted chromatin can be purified efficiently and rapidly in a magnetic field and is recovered in small volumes allowing the establishment of optimal conditions for subsequent *in vitro* transcription. Chromatin proteins can be selectively extracted from purified chromatin by salt and other reagents for further analysis.

We first applied the system to define the molecules that are responsible for transcriptional repression in reconstituted chromatin. Using diagnostic salt extractions of chromatin we conclude that nucleosome cores are dominant repressors at physiological repeat lengths. Histone H1, when incorporated in sufficient amounts to increase the linker lengths to 200–220 bp, does not contribute significantly to transcriptional repression. The discrepancies between our data and previous results (Shimamura *et al.*, 1989; Laybourn

© Oxford University Press

and Kadonaga, 1992) preclude generalizations on the role of H1 as the dominant repressor of transcription, and may reveal additional mechanisms of repression that act in a preblastoderm embryo.

Results

Chromatin reconstitution on long linear immobilized DNA

We chose the *Drosophila* hsp70 gene for our initial studies because its promoter and chromatin structure have been studied intensely in the past (Wu, 1980, 1984; Udvardy and Schedl, 1984; Rougvie and Lis, 1988) and since preliminary experiments had indicated that at least some aspects of the *in vivo* regulation could be reconstituted (Becker *et al.*, 1991). To avoid end effects and in order to minimize presumed interference by prokaryotic vector sequences with correct nucleosome positioning we decided to analyse the promoter within 3.2 kb of native sequence. Figure 1 illustrates the strategy to immobilize ~6 kb of linearized plasmid DNA with one end on paramagnetic beads. Immobilized DNA was assembled into chromatin using the fly embryo extract. When the quality of the reconstitution was checked by partial digestion with micrococcal nuclease (MNase) a ladder of resistant fragments representing mono- and oligo-nucleosomal DNA was readily apparent (Figure 2A, panel 1). The pattern persisted when the bead chromatin was subjected to salt extractions with NaCl concentrations of up to 600 mM (panels 2–6). When immobilized chromatin was extracted with 2 M NaCl, i.e. a salt concentration that strips histones off the DNA, the periodic resistance towards MNase was lost and DNA was rapidly degraded (panel 7). The digestion profiles in Figure 2 also demonstrate that, following extraction with increasing salt concentrations, chromatin is rendered more sensitive towards nuclease digestion, indicating the removal of nonhistone proteins from the DNA. In order to visualize the nonhistone proteins that copurify with *in vitro* assembled chromatin we separated the salt-eluted proteins by gel electrophoresis (Figure 2B). Whilst few proteins stick to the bead matrix *per se* (Figure 2B, lanes 3 and 4), numerous proteins are extracted with 0.6 M salt from chromatin (lane 5). The remaining proteins, stripped off with 3 M salt, are mainly core histones in the appropriate stoichiometry (lane 6). We were surprised that a large number of nonhistone proteins were extracted with 600 mM KCl since previous chromatin purifications via sucrose gradients had failed to reveal a corresponding complexity (Becker and Wu, 1992). We assume this difference is due to dissociation of these proteins during the lengthy gradient centrifugation step. Whether these proteins identify relevant components of early embryonic chromatin *in vitro* remains to be determined.

Inhibition of transcription is due to nucleosome cores

We next analysed the transcriptional potential of reconstituted chromatin templates after diagnostic salt extraction (Figure 3A). Immobilized DNA was assembled into chromatin for increasing periods of time, separated magnetically from the reaction mix and washed twice with transcription buffer containing variable salt concentrations prior to equilibration in transcription buffer. A control template (free template) was introduced into the transcription reaction mixture just prior to the addition of the washed bead template. As a further control for RNA recovery and primer extension a 'spike RNA' was added to the reaction with the stop mix (Becker *et al.*, 1991). The transcriptional activity of the template was repressed by incubation in the chromatin assembly reaction (Figure 3A, compare lanes 1–5 with lane 10), in a time course paralleling nucleosome assembly. When the chromatin formed after 6 h was subjected to salt washes, extraction of the non-histone proteins with 650 mM NaCl did not relieve inhibition (lane 7). Removing the core histones (Figure 3A, lane 8) resulted in a near-complete activation of transcription, indicating that nucleosome cores were critically responsible for transcriptional inhibition. Salt extractions did not restore the full activity but yielded transcription levels similar to the ones obtained from templates after incubation in the assembly extract for 2 min (compare lanes 9, 8 and 1). Apparently, a small fraction of template is rendered inactive within the first minutes of incubation in the chromatin assembly reaction by a mechanism that cannot be reversed by salt extractions and thus is unrelated to the continued binding of chromatin proteins.

An identical profile of transcriptional inhibition and reactivation after salt washes was also observed when the transcription reactions were performed in the absence of free magnesium (see Materials and methods), suggesting that chromatin folding of the type described by Hansen and

