Remodeling of regulatory nucleoprotein complexes on the *Xenopus* hsp70 promoter during meiotic maturation of the *Xenopus* oocyte

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Transcriptional repression occurs during meiotic maturation of *Xenopus* oocytes. Injection of a DNA template containing an hsp70 promoter into *Xenopus* oocytes, followed by progesterone-induced maturation has been used to demonstrate a dynamic competition between the assembly of transcription factor-containing nucleoprotein complexes and repressive nucleosomal arrays during the maturation process. In particular, it is shown that increased levels of injected heat shock protein, the transcriptional activator Gal4–VP16 or the DNA template itself all lead to reduced repression of transcription on maturation. Conversely, injection of additional histone increases repression. Repression of transcription is shown to be accompanied by the formation of a more regular array of nucleosomes and by an increase in the efficiency of nucleosome assembly on the injected plasmid. Meiotic maturation is therefore accompanied by replacement of transcription factor complexes by a repressive chromatin environment.

Keywords: heat shock/meiosis/nucleoprotein/transcription repression/*Xenopus*

Introduction

Dramatic differences exist in the regulation of gene expression between oocytes and eggs (Davidson, 1986). Within the oocyte, the maternal genome directs the complex differentiative process by which stores of macromolecules necessary for early development accumulate. Within an egg, these stores are utilized immediately following fertilization to initiate the assembly of the embryo. In striking contrast to the oocyte, the chromosomes of the vertebrate egg or embryo are usually transcriptionally silent at least until after the first cleavage division. The transition from an oocyte to an egg is a major developmental step.

In *Xenopus laevis*, the oocyte genome has remarkably high transcriptional activity (Brown and Littna, 1964; Davidson *et al.*, 1964; La Marca *et al.*, 1973). Exogenous somatic nuclei introduced into oocytes increase the transcription of a variety of genes (De Robertis and Gurdon, 1977; Wakefield *et al.*, 1983). In contrast the *Xenopus* egg genome is transcriptionally silent (Brown and Littna, 1966; Newport and Kirshner, 1982), and somatic nuclei introduced into eggs replicate, but do not efficiently

transcribe their DNA (Graham *et al.*, 1966; Wakefield and Gurdon, 1983). Many aspects of the physiological transition from an oocyte to an egg can be induced *in vitro* by incubating fully developed stage VI oocytes with progesterone (Wassarman and Smith, 1978; Maller and Krebs, 1980). We wished to identify the molecular mechanisms directing the dominant repression of transcription during the meiotic maturation process (Masui and Shibuya, 1987).

The *Xenopus* oocyte has proven useful in defining the role of chromatin proteins in the regulation of transcription *in vivo* (Gargiulo and Worcel, 1983; Gargiulo *et al.*, 1985; Perlman and Wrangé, 1991; Almouzni and Wolffe, 1993a,b; Landsberger and Wolffe, 1995; Wong *et al.*, 1995). Clear differences exist between the transcription of exogenous templates microinjected into oocyte nuclei and the same templates introduced into eggs (Almouzni and Wolffe, 1993, 1995; Prioleau *et al.*, 1994, 1995). Transcription of exogenous DNA in an oocyte is much more efficient than in an egg. Chromatin proteins exert repressive effects on transcription in both eggs and oocytes (Prioleau *et al.*, 1994; Almouzni and Wolffe, 1995) and components of the basal transcriptional machinery appear to be limiting in eggs and early embryos (Almouzni and Wolffe, 1995; Prioleau *et al.*, 1995). However, the exact molecular basis for the transcriptional activity of the oocyte compared with the transcriptional quiescence of the egg and early embryo has not been determined.

In this work we describe the assembly of the *Xenopus* hsp70 promoter (Bienz, 1984) into a transcription complex that can be made dependent for activity on the transactivator's heat shock transcription factor (HSF) and Gal4–VP16 (Landsberger and Wolffe, 1995). We follow the transcriptional activity and nucleoprotein organization of the hsp70 promoter through meiotic maturation. Both transcriptional activity and the maintenance of the regulatory nucleoprotein complex on the hsp70 promoter during this developmental transition are found to be dependent on the abundance of transcriptional activators and the efficiency with which the template is assembled into nucleosomes. Our results provide direct evidence for the regulation of gene expression through a mechanism in which the transcriptional machinery exists in a continual competition with chromatin proteins for association with regulatory DNA.

Results

Remodeling of nucleoprotein complexes during meiotic maturation

In earlier work we have defined the essential role of chromatin structure in heat inducible transcription from the *Xenopus* hsp70 promoter (Landsberger and Wolffe, 1995; Landsberger *et al.*, 1995). The *Xenopus* hsp70

Fig. 1. The nucleoprotein complexes assembled on the *hsp70* promoter are remodeled during meiotic maturation. Mapping by indirect end labeling of the DNase I hypersensitive sites present on the *hsp70* promoter (A) or the *G5hsp70* promoter (B). (A) XHSF1 mRNA was not injected (lanes 4–11) or injected (lanes 12–19) into stage VI oocytes. After oocyte incubation for 16 h, 1 ng of HSPCAT DNA was injected. The oocytes were incubated as described in Materials and methods. Groups of 25 oocytes incubated in the absence (lanes 4–7 and 12–15) or presence of progesterone (lanes 8–11 and 16–19) were collected, treated with DNase I and further processed as described in Materials and methods. Control naked DNA (lanes 1–3) was also digested. Distances in base pairs relative to the linearization site (*Nco*I) are indicated on the left. The position of the promoter and the transcriptional start site relative to the *Nco*I site are also represented on the left of the panel. DNase I hypersensitive sites are indicated on the right by arrows. On top of the panel a map of the *Xenopus hsp70* promoter is represented. (B) GAL4VP16 mRNA was either not injected (lanes 4–9) or injected (lanes 10–15) into oocytes. After 16 h of incubation, the oocytes were injected with 1 ng of G5HSPCAT DNA and incubated as described above. Groups of 25 oocytes incubated in the absence (lanes 4–6 and 10–12) or in the presence (lanes 7–9 and 13–15) of progesterone were collected and processed as described in (A). Control naked DNA (lanes 1 to 3) was also digested. As above, the position of the promoter, the transcriptional start site and distances in base pairs relative to the *Nco*I site are indicated on the left of the panel. A map of the *G5HSP70* promoter is represented on top of the panel. On the right, the arrow indicates a DNase I hypersensitive site. A region of protection from the nuclease digestion, evident only in the presence of the exogenous factor, is indicated by a solid bar on the left hand side of the panel.