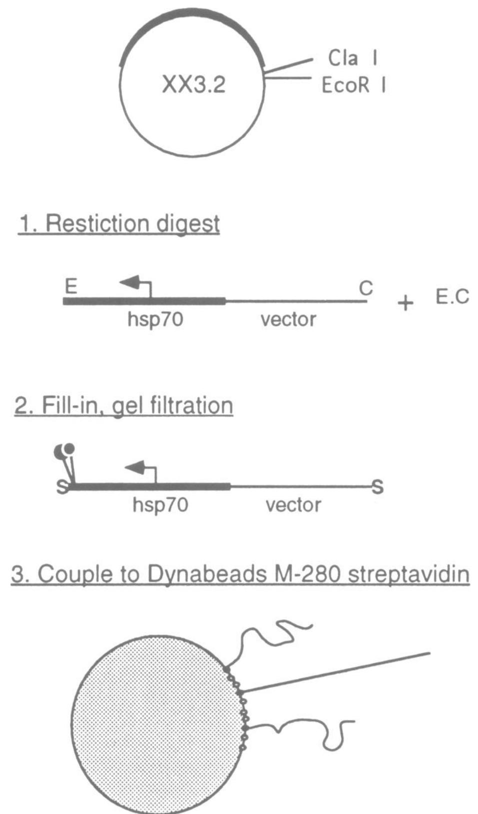

Fig. 1. Strategy for coupling of long linear DNA to paramagnetic beads. The 6 kb plasmid containing the hsp70 gene was cleaved with *Eco*RI (E) and *Cla*I (C) to yield a long and a short (21 bp) fragment. The 5′ protruding ends were filled in with biotin-14-dATP and α-thio-dNTPs (S) to selectively furnish the *Eco*RI site with biotins (filled circles) and to shield both ends from exonuclease attack. Unincorporated nucleotides and the small fragment were removed by gel filtration prior to coupling to paramagnetic beads as described in the text.

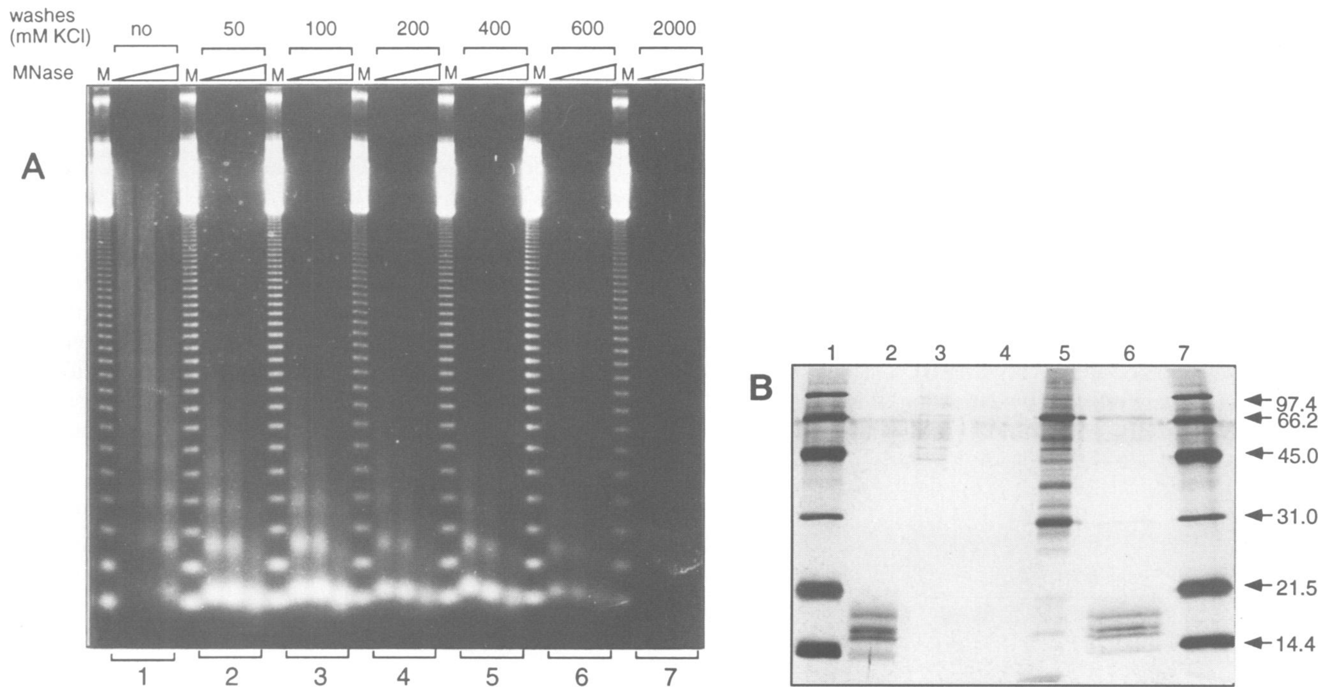

Fig. 2. (A) MNase analysis of immobilized chromatin. Immobilized linear plasmid DNA was assembled into chromatin in the preblastoderm embryo extract. Chromatin beads were washed with buffer containing the indicated amounts of KCl prior to MNase digestion for increasing times. Solubilized DNA fragments were purified and resolved on 1.3% agarose gels and stained with ethidium bromide. M: 123 bp size markers (BRL). (B) Protein composition of reconstituted chromatin. 1 μg of immobilized DNA was reconstituted into chromatin and washed with buffer containing 50 mM KCl. Proteins were eluted from chromatin beads with either 650 mM KCl (lane 5) or 3 M KCl (lane 6). Proteins that nonspecifically bind to control beads lacking DNA were eluted with 650 mM KCl and 3 M KCl (lanes 3 and 4, respectively). Lanes 1 and 7: protein size markers, molecular weights indicated to the right. Lane 2: purified core histone marker. Proteins were resolved by 15% SDS−PAGE and visualized by silver staining.

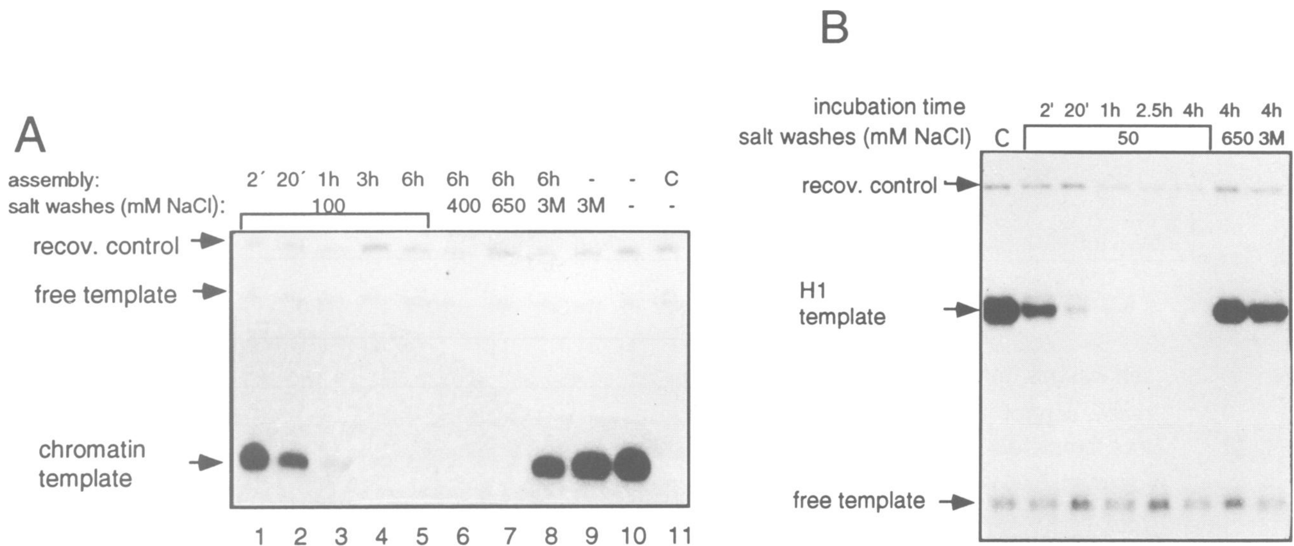

Fig. 3. (A) Inhibition of transcription by nucleosome assembly and reactivation by salt extraction of chromatin proteins. Chromatin was reconstituted on 200 ng of immobilized DNA for the indicated times, washed with buffer containing various salt concentrations and assayed for transcriptional activity *in vitro* (lanes 1−8). 25 ng of naked plasmid containing the hsp26 gene was added to the transcription reaction as an internal control (free template). Transcripts were detected by primer extension and separated by PAGE. Lanes 9 and 10: 200 ng of the free immobilized template with or without wash with 3 M salt. Lane 11 (C): transcription in the absence of bead template. (B) Inhibition of transcription by histone H1. 10 U of H1 were incubated with 200 ng of immobilized template for the indicated times. The template was then washed with the given salt concentrations prior to *in vitro* transcription as in (A). C: control reaction for unrepressed transcription.

Wolffe (1992) does not contribute to the observed inhibition of transcription by chromatin. We note, however, that even at those concentrations of monovalent cations unavoidable for *in vitro* transcription experiments (~50 mM) a significant folding of nucleosomal DNA had been observed (Hansen *et al.*, 1989). Chromatin folding in this study was observed in a system comprising only histones and DNA. It is not clear whether the sites of core−core interactions necessary for folding would be available after assembly in crude extracts as described here.

Histone H1 does not repress transcription when incorporated into preblastoderm chromatin at physiological ratios

Histone H1 has been suggested to be a potent and dominant inhibitor of transcription (Shimamura *et al.*, 1989; Croston *et al.*, 1991; Laybourn and Kadonaga, 1992). Since H1 can be eluted from native chromatin with 650 mM monovalent cations (Ohlenbusch *et al.*, 1967; Tatchell and van Holde, 1977) we tested whether we could identify the contribution of H1 to transcriptional inhibition by a diagnostic salt extraction. When DNA beads were incubated with purified H1 in the absence of chromatin assembly, efficient inhibition of transcription occurred (Figure 3B). This inhibition could be quantitatively relieved by extraction of H1 with 650 mM NaCl (Figure 3B, compare 650 mM with 3 M NaCl).

We next tested whether histone H1, when incorporated into chromatin with proper spacing *in vitro*, would contribute to transcriptional inhibition. We previously demonstrated that the chromatin assembly extract from fly embryos is apparently devoid of the major H1 linker histone found in late embryo chromatin (Becker and Wu, 1992). Chromatin assembled in the extract is characterized by a repeat length (RL) of ~180 bp (the exact value varies between 175 and 185 depending on the individual extract used) and can clearly be distinguished from closely packed nucleosomes (RL = 150) or short spacing (RL = 165). Purified H1 is incorporated into chromatin resulting in a characteristic change in RL to ~200 bp. The amounts of H1 required to shift the RL clearly to 200 bp were determined empirically (Figure 4A) and defined as 1 unit of H1 for the purposes