promoter exists in a DNase I hypersensitive site following the assembly of chromatin in the *Xenopus* oocyte nucleus (Landsberger and Wolffe, 1995). This DNase I hypersensitive site is present even in the absence of fully induced levels of transcription, since functional components of the basal transcriptional machinery associate with the promoter even in the absence of heat shock (Landsberger and Wolffe, 1995, see also Lis and Wu, 1993). Thus the chromatin structure of the *hsp70* promoter is preset, most probably in order to facilitate the rapid and efficient association of heat shock transcription factor (HSF) as an essential component of transcriptional activation following heat shock (Becker, 1994; Wallrath *et al.*, 1994).

A DNase I hypersensitive site is present at the *hsp70* promoter following the assembly of a double-stranded DNA template into chromatin within the oocyte nucleus (Figure 1A, compare lanes 1–3 with lanes 4–7, the hypersensitive site is indicated by the large horizontal arrow). Maturation of these oocytes with progesterone leads to the disappearance of the DNase I hypersensitive site (Figure 1A, compare lanes 4–7 with 8–11). Thus meiotic maturation leads to the remodeling of regulatory nucleoprotein complexes assembled under normal physio-

logical conditions at the *Xenopus hsp70* promoter. Synthesis of exogenous HSF in *Xenopus* oocytes leads to constitutive occupancy of the *Xenopus hsp70* promoter with HSF and an increase in transcriptional activity (Landsberger and Wolffe, 1995, see Figure 2). Under these conditions the pattern of DNase I cleavage over the *Xenopus hsp70* promoter changes to reflect the association of HSF with three binding sites (heat shock response elements, HSEs) within the promoter (Figure 1A, compare lanes 4–7 with lanes 12–15, three new DNase I hypersensitive sites are indicated by the small horizontal arrows). Maturation of the oocytes in the presence of exogenous HSF does not lead to any significant change in the DNase I hypersensitive site (Figure 1A, compare lanes 12–15 with 16–19). Thus an excess of a sequence-specific transcriptional regulator such as HSF can prevent the loss of DNase I hypersensitivity and can potentially sustain the association of that regulator and other components of the transcriptional machinery with a promoter through meiotic maturation (see Figure 2).

We next examined whether the capacity of a transcriptional regulator to remain associated with a promoter was restricted to *Xenopus* HSF or whether a heterologous

Fig. 2. Meiotic maturation leads to the loss of transcriptional competence under physiological conditions. (A) XHSF1 mRNA was not injected (lanes 1 and 2) or injected (lanes 3 and 4) into oocytes. After 14 h of incubation, 1 ng of HSPCAT template was injected. The oocytes were incubated 2 h, divided and incubated a further 16 h in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of progesterone. Healthy oocytes were collected and analyzed by primer extension as described in Materials and methods. The position of accurately initiated transcripts from the hsp70 promoter (Hsp70) is indicated. The transcriptional signals were quantified with a PhosphorImager, and the levels of transcription relative to DNA mass injected (estimated from Southern blotting, data not shown) are indicated in the bar graph. A.U., arbitrary units. An experimental scheme is represented on top of the panel. (B) A scheme of the experiment is shown in the upper part of the panel. The protocol is as in the previous panels. Gal4VP16 mRNA was either not injected (lanes 1–3) or injected (lanes 4–6) into stage VI oocytes. Healthy oocytes were collected 2 h after the DNA injection (lanes 1 and 4), divided and incubated in the absence (lanes 2 and 5) or presence of progesterone (lanes 3 and 6). Lanes 1 and 4 (marked C for control) show the amount of RNA synthesized in the 2 h after injection, prior to the addition of progesterone or control buffer. The levels of transcription, evaluated as previously described, are indicated in the bar graph.

DNA binding protein such as Gal4–VP16 could also retain association with chromosomal DNA. To resolve this issue we modified the hsp70 promoter to contain five binding sites for Gal4 immediately upstream of the binding sites for HSF (Figure 1B). The presence of these sites does not

affect the generation of a DNase I hypersensitive site on the hsp70 promoter (Figure 1B, compare lanes 1–3 with 4–6, the hypersensitive site is indicated by the horizontal arrows), nor do they protect the DNase I hypersensitive site from remodeling following meiotic maturation (Figure

1B, compare lanes 4–6 with 7–9). Expression of Gal4–VP16 in the oocyte leads to very different results. The association of Gal4–VP16 with the promoter is apparent both before and after meiotic maturation (Figure 1B, compare lanes 4–9 with 10–15, the region of protection from DNase I cleavage is indicated by the vertical bar). The presence of bound Gal4–VP16 leads to sustained changes in DNase I cleavage in the vicinity of the hsp70 promoter in spite of meiotic maturation (Figure 1B, compare lanes 4–9 with 10–15). Thus the retention of DNase I hypersensitive sites during meiotic maturation depends in part on the abundance of sequence-specific transcriptional regulatory proteins.