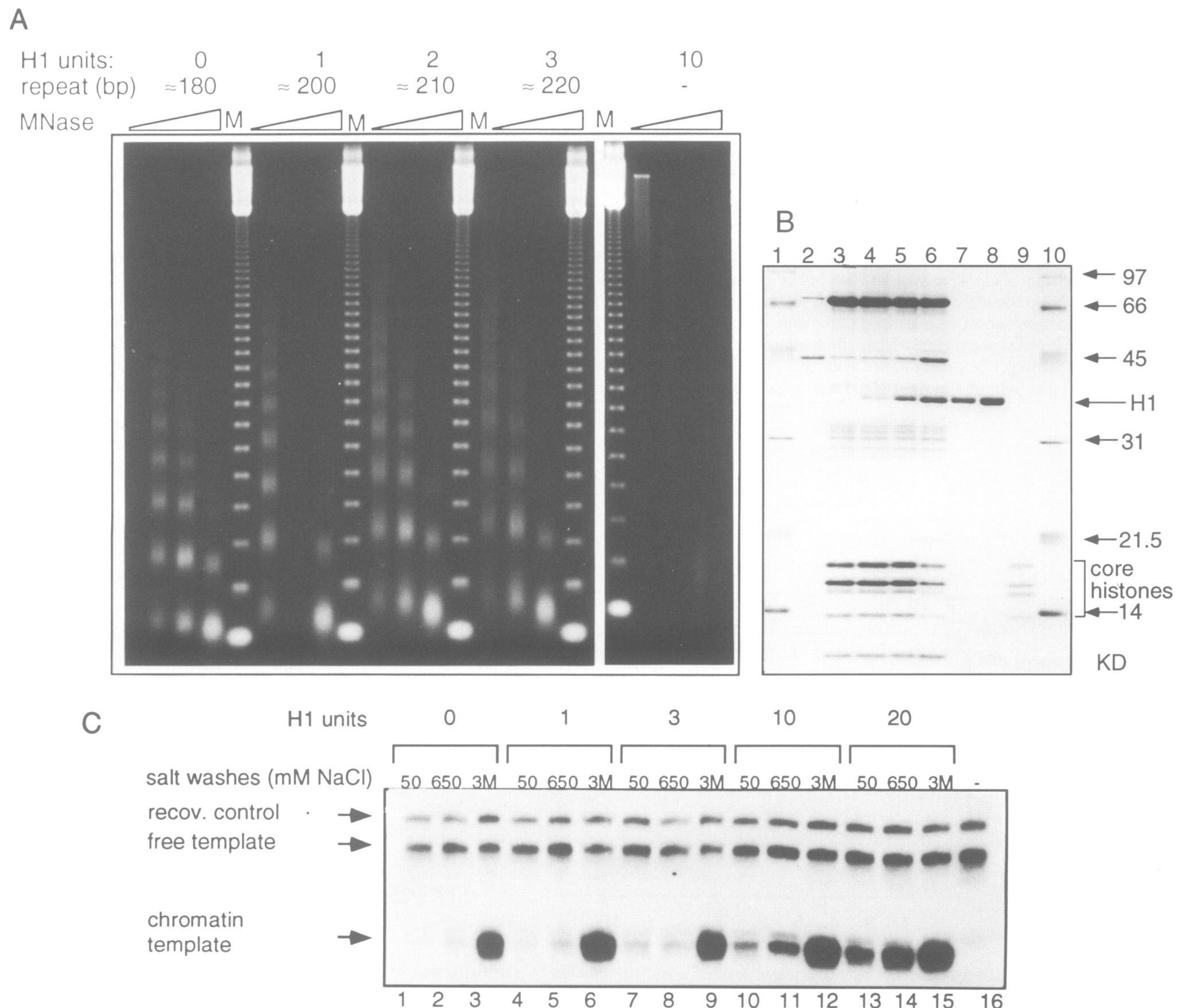

Fig. 4. (A) H1 titration to determine input units. Chromatin was assembled with increasing amounts of histone H1. Reconstituted chromatin was digested with MNase for 0.5, 1 or 5 min. Purified DNA was analyzed on a 1.3% agarose gel, and stained with ethidium bromide. M: 123 bp ladder (BRL). (B) Acid extraction of histones from immobilized chromatin. Chromatin was assembled on immobilized DNA in the presence of 0, 1, 3 or 10 U of H1 (lanes 3–6). Chromatin beads were extracted with 0.25 M HCl. Soluble proteins were acetone precipitated and analysed by 15% PAGE and Coomassie Blue staining. Lanes 1 and 10: marker proteins with sizes indicated to the right. Lane 2: background proteins purified from assembly reactions containing beads without DNA. Lanes 7 and 8: H1 standards. Lane 9: core histone standards. (C) Transcriptional analysis of H1-containing chromatin. Immobilized DNA was assembled into chromatin in the presence of 0, 1, 3, 10 or 20 U of histone H1. Chromatin beads were subjected to diagnostic salt extractions prior to *in vitro* transcription as in Figure 3. The contribution of H1 to transcriptional inhibition, derived from the ratio between transcription levels after 650 mM and 50 mM washes was 1.6-fold in the presence of 1 and 3 U of H1. Upon extraction with 3 M salt, a further 13- and 7-fold reactivation was observed. The lower level of antirepression correlates with *decreased* inhibition as more H1 is added: increasing fractions of the inhibition can be relieved with a 50 mM salt wash.

of the following experiments. This titration was done on plasmids in solution since the higher quality of the MNase ladder obtained allows a more precise determination of the repeat length. A corresponding shift in RL was also observed upon incorporation of H1 into bead chromatin. Addition of 2 or 3 U of H1 to the assembly reaction resulted in further increases to 210 or 220, respectively (Figure 4A). We have not observed longer repeat lengths under any circumstance and, indeed, upon addition of further H1 the regular MNase pattern is lost indicating that an excess of H1 compromises the assembly reaction (Figure 4A, 10 U). To verify that H1 was incorporated into the immobilized chromatin at the expected stoichiometries we extracted histones with 0.25 M HCl and visualized them by PAGE and Coomassie Blue staining (Figure 4B). Upon addition of 3 U of the linker histone, H1 is incorporated approximately with the expected stoichiometry of about one H1 per nucleosome core (van Holde, 1988) (lane 5), and excess H1 is incorporated into chromatin at the expense of core histones when 10 U are added (lane 6).

The transcriptional activity of chromatin templates containing varying amounts of H1 was analysed (Figure 4C). When H1 was added in quantities that yield a regular repeat pattern in a MNase assay (1–3 U, lanes 4–9), inhibition of transcription was substantial but could not be reversed by 650 mM NaCl extraction indicating that under those conditions H1 does not significantly contribute to transcriptional repression. When H1 was added in amounts which are incompatible with the assembly of spaced chromatin (10 or 20 U), an increasing fraction of the inhibition could be reversed by a 650 mM salt wash and hence was apparently due to H1 binding (lanes 11 and 14). It is also apparent from this experiment that with increasing amounts of H1 a corresponding proportion of the inhibition could be reversed by a low salt wash (50 mM, lanes 10 and 13), indicating that inhibition of transcription by H1 was not entirely due to DNA-bound molecules.

We conclude that histone H1, when incorporated with the appropriate stoichiometry into regularly spaced chromatin by a preblastoderm embryo extract, is not a dominant repressor of transcription in our system, but inhibits transcription only when added in excess under conditions that compromise the establishment of chromatin with regular repeat units. In contrast, nucleosome cores were responsible for significant (generally 10- to 15-fold) repression of pol II-directed transcription.

Discussion

The observation that histone H1 did not obviously contribute to transcriptional inhibition when incorporated into preblastoderm chromatin with regular repeat length is surprising in light of the fact that H1 had previously been assigned a dominant role in transcriptional repression (Shimamura *et al.*, 1989; Laybourn and Kadonaga, 1992). The discrepancies between our data and previous results preclude generalizations on the role of H1 as the dominant repressor of transcription and may reveal additional mechanisms of repression that act in a preblastoderm embryo.

The molecular nature of the transcriptional repression that governs zygotic gene activity in preblastoderm *Drosophila* embryos and the acquisition of transcriptional competence of individual genes in cycles 11–14 is not understood (Yasuda *et al.*, 1991). Little is known about how the chromatin of rapidly dividing nuclei differs from chromatin of later, transcriptionally competent stages and whether the activation of the zygotic transcription is accompanied by a transition in chromatin content or structure (Elgin and Hood, 1973). It has been noted that early *Drosophila* embryos are devoid of the main linker histone H1 (Elgin and Hood, 1973) which is consistent with the fact that we do not find H1 in early embryo extracts (Becker and Wu, 1992). Similarly, H1 is not present in early *Xenopus* embryo extracts; however, a cleavage stage linker histone (B4) has recently been identified in the chromatin of early embryonic stages which is gradually replaced by the adult H1 during development (Smith *et al.*, 1988; Dimitrov *et al.*, 1993). It is presently unclear whether an equivalent protein exists in cleavage stages of flies and there is no evidence so far that such a protein contributes to the regular nucleosome spacing in the absence of H1.

The addition of H1, purified from chromatin of late embryos, to the assembly reaction results in a characteristic increase in repeat length to ~200 bp which is the basis for our conclusion that H1 is incorporated into chromatin with appropriate stoichiometry. Upon further addition of H1, repeat lengths of up to 220 bp are observed before all regularity is lost. Similar results have been obtained by Worcel and coworkers (Rodriquez-Campos *et al.*, 1989) in analogous experiments using *Xenopus* oocyte extracts. The repeat lengths beyond 200 bp may reflect the binding of a second H1 molecule to the nucleosome core (Nelson *et al.*, 1979; Bates and Thomas, 1981). The removal of H1 from chromatin having increased repeat lengths assembled in a *Xenopus* oocyte extract resulted in transcriptional activation of a 5S RNA gene by polymerase III (Shimamura *et al.*, 1989). A related effect was observed by Wolffe (1989) who demonstrated that the addition of purified H1 to *Xenopus* sperm chromatin, which is naturally deficient in H1, resulted in a transcriptional repression of oocyte 5S RNA genes. Transcription of the somatic 5S RNA and that of tRNA genes, however, was not repressed under identical conditions, demonstrating that the presence of H1 plays a more decisive role for some promoters than for others. The *Xenopus* system has so far not been used to determine the activity of chromatin templates transcribed by pol II. It is possible that among pol II genes a similar spectrum of sensitivity towards H1 will be found. In this context it should be emphasized that for our study we have used the hsp70 promoter which is known to be free of nucleosomes and H1 and thus accessible to heat shock factor early in development and prior to heat shock (Lowenhaupt *et al.*, 1983; Wu, 1980; Nacheva *et al.*, 1989). The absence of nucleosomes from the promoter elements under virtually all circumstances suggests that it may be particularly sensitive to inhibition by nucleosome cores requiring an active mechanism to prevent occlusion of sensitive sites by nucleosomes, a mechanism which clearly does not operate under our reconstitution conditions. A contrasting example where removal of histone H1 may play a more decisive role is the MMTV LTR where transcriptional activation by glucocorticoid results in both nucleosome destabilization and a decreased presence of H1 at regulatory elements (Bresnick *et al.*, 1992). Despite many attempts to determine the role of H1 in transcriptional regulation *in vivo*, no unifying model has been established that accounts for all experimental observations (reviewed by Zlatanova, 1990).