Transcriptional regulation during meiotic maturation

Earlier work had indicated that the endogenous oocyte genome was transcriptionally silenced following meiotic maturation (La Marca *et al.*, 1975). We examined the transcription of the *Xenopus* hsp70 promoter dependent on meiotic maturation (Figure 2A). Under physiological conditions in the absence of exogenous HSF, transcription from the hsp70 promoter is repressed by the maturation process (Figure 2A, compare lanes 1 and 2). Quantitation of relative transcriptional activities relative to DNA recovery indicates a 10-fold repression. In the presence of exogenous HSF, transcription from the hsp70 promoter is stimulated 9-fold at 22°C (Figure 2A, compare lanes 1 and 3; Landsberger and Wolffe, 1995). We have documented in detail how the increase in HSF abundance allows transcriptional activation of the *Xenopus* hsp70 promoter at non heat shock temperatures in earlier work (Landsberger and Wolffe, 1995). Meiotic maturation under these conditions of elevated transcription factor concentration, leads to a 2-fold repression of hsp70 promoter activity (Figure 2A, compare lanes 3 and 4). Transcription persists at a level which exceeds that of the promoter in the absence of exogenous HSF and maturation (Figure 2A, compare lanes 1 and 4). Thus consistent with the loss of DNase I hypersensitivity (Figure 1A, lanes 4–11), meiotic maturation leads to the loss of transcriptional competence from the hsp70 promoter under physiological conditions. An increase in the abundance of HSF prevents the loss of DNase I hypersensitivity (Figure 1A, lanes 12–19) and allows transcription to be sustained, albeit at a reduced level to that preceding maturation.

We have described how exogenous Gal4–VP16 can remain associated with a synthetic hsp70 promoter through meiotic maturation and sustain DNase I hypersensitivity at the promoter (Figure 1B). We find that exogenous Gal4–VP16 also sustains vigorous transcriptional activity at the hsp70 promoter (Figure 2B, lanes 5 and 6). This result is consistent with the capacity of exogenous Gal4–VP16 to prevent the remodeling of the DNase I hypersensitive site at the hsp70 promoter (Figure 1B, lanes 10–15). We suggest that the loss of transcriptional activity associated with meiotic maturation depends on the abundance of the sequence-specific transcription factor required for transcription.

Our measurements of the transcription directed by the hsp70 promoter before and after meiotic maturation examine the accumulation of transcripts that are initiated at a specific site within a promoter (Figure 2). We wished

to refine our definition of transcriptional repression by determining both the stability of transcripts and the absolute transcription rate. To assess the effect of meiotic maturation on transcript stability we made use of the protocol illustrated in Figure 3A, in which transcripts were allowed to accumulate for 6 h before oocytes were incubated with either buffer, actinomycin D, or actinomycin D and progesterone. The concentration of actinomycin D necessary to inhibit transcription by RNA polymerase II was determined empirically (data not shown). We find that the stability of transcripts is not significantly affected by the maturation process (Figure 3B). The similarity in hsp70 transcript levels in the oocytes treated with progesterone alone (Figure 3B, lanes 5 and 6) compared with those treated with actinomycin D and progesterone (Figure 3B, lanes 7 and 8) suggests that transcription directed by the hsp70 promoter is completely inhibited by progesterone treatment.

We next examined whether RNA polymerase II molecules engaged on the *Xenopus* hsp70 promoter would be displaced on meiotic maturation. Run-on experiments with the *Xenopus* hsp70 promoter in which hybridization to regions at the 5' end, middle and 3' end of transcribed region takes place shows that incubation with the detergent Sarkosyl does not facilitate transcription before or after oocyte maturation (data not shown; Landsberger and Wolffe, 1995). We conclude that the inhibition of transcription that occurs on oocyte maturation involves the displacement of template-engaged RNA polymerase II.

A role for chromatin assembly in the repression of transcription on meiotic maturation

The transition from an oocyte to an egg involves the programmed release of the stores of macromolecules necessary for the assembly of the embryo (Almouzni and Wolffe, 1993b). This transition involves the phosphorylation and capacitation of molecular chaperones involved in chromatin assembly, such as nucleoplasmin (Sealy *et al.*, 1986; Leno *et al.*, 1995). Thus, although the mass of chromatin proteins present in the cell does not change on meiotic maturation, their availability for chromatin assembly increases significantly. Chromatin is not necessarily a passive regulatory component for transcription: changes in the type of histone (Bouvet *et al.*, 1994; Kandolf, 1994), the pathway of chromatin assembly (Almouzni and Wolffe, 1993a; Kamakaka *et al.*, 1993; reviewed by Paranjape *et al.*, 1994) or the excess of histones (Han *et al.*, 1988; Shimamura *et al.*, 1988; Grunstein *et al.*, 1995) can all exert dominant effects on the transcription process. Transcription of the *Xenopus* hsp70 promoter is known to be sensitive to chromatin structure. We next examined the role of chromatin assembly in the repression of transcription observed on meiotic maturation.