There is ample evidence that nucleosome cores at densities that render the TATA box and/or initiator element inaccessible inhibit transcription by polymerase II (Sergeant *et al.*, 1984; Knezetic and Luse, 1986; Workman *et al.*, 1991; Lorch *et al.*, 1992). By contrast, Laybourn and Kadonaga (1992) reached the conclusion that nucleosome cores play only a marginal role in transcriptional inhibition but that H1 was the dominant repressor. It is difficult to compare their study with ours because of the differences in experimental design. In their studies nucleosomes were assembled from late embryo histones using polyglutamic acid as a carrier, a procedure that typically yields closely packed nucleosomes. H1 was introduced into the sucrose gradient-purified nucleosomal template by dialysis from 0.6 M salt. Hayes and Wolffe (1993) have recently shown that the interaction of H1 with nucleosomes requires linker DNA to either side of the core particle. Binding of H1 to closely packed nucleosomes may thus not result in faithful chromatin reconstitution.

It is noteworthy that actively transcribed genes *in vivo* are not generally devoid of H1, but show at best a partial depletion of H1, indicating that the presence of H1 *per se* is not an obstacle for transcription (Weintraub, 1984; Kamakaka and Thomas, 1990). Weintraub (1984) has suggested that the main difference between active and inactive gene sequences may not be the presence or absence of H1 but rather the mode of its association with DNA. Similar conclusions have been reached through *in vivo* crosslinking studies where the binding of H1 to chromatin via the globular domain was disrupted in the active gene, but association via the N-terminal lysines was maintained (Nacheva *et al.*, 1989). Although H1 incorporation results in an increased nucleosomal repeat length in our experiments the precise interactions of H1 with the histone octamer which may influence the transcriptional potential of the resulting chromatin are unknown. H1 binding stabilizes the nucleosomal core (van Holde, 1988), and hence its role as a transcriptional repressor may involve core nucleosome stabilization. The action of H1 as a transcriptional repressor may be influenced by other factors that also contribute to core stability and thus by the experimental details of the reconstitution procedure employed.

Our future efforts will be directed towards characterization of the transition that leads to transcriptionally active promoters in a chromatin context. The synthesis of chromatin on immobilized templates should prove a useful tool for these studies.

Materials and methods

Template immobilization

Plasmid pdHSP70 XX3.2 contains 3.2 kb of hsp70 gene sequences (locus 87A) between the *Xba*I sites at −1.4 kb and +1.8 kb (with respect to the transcriptional start site) isolated from plasmid 122X14 (Mason *et al.*, 1982) and cloned into the *Xba*I site of pBluescript SK M13+ (Strategene). For immobilization the plasmid was first completely linearized with *Cla*I and then the linearized fragment was further cleaved with *Eco*RI, generating a long (~6.2 kb) and a short fragment (21 bp). 5′ overhangs were filled in with Klenow polymerase (Boehringer, Mannheim) using biotin-14-dATP, α-thio-dCTP, α-thio-dGTP and α-thio-dTTP (Boehringer, Mannheim). Thus both fragments were biotinylated at the 3′ end of the *Eco*RI site. α-thio-dNTPs were used to seal the ends against any exonuclease activity. Unincorporated dNTPs and the short fragment were removed by gel filtration through a Chroma spin+TE-100 column (Clontech, Palo Alto). The long biotinylated fragment was then coupled to Dynabeads M-280 (Dynal SA, Oslo, Norway). Beads were washed according to the manufacturer's instructions. Coupling was done in 2 M NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5 at 30 ng DNA per μl of bead suspension overnight at room temperature on a rotating wheel. Routinely 4.2 μg were coupled to each mg of beads.

Chromatin assembly on immobilized DNA

Chromatin assembly extract was prepared from *Drosophila* embryos 0−90 min after egg laying as described by Becker and Wu (1992). The assembly reactions were according to Becker and Wu (1992) with the following modifications: incubations were in 250 μl tubes (Bio-Rad cat. no. 223-9471) at 26°C with constant rotation to avoid settling of the beads while keeping the reaction mixture at the bottom of the tube. 0.05% Nonidet P40 was included to avoid bead clumping during assembly and in all wash buffers and solutions (see below). Histone H1 was mixed into the assembly extract prior to addition of the DNA.

Chromatin washes and protein elution

Reconstituted chromatin was concentrated on a Magnetic Particle Concentrator (Dynal) and the supernatant was removed. The chromatin beads were resuspended in 100 μl of extract buffer/NP40 (EX-N) containing appropriate concentration of NaCl or KCl. For the analysis of chromatin-associated proteins, chromatin beads equivalent to 1 μg of template were washed twice with 100 μl of extract buffer, NP40, 50 mM KCl (EX-N-50). Then chromatin was suspended in 7.5 μl of EX-N-600 and concentrated again, and the supernatant was kept. This elution was repeated with a further 7.5 μl EX-N-600 and the two supernatants were pooled. The beads were then washed twice with 100 μl of EX-N-600 followed by suspension in 15 μl of EX-N-2000. After incubation at room temperature for 5 min the beads were concentrated again and the supernatant recovered. The supernatants containing the eluted proteins were analysed on 15% PAGE and visualized by silver staining (Wray *et al.*, 1981).