We measured the number of nucleosomes present on the minichromosome containing the *Xenopus* hsp70 promoter before and after meiotic maturation. This is most effectively done by assaying the number of negative superhelical turns within the minichromosome after deproteinization (Germond *et al.*, 1975). We find that before maturation there is an average of 25 nucleosomes assembled on the 4685 bp long template DNA molecule used at these low DNA concentrations (see Figure 6B

Fig. 3. hsp70 transcription is completely inhibited by progesterone treatment. (A) A scheme of the experiment shown in (B) is presented. 3 ng of HSPCAT template was injected into stage VI oocytes. After injection, the oocytes were allowed to transcribe the template for 6 h. Oocytes were then incubated 4 h either in buffer, actinomycin D (60 μ g/ml), or actinomycin D (60 μ g/ml) and progesterone. Groups of 15 healthy oocytes were collected. The remaining oocytes were incubated a further 12 h before collection. (B) For transcription analysis, a primer extension was performed as described in Materials and methods. The position of accurately initiated transcripts from the hsp70 promoter (Hsp70) is indicated. The transcription signals were quantified with a PhosphorImager, and the amounts of hsp70 mRNA accumulated relative to DNA template (quantified by Southern blotting, data not shown) are indicated in the bar graph. A.U., arbitrary units.

later). However on maturation an average of four more nucleosomes are accommodated on the minichromosome (Figure 4A, compare lanes 4 and 5, bars indicate the center of topological distribution). This could occur by nucleosome assembly at sites vacated by transcription factors and/or by increasing the density of nucleosomes on each template irrespective of DNA sequence.

We repeated this experiment using the G5HSPCAT construct, once again detecting a significant increase in nucleosome number following oocyte maturation (Figure 4B, compare lanes 4 and 6 with lanes 5 and 7, see scans of lanes 6 and 7). Expression of Gal4-VP16 reduces both the number of nucleosomes on the template prior to oocyte maturation (Figure 4B, lanes 6 and 7). This result is consistent with previous observations that Gal4-VP16 will disrupt nucleosomes (Laybourn and Kadonaga, 1991; Lorch *et al.*, 1992; Workman and Kingston, 1992; Morse, 1993) and the maintenance of the DNase I hypersensitive site and transcription (Figure 1B), indicating that Gal4-VP16 can interfere with histone association *in vivo* during this physiological transition. Although the addition of Gal4-VP16 might be expected to displace a single nucleosome through DNA binding alone, a topological change equivalent to a loss of four nucleosomes is observed (Figure 4B, compare lanes 4 and 6). This suggests that Gal4-VP16 is participating in nucleosome disruption through mechanisms in addition to DNA binding alone (see Wong *et al.*, 1997). Control experiments indicate that specific DNA binding is however, a prerequisite for the

Gal4-VP16 dependent topological change, since removal of the Gal4 binding sites from the template prevents the change in topology from occurring in the presence of Gal4-VP16 (J.Strouboulis and A.P.Wolffe, data not shown). Our next experiments examined the molecular mechanisms that account for changes in nucleosome number following oocyte maturation by determination of the efficiency of nucleosome assembly, general nucleosome spacing and chromatin organization over the promoter itself following oocyte maturation.

Microinjection of DNA templates into oocytes before (Figure 4C, lanes 3–8) and after (Figure 4C, lanes 9–14) maturation reveals a much more rapid assembly of nucleosomes in mature oocytes as shown through DNA supercoiling. This result is consistent with the stimulation/enhancement of the chromatin assembly machinery during maturation (Sealy *et al.*, 1986). Note that the gel used to resolve topoisomers in Figure 4C lacks chloroquine, whereas the gels used in Figure 4A and B contain chloroquine. Surprisingly, micrococcal nuclease digestion reveals that the general density of nucleosomes over the entire minichromosome appears to decrease slightly on meiotic maturation (Figure 4D, compare lanes 1–4 and 9–12, dots show the major protected DNA fragment sizes indicative of the spacing of nucleosomes). If the four additional nucleosomes had been accommodated entirely on the minichromosome by sliding adjacent nucleosomes closer together, the nucleosome repeat should have decreased from 188 to 161 bp, a difference easily detected

Fig. 4. Analysis of chromatin assembly on plasmid DNA before and after meiotic maturation. (A) DNA from oocytes injected with 1 ng of HSPCAT DNA and incubated in the presence (lane 4) or absence (lane 5) of progesterone was extracted. The purified DNA was resolved on a 1% agarose gel containing chloroquine (10 μ g/ml). Lane 1, supercoiled marker DNA; lane 2, linear marker DNA; lane 3, plasmid template previously relaxed with topoisomerase I. The positions of supercoiled (I), nicked closed circular (II) and linear DNA (III) are indicated. The bars indicate the centers of topoisomer distributions in lanes 4 and 5. (B) As in (A) except that 1 ng of G5HSPCAT was microinjected into oocytes. Lane 1, supercoiled marker DNA; lane 2, linear marker DNA; lane 3, plasmid template previously relaxed with topoisomerase I. In lanes 4 and 5 G5HSPCAT is shown in the presence (lane 4) or absence (lane 5) of progesterone. In lanes 6 and 7, G5HSPCAT is microinjected into oocytes expressing Gal4-VP16 in the presence (lane 6) or absence (lane 7) of progesterone. The positions of supercoiled (I), nicked closed circular (II) and linear DNA (III) are indicated. Densitometric scans of lanes 6 and 7 are shown together with markers and the centers of distribution of the topoisomers reflecting the experimental sample. (C) Comparison of the efficiency of DNA supercoiling in non matured (-PG) and matured (+PG) oocytes. DNA from oocytes injected with 3 ng of HSPCAT is shown after extraction at various times after injection as indicated. Lane 1 contains supercoiled DNA and lane 2 linear DNA as markers. The position of supercoiled (I), nicked closed circular (II) and linear DNA (III) are indicated. DNA was resolved on a 1% agarose gel in the absence of chloroquine. (D) 1 ng of HSPCAT DNA was injected into oocytes. They were incubated in the absence (lanes 1-4) or presence (lanes 5-12) of progesterone. At the end of the incubation period, minichromosomes were digested with increasing concentrations of micrococcal nuclease. 10 U (lanes 1 and 5), 20 U (lanes 2 and 6), 40 U (lanes 3 and 7) and 60 U (lanes 4 and 8) are used. In lanes 9-12 the MNase concentration has been increased to 40 U, 80 U, 120 U and 160 U respectively. The DNA was purified, resolved on a 1.5% agarose gel and transferred as described in Materials and methods. The hybridization probe is a random primed HSPCAT template. On the left markers are labeled in base pairs. Dots highlight the spacing of protected DNA fragments after micrococcal nuclease digestion.