Acid extraction of histones

5 μg of immobilized DNA was assembled into chromatin in the presence of 0, 1, 3 or 10 U of H1 under standard conditions. Immobilized chromatin was washed three times with 500 μl EX-N-50. Chromatin beads were extracted with 30 μl 0.25 M HCl for 30 min on ice. After removal of beads, the precipitated proteins were pelleted for 30 min in an Eppendorf centrifuge at 4°C. The soluble proteins were precipitated with 6 vol of acetone overnight at −20°C. The pellet was washed three times with 90% acetone, dried and dissolved in SDS loading buffer. Proteins were separated by PAGE and stained with Coomassie Brilliant Blue.

Micrococcal nuclease analysis

Chromatin from 900 ng of immobilized DNA was washed in 120 μl of EX-N-50 and finally resuspended in the original volume of EX-N-500. 180 μl of EX buffer containing 5 mM $CaCl_2$ and 50 U of MNase (Boehringer, Mannheim) were added. After 0.5, 1 and 5 min at room temperature, 100 μl of the reaction was stopped (Becker and Wu, 1992). Beads were concentrated and the supernatant was subjected to RNase treatment and SDS−proteinase K treatment as described by Becker and Wu (1992). Gel electrophoresis of the MNase digestion products was as described by Shimamura *et al.* (1988) using BRL 123 bp markers. For MNase digests without removal of the reaction mix or washes, three times more MNase was used. Repeat lengths were determined as described by Rodriquez-Campos *et al.* (1989). For reasons that are not clear, MNase assays on immobilized DNA did not yield as extensive oligonucleosomal ladders on agarose gels as are usually obtained from plasmids in solution. Since repeat lengths are best determined on larger oligonucleosome fragments where the contribution of end trimming is minimized (Rodriquez-Campos *et al.*, 1989), repeat lengths on immobilized DNA tend to be underestimated.

Transcription extract and transcription reaction

Transcription extracts were prepared from 0 to 12 h *Drosophila* embryos (Oregon R) as described previously (Soeller *et al.*, 1988; Kadonaga, 1990). Reconstituted chromatin was washed once with EX-N-50, once with 100 μl of 25 mM HEPES (pH 7.6), 0.1 mM EDTA, 10% glycerol, 50 mM KCl and 0.05% NP40. To 200 ng of washed chromatin beads, 25 μl of transcription premix were added, consisting of 7.5 μl of transcription extract, 5 μl of HEMG100 (25 mM HEPES pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol and 100 mM KCl), 7.5 μl 13 mM HEPES pH 7.6, 0.25% NP40, 0.34 mM DTT, 13.3 mM creatine phosphate, 10 ng creatine phosphokinase, 0.25 U of Inhibit-Ace (5 prime-3 prime), 3.3 mM each of ATP, CTP, GTP and UTP, 5 μl 10 mM Tris (pH 7.6), 0.1 mM EDTA, 1 μg pUC DNA as competitor for soluble inhibitors (Becker *et al.*, 1991) and 25 ng pHSP26HH4.8 which contains the hsp26 gene as internal control. Transcription reactions were rotated for 25 min at 26°C. Reactions were

terminated by addition of 250 μl of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1 M β-mercaptoethanol containing 10 μg of yeast total RNA and 1−10 fmol of spike RNA as recovery control (Becker *et al.*, 1991). Transcriptions were done either under standard conditions (2.25 mM free Mg^{2+}) or with increased input of NTPs and reduced magnesium levels such that no free magnesium was present by the criteria of Hansen and Wolffe (1992). For RNA purification we used the guanidinium thiocyanate method as described by Chomczynski (1987), scaled down appropriately. Transcripts were analysed by primer extension as described by Becker *et al.* (1991) using the following primers: P119: 5′-GCAGATTGTTTAGCTTGTTC-3′ (complementary to hsp70 RNA between positions 64 and 84); P204: 5′-CGCAAAGTTGCTTTGAG-TTGTTCACTGCTC-3′ and P214: 5′-GAATGAACTTGTTTGACT-TGTAAGCAAAGG-3′ (complementary to hsp26 RNA between positions 21/51 and 91/120, respectively). Quantitation of radioactivity was performed using a PhosphorImager and Molecular Dynamics software.

Purification and incorporation of H1

H1 was purified according to the procedure of Croston *et al.* (1991b) and its identity and integrity verified by Western blotting using an antibody kindly provided by Drs R.Kamakaka and J.T.Kadonaga. For incorporation into chromatin, H1 was mixed with the chromatin assembly extract prior to addition of the other components, DNA was added last.

Acknowledgements

We thank Drs R.Kamakaka and J.T.Kadonaga for a gift of H1 antibody and Drs I.W.Mattaj, A.F.Stewart and J.-P.Quivy for critical reading of the manuscript. R.S. is the recipient of an EMBO predoctoral fellowship. T.B. was supported by a grant from the Deutsche Forschungsgemeinschaft to P.B.