in this assay. In fact nucleosomes appear to be more clearly defined and widely spaced after oocyte maturation; this is consistent with a requirement for significantly more micrococcal nuclease to digest chromatin isolated from matured oocytes suggesting a more stable or less accessible chromatin structure (Figure 4D, compare lanes 1–4 with 5–8). The reasons for this restriction in micrococcal nuclease accessibility are unknown, but might reflect the association of additional non-histone proteins with chromatin, or a change in histone chemistry or the release of inhibitors of micrococcal nuclease. Our results demonstrate that although the rate of nucleosome deposition is increased following oocyte maturation (Figure 4C), the

overall spacing of nucleosomes is not increased (Figure 4D). Therefore, we next examined the remaining hypothesis that a more efficient assembly of nucleosomes onto regions of the promoter that had previously been occupied by the transcriptional machinery, might account for the increase in nucleosome number per template. Such a possibility might also explain the capacity for Gal4–VP16 to reduce the increase in the number of nucleosomes following oocyte maturation (Figure 4B).

Our experimental strategy to examine the nucleosomal organization of promoter DNA was to digest chromatin assembled *in vivo* with micrococcal nuclease and then to hybridize the Southern blot of this digest with both a promoter specific probe (Figure 5A, C and E) and a vector probe (B, D and E). By comparing the quality of the nucleosomal array before and after oocyte maturation on the promoter and on vector DNA, we could detect promoter-specific transitions in chromatin structure. The *Xenopus* hsp70 promoter is not assembled into a defined nucleosomal array under normal physiological conditions in the oocyte (Landsberger and Wolffe, 1995). This is apparent in the presence of a DNase I hypersensitive site (Figure 1A, lanes 4–7) and in the absence of a regular nucleosomal array, when a Southern blot of minichromosomal DNA digested using micrococcal nuclease is probed with a radiolabelled DNA fragment specific for the hsp70 promoter (Figure 5A, lanes 3 and 4). However, on maturation of the oocyte, the hsp70 promoter is incorporated into a well defined nucleosomal array (Figure 5A, lanes 5–7). That this change in nucleoprotein organization is specific for the promoter is revealed by control hybridizations to micrococcal digests of minichromosomes using a fragment containing the CAT transcription unit (Figure 5B, lanes 3–10). The transcription unit is within a nucleosomal array before and after meiotic maturation, although the definition of the array improves slightly on maturation perhaps due to the elimination of transcriptional activity from the promoter (Figure 2). If an equilibrium mechanism

Fig. 5. The hsp70 promoter is included in a canonical nucleosomal array after meiotic maturation, transcriptional activators interfere with this inclusion. A comparison is made between the assembly of the promoter into a nucleosomal array (A, C and E) and the assembly of vector DNA into nucleosomal array (B, D and F). 1 ng of HSPCAT template DNA was injected and the oocytes incubated either in the absence (A, lanes 3 and 4; B, lanes 3–6) or presence (A, lanes 5–7; B, lanes 7–10) of progesterone. Minichromosomes were digested with micrococcal nuclease and processed as described in Figure 4. In lanes 1 and 2 naked DNA was digested as control. On the left markers are labeled in base pairs. (A) Hybridization with a probe specific for the hsp70 promoter. (B) Hybridization with a labeled portion of the CAT gene. In (C and D) Gal4–VP16 mRNA was injected into oocytes. After 14 h of incubation, 1 ng of G5HSPCAT template was injected and oocytes incubated either in the absence (C and D, lanes 1–4) or presence (C and D, lanes 5–8) of progesterone. Minichromosomes were digested with micrococcal nuclease and processed as described in Figure 4. In lanes 9–11 naked DNA was digested as a control. The position of markers is indicated in bp. The position of sub-nucleosomal material is indicated. (C) Hybridization with a probe specific for the hsp70 promoter. (D) Hybridization with a labeled portion of the CAT gene. (E and F) HSF mRNA was injected, the oocytes incubated for 14 h and then injected with 1 ng of HSPCAT DNA. The oocytes were then incubated in the absence (lanes 3–6) or in the presence (lanes 7–10) of progesterone. Minichromosomes were processed as described above. Lanes 1 and 2, naked DNA. The position of markers is indicated in bp. The sub-nucleosomal particles are indicated. (E) Hybridization with a probe specific for the promoter. (F) Hybridization with a labeled portion of the CAT gene.

determines the assembly of regulatory nucleoprotein complexes on the promoter then an increase in transcription factor concentration would be expected to disrupt nucleosome structure on the promoter even after meiotic maturation (see Figures 1A, lanes 11–19, 1B, lanes 10–15 and 4B). Expression of Gal4–VP16 or HSF selectively disrupts nucleosome assembly over the promoter (Figure 5C and E). The nucleosomal array is weakly defined with large amounts of sub-nucleosomal material present before oocyte maturation (Figure 5C, lanes 1–4 and 5E, lanes 3–6). After maturation substantial sub-nucleosomal material remains indicative of continued association of transcription factors (Figure 5C, lanes 5–8 and 5E, lanes 7–10). This sub-nucleosomal material is absent when the same filter is rehybridized with a probe outside of the promoter (Figure 5D, lanes 1–8, and 5F, lanes 3–10). Note that there is more sub-nucleosomal material on digestion of promoter DNA in panels C and E than in panel A. This is because in panel A, only endogenous transcription factors are bound to DNA whereas in panels C and E, high levels of exogenous factors protect promoter DNA from micrococcal nuclease digestion much more effectively. Thus the expression of exogenous factors can interfere with the repression of transcription mediated by nucleosome assembly following oocyte maturation (see also Figures 1, 2 and 4). A prediction from this result is that if nucleosome assembly has a causal role in directing the displacement of the transcriptional machinery from the hsp70 promoter, then the presence of additional exogenous DNA should alleviate this repression.

We find that the injection of an increased mass of template DNA (15 ng) reduces nucleosome density relative to the low mass of DNA (1 ng) used in our earlier experiments (Figure 6A, compare lanes 3 and 4). Quantitation of the number of nucleosomes in oocytes before maturation by two dimensional gel electrophoresis reveals there to be an average of eight fewer nucleosomes assembled on the template injected at higher concentration (15 ng per oocyte) compared with the average on DNA at lower concentration (1 ng per oocyte) (Figure 6B, compare Low and High DNA). This reduction in nucleosome density increases transcription both before (Figure 6C, compare lanes 3 and 5) and after oocyte maturation (Figure 6C, compare lanes 4 and 6). However, the relative increase in transcription after maturation is much greater (10-fold) than before maturation. This experiment uses the same protocol as in Figure 2 in which oocytes are allowed 2 h of recovery following injection of template, before 16 h of further incubation in the presence or absence of progesterone. As a control we show the hsp70 mRNA levels that accumulate in oocytes following the injection of low and high DNA concentrations during a 2 h incubation in the absence of progesterone (Figure 6C, lanes 1 and 2). On meiotic maturation, the number of nucleosomes on DNA templates increases by three or four (Figure 4A and data not shown); however, this is insufficient to establish a completely repressive environment at high DNA concentrations (Figure 6C). We conclude that the change in the efficiency of nucleosome assembly that occurs on meiotic maturation has a causal role in directing the repression of transcription.

A dynamic competition between transcription factors and chromatin determines transcriptional activity during meiotic maturation

Our evidence suggests that the remodeling of regulatory nucleoprotein complexes during meiotic maturation depends on transcription factor abundance (Figures 1–3) and the efficiency of nucleosome assembly (Figures 4–6). A prediction from this work is that the late addition of histones or of transcription factors after oocyte maturation should lead to transcriptional repression or activation respectively. We tested this possibility in the experiments shown in Figure 7. The G5HSPCAT template was transcriptionally activated by Gal4–VP16 prior to maturation of the oocyte (Figure 7A, lanes 1 and 2, see also Figure 2B, lanes 5 and 6). At the levels of Gal4–VP16 used very little repression of transcription is observed on oocyte maturation, however, the late addition of core histones to mature oocytes represses transcription (Figure 7A, lanes 3 and 4). This is consistent with our prediction that chromatin assembly can exert a dominant-repressive effect on preassembled transcription complexes. Conversely the late addition of Gal4–VP16 (Figure 7B) or HSF (Figure 7C) to matured oocytes can activate transcription depending on the abundance of the transcription factor. In these two experiments oocyte maturation initially leads to a repression of transcription (see Figure 2B, lanes 2 and 3 and Figure 7C, lanes 1 and 2) that is subsequently reversed by increases in transcription factor abundance (Figure 7B, lanes 2–4 and 6–8; and 7C, lanes 3 and 4). Thus states of gene activity are unstable in the mature oocyte. Our results provide evidence that a continual dynamic equilibrium between transcription factors and chromatin assembly/disassembly determines transcriptional activity following meiotic maturation.

Discussion

We have investigated the molecular mechanisms responsible for transcriptional repression during meiotic maturation of *Xenopus* oocytes. The major conclusion is that transcription states show an unexpectedly high level of instability. Regulatory nucleoprotein complexes are reorganized as a consequence of oocyte maturation (Figure 1). This remodeling leads to transcriptional repression under normal physiological conditions (Figures 2 and 3). Our results indicate that a regulated enhancement in the efficiency of chromatin assembly concomitant with meiotic maturation (Figure 4) directs the displacement of the transcriptional machinery and an increase in the incorporation of regulatory DNA into nucleosomes (Figure 5). Either an increase in the abundance of transcriptional activators (Figures 1, 2 and 7) or a decrease in the efficiency of chromatin assembly (Figure 6) can interfere with the repression of transcription normally seen on meiotic maturation. Addition either of repressive components of chromatin (histones) or of transcriptional activators can reversibly influence transcription even after oocyte maturation is complete (Figure 7). Thus our results provide evidence for the existence of a dynamic competition driving the restructuring of the chromosomal environment towards the repression or activation of transcription.

Fig. 6. The titration of repressive chromatin components alleviates the transcriptional repression obtained after oocyte maturation. (A, B and C) Oocyte nuclei were injected with either a low mass (1 ng) or a high mass (16 ng) of HSPCAT DNA. The oocytes were incubated as previously described in the legend of Figure 2. (A) DNA was extracted from the injected oocytes and resolved on a 1% agarose gel. After transfer, DNA was probed with a random-primed labeled HSPCAT vector. Markers of supercoiled and linear DNA are resolved respectively in lanes 1 and 2. Lane 3 shows the DNA extracted from oocytes injected with a low mass. Lane 4 contains DNA purified from oocytes injected with a high mass. The positions of supercoiled (I), nicked closed circular (II) and linear DNA (III) are indicated. (B) The purified DNAs (Low DNA and High DNA) were resolved on a two-dimensional agarose gel containing chloroquine (see Materials and methods). DNA was transferred and probed as described in (A). The arrows indicate the two dimensions of electrophoresis. In each panel the number indicates the average number of negative supercoils. (C) RNA was extracted from the injected oocytes and analyzed by primer extension. Lanes 1 and 2 (C, control) show the amount of transcripts accumulated in the first 2 h after the DNA injection in the absence of progesterone. In lanes 3 and 5 oocytes were incubated in the absence of progesterone. Lanes 4 and 6 contain oocytes incubated in the presence of progesterone. The position of accurately initiated transcripts from the hsp70 promoter (Hsp70) is indicated.

Transcriptional repression as a consequence of meiotic maturation

A regulatory nucleoprotein complex assembled on the *Xenopus* hsp70 promoter is remodeled and transcription repressed during meiotic maturation (Figures 1 and 2). In addition RNA polymerase II is released from the transcription unit. This result is very similar to events that occur during mitosis (Shermoen and O'Farrell, 1991; Hershkowitz and Riggs, 1995; Martinez-Balbas *et al.*, 1995; Segil *et al.*, 1996). However, an increase in the abundance of a sequence-specific transcription factor (HSF, Gal4-VP16) substantially overcomes this repression (Figures 1, 2 and 7). The activation of transcription can even occur once oocytes have completed the maturation process (Figure 7). Thus although meiotic maturation can direct the dominant repression of genes, the efficiency of repression will necessarily depend on the abundance of

the regulatory factors and their affinity for a specific gene. Moreover transcriptional repression is reversible (Figure 7). In the absence of abundant regulatory factors, the basal transcriptional machinery and RNA polymerase II is functionally compromised following meiotic maturation (Figure 3) presumably because of exclusion by the increased assembly of the promoters into nucleosomes (Figures 4–6, see later). However, since excess Gal4-VP16 almost completely alleviates repression of transcription from a synthetic hsp70 promoter containing the binding sites for this regulatory protein and excess HSF also recovers more than 50% of transcriptional activity (Figures 2A and 7C), this demonstrates that the basal machinery and RNA polymerase II can be recruited in a functional form to promoters after maturation (Figures 2 and 7). Thus any impairment in the function of the basal machinery associated with meiosis can be overcome in

the presence of an excess of a transcriptional regulator (see also Almouzni and Wolffe, 1995). Transactivators such as HSF and Gal4-VP16 may allow access to molecular mechanisms that allow the basal transcriptional machinery to evade the repressive effects of chromatin structure (see Kingston *et al.*, 1996).

We suggest that a major contributory factor towards the general repression of transcription during meiotic maturation in oocytes is an increase in the efficiency of

chromatin assembly (Figure 4). Oocytes contain large stores of histones in their nuclei, sufficient to assemble 20 000 somatic nuclei (Woodland and Adamson, 1977), however, these histones are constrained for nucleosome assembly by association with the molecular chaperones nucleoplasmin (Dilworth *et al.*, 1987) and N1/N2 (Kleinschmidt *et al.*, 1990). Biochemical differences between oocytes and eggs include the phosphorylation of nucleoplasmin in eggs, a modification which facilitates the process of nucleosome assembly (Sealy *et al.*, 1986). Thus an oocyte acquires the capacity for more efficient nucleosome assembly during meiotic maturation (Figures 4 and 5). This increase in the efficiency of nucleosome assembly will provide a more competitive chromatin environment within which the transcriptional machinery has to function. This leads to a more efficient incorporation of promoter elements into canonical nucleosomes (Figure 5) and a concomitant increase in the number of nucleosomes per minichromosome (Figure 4A and B). It should be emphasized that considerable evidence supports the continued presence of histones on actively transcribed genes *in vivo* even after transcriptional activation (see Solomon *et al.*, 1988) although certain regions such as the *Drosophila* hsp70 promoter are histone-free at all times (see Nacheva *et al.*, 1989). Therefore we suggest that meiotic maturation directs an increase in the extent to which the *Xenopus* hsp70 promoter is assembled into canonical nucleosomes, rather than a simple all or none transition. Two independent means to relieve the repression of transcription are to provide more transcription factors (Figure 2), which is disruptive to nucleosome assembly (Figure 4B) or to titrate nucleosome assembly directly (Figure 6A–C). This leads to the hypothesis that the

Fig. 7. Reversibility of transcriptional repression obtained after oocyte maturation. **(A)** The experimental scheme is represented in the upper part of the figure. Gal4-VP16 mRNA was injected into oocytes. After 14 h of incubation, 1 ng of G5HSPCAT DNA template was injected. The oocytes were incubated for 2 h, divided and incubated a further 10 h in the absence or presence of progesterone. Healthy matured oocytes were sorted and either not injected (lane 2) or injected with 4 ng (lane 3) or 8 ng (lane 4) of core histone proteins (see Materials and methods). After 6 h of incubation, the eggs were collected and the level of transcription analyzed by primer extension. In lane 1 the oocytes were injected with Gal4-VP16 mRNA, incubated for 14 h, injected with G5HSPCAT DNA and incubated for 18 h in the absence of progesterone. **(B)** A scheme of the experiment is shown in the upper part of the panel. G5HSPCAT DNA (1 ng) was injected into oocytes. After 6 h of incubation in buffer, progesterone was added to the buffer and the oocytes incubated for a further 10 h. Healthy matured oocytes were sorted and not injected (lanes 1 and 5) or injected respectively with 0.5 ng (lanes 2 and 6), 1 ng (lanes 3 and 7) and 2.5 ng (lanes 4 and 8) of purified recombinant Gal4-VP16 protein (see Materials and methods). The eggs were then incubated for a further 6 h (lanes 1–4) or 16 h (lanes 5–8), collected and analyzed by primer extension. **(C)** A scheme of the experiment is shown. 1 ng of HSPCAT DNA was injected into oocytes. After 6 h of incubation, progesterone was added and the oocytes incubated for a further 8 h. Healthy matured oocytes were not injected (lane 2) or injected with 0.5 ng (lane 3) and 2.5 ng (lane 4) of recombinant *Drosophila* HSF protein (a kind gift of Dr Min Zhang and Carl Wu). The eggs were then incubated for a further 16 h, collected and analyzed by primer extension. Lane 1 shows the amount of transcripts accumulated in 6 h from oocytes injected with the HSPCAT DNA and incubated in the absence of progesterone. The position of accurately initiated transcripts from the hsp70 promoter (Hsp70) is indicated. The transcription signals were quantified with a PhosphorImager, and the levels of hsp70 transcription are indicated in the bar graphs. A.U., arbitrary units.

regulation of transcription from a particular promoter in an oocyte during meiotic maturation depends on competitive and reversible association of sequence-specific transcription factors and the transcriptional machinery versus the association of chromatin proteins with the promoter. This concept is further supported by both the repression of transcription by injected histones (Figure 7A) and the activation of transcription by injected Gal4-VP16 (Figure 7B) or HSF (Figure 7C). Since these proteins were introduced into oocytes after the oocyte maturation process was complete, states of transcriptional repression or activation must be reversible *in vivo*.

Kinetic versus reversible/competitive models for transcriptional regulation in a chromatin context

Early models for gene regulation in a chromatin context considered nucleosomes and transcription complexes as stable structures (Brown, 1984; Weintraub, 1985). Transcriptional activation versus repression was proposed to be largely determined by the rate at which transcription factors assembled a complex versus the rate at which histones assembled a nucleosome on regulatory DNA. Whichever structure came first established a stable state of gene expression. These concepts, although very useful and applicable to numerous genes (Cizewski and Sollner-Webb, 1983; Wandelt and Grummt, 1983; Mattaj *et al.*, 1985; Knezetic and Luse, 1986; Workman and Roeder, 1987; Workman *et al.*, 1988), represent a static view of the role of chromatin in gene regulation that is at variance with many more recent observations.

Transcription complexes are not necessarily stable through the cell cycle (Segil *et al.*, 1996); they can be erased by replication (Wolffe and Brown, 1986), at mitosis (Shermoen and O'Farrell, 1991; Herskovitz and Riggs, 1995; Martinez-Balbas *et al.*, 1995) and during meiotic maturation (this work). The intrinsic instability of individual transcription complexes plays an important role in gene regulation (Wolffe and Brown, 1987, 1988; Kadonaga, 1990; Auble and Hahn, 1993; Auble *et al.*, 1994). Likewise nucleosomes are unstable (Meersseman *et al.*, 1992; Studitsky *et al.*, 1994; Ura *et al.*, 1995), components such as H2A/H2B and H1 exchange in and out of chromatin (Louters and Chalkley, 1985) and molecular machines exist that might facilitate chromatin remodeling (Côté *et al.*, 1994; Tsukiyama *et al.*, 1994; Cairns *et al.*, 1996; Kingston *et al.*, 1996). In light of the dynamic nature of regulatory nucleoprotein complexes it is not surprising that chromatin proteins influence transcriptional efficiency through their association with a promoter in competition with transcription factors. For the oocyte 5S rRNA genes in *Xenopus*, transcriptional efficiency during development depends on the competitive binding of TFIIIA versus histone H1 to a nucleosomal template (Bouvet *et al.*, 1994; Kandolf, 1994). A developmentally regulated increase in the abundance of histone H1 can drive the specific and dominant repression of the oocyte 5S rRNA genes.

The events of meiotic maturation on the hsp70 promoter can be explained by nucleosome-directed displacement of transcription factors in a process that is both competitive and reversible. The general conclusion is that the chromosomal environment is not necessarily passive, but in fact has a continual active role in transcriptional regulation.

Materials and methods

Plasmid constructs

The *Xenopus* hsp70 promoter cloned into the *Xba*I–*Hind*III site of pCAT basic (HSP-CAT; Promega) has been previously described (Ranjan *et al.*, 1993). To generate pG5-HSPCAT, a 152 bp fragment, containing five Gal4 binding sites, was amplified by PCR from pG5E4T (Carey *et al.*, 1990) and cloned into the *Hind*III site of HSP-CAT. The plasmid XHSF-pSP64, containing the coding region of the XHSF1 cDNA (Stump *et al.*, 1995) has been previously described (Landsberger and Wolffe, 1995). The Gal4(1–147)–VP16 fragment was cloned by PCR from plasmid pET15GAL4-VP16 (a kind gift from Dr Yoshihiro Nakatani, National Institute of Child Health and Human Development, Bethesda, MD). The fragment was subcloned into the *Hind*III and *Bam*HI sites of pSP64PA vector (GAL4VP16-pSP64; Promega).

Oocyte microinjection and maturation

The preparation of defolliculated *Xenopus* stage VI oocytes and the microinjection procedure were as previously described (Almouzni and Wolffe, 1993a; Landsberger and Wolffe, 1995). Oocytes were injected into the nucleus with 30 nl of a DNA solution. The different DNA masses utilized are indicated in the text and figure legends. After injection, the oocytes were allowed to recover at 18°C for 2 h, divided into two samples and incubated for a further 14 h in the presence or absence of progesterone (1 µg/ml; Sigma). Oocyte maturation was detected by observation of the breakdown of the oocyte nucleus. Control