References

Almouzni,G. and Mechali,M. (1988) *EMBO J.*, **7**, 665−672.
Anderson,K.V. and Lengyel,J.A. (1979) *Dev. Biol.*, **70**, 217−231.
Anderson,K.V. and Lengyel,J.A. (1981) *Dev. Biol.*, **82**, 127−138.
Bates,D.L. and Thomas,J.O. (1981) *Nucleic Acids Res.*, **9**, 5883−5894.
Becker,P.B. and Wu,C. (1992) *Mol. Cell. Biol.*, **12**, 2241−2249.
Becker,P.B., Rabindran,S.K. and Wu.C. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 4109−4113.
Bresnick,E.H., Bustin,M., Marsaud,V., Richard-Foy,H. and Hager,G.L. (1992) *Nucleic Acids Res.*, **20**, 273−278.
Chomczynski,P. and Sacchi,N. (1987) In Ausubel,F., Brent,R., Kingston,R., Moore,P., Seidman,J., Smith,J. and Struhl,K. (eds), *Current Protocols in Molecular Biology*. Wiley Interscience, New York.
Croston,G.E. and Kadonaga,J.T. (1993) *Curr. Opin. Cell Biol.*, **5**, 417−423.
Croston,G.E., Kerrigan,L.A., Lira,L.M., Marshak,D.R. and Kadonaga,J.T. (1991a) *Science*, **251**, 643−649.
Croston,G.E., Lira,L.M. and Kadonaga,J.T. (1991b) *Protein Expression Purif.*, **2**, 162−169.
Dimitrov,S., Almouzni,G., Dasso,M. and Wolffe,A.P. (1993) *Dev. Biol.*, **160**, 214−227.
Edgar,B.A. and Schubinger,G. (1986) *Cell*, **44**, 871−877.
Elgin,S.C.R. and Hood,L.E. (1973) *Biochemistry*, **12**, 4984−4991.
Felsenfeld,G. (1992) *Nature*, **355**, 219−224.
Grunstein,M. (1990) *Annu. Rev. Cell Biol.*, **6**, 643−678.
Hansen,J.C. and Wolffe,A.P. (1992) *Biochemistry*, **31**, 7977−7988.
Hansen,J.C., Ausio,J., Stanik,V.H. and van Holde,K.E. (1989) *Biochemistry*, **28**, 9129−9136.
Hayes,J.J. and Wolffe,A.P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 6415−6420.
Kadonaga,J.T. (1990) *J. Biol. Chem.*, **265**, 2624−2631.
Kamakaka,R.T. and Thomas,J.O. (1990) *EMBO J.*, **9**, 3997−4006.
Knezetic,J.A. and Luse,D.S. (1986) *Cell*, **45**, 95−104.
Kornberg,R.D. and Lorch,Y. (1992) *Annu. Rev. Cell Biol.*, **8**, 563−587.
Laybourn,P.J. and Kadonaga,J.T. (1992) *Science*, **257**, 1682−1685.
Lorch,Y., LaPointe,J.W. and Kornberg,R.D. (1992) *Genes Dev.*, **6**, 2282−2287.
Lowenhaupt,K., Cartwright,I.L., Keene,M.A., Zimmerman,J.L. and Elgin,S.C.R. (1983) *Dev. Biol.*, **99**, 194−201.
Mason,P.J., Török,I., Kiss,I., Karch,F. and Udvardy,A. (1982) *J. Mol. Biol.*, **156**, 21−35.
Nacheva,G.A., Guschin,D.Y., Preobrazhenskaya,O.V., Karpov,V.L., Ebralidse,K.K. and Mirzabekov,A.D. (1989) *Cell*, **58**, 27−36.
Nelson,P.P., Albright,S.C., Wiseman,J.M. and Garrard,W.T. (1979) *J. Biol. Chem.*, **254**, 11751−11760.
Ohlenbusch,H.H., Olivera,B.M., Tuan,D. and Davidson,N. (1967) *J. Mol. Biol.*, **25**, 299−315.
Rodriquez-Campos,A., Shimamura,A. and Worcel,A. (1989) *J. Mol. Biol.*, **209**, 135−150.
Rougvie,A.E. and Lis,J.T. (1988) *Cell*, **54**, 795−804.
Sergeant,A., Bohmann,D., Zentgraf,H., Weiher,H. and Keller,W. (1984) *J. Mol. Biol.*, **180**, 577−600.
Shimamura,A., Tremethick,D. and Worcel,A. (1988) *Mol. Cell. Biol.*, **8**, 4257−4269.
Shimamura,A., Sapp,M., Rodriquez-Campos,A. and Worcel,A. (1989) *Mol. Cell. Biol.*, **9**, 5573−5584.
Smith,R.C., Dworkin-Rastl,E. and Dworkin,M.B. (1988) *Genes Dev.*, **2**, 1284−1295.
Soeller,W.C., Poole,S.J. and Kornberg,T. (1988) *Genes Dev.*, **2**, 68−81.
Tatchell,K. and van Holde,K.E. (1977) *Biochemistry*, **16**, 5295−5303.
Udvardy,A. and Schedl,P. (1984) *J. Mol. Biol.*, **172**, 385−403.
van Holde,K.E. (1988) *Chromatin*. Springer Verlag, New York.
Weintraub,H. (1984) *Cell*, **38**, 17−27.
Wolffe,P.A. (1989) *EMBO J.*, **8**, 527−537.
Wolffe,P.A. (1990) *New Biol.*, **2**, 211−214.
Workman,J.L. and Buchman,A. (1993) *Trends Biochem. Sci.*, **18**, 90−95.
Workman,J.L., Taylor,I.C.A. and Kingston,R.E. (1991) *Cell*, **64**, 533−544.
Wray,W., Boulikas,T., Wray,V.P. and Hancock,R. (1981) *Anal. Biochem.*, **118**, 197−203.
Wu.C. (1980) *Nature*, **286**, 854−860.
Wu,C. (1984) *Nature*, **309**, 229−234.
Yasuda,G.L., Baker,J. and Schubinger,G. (1991) *Genes Dev.*, **5**, 1800−1812.
Zlatanova,J. (1990) *Trends Biochem. Sci.*, **15**, 273−276.

Received on October 12, 1993; revised on November 2, 1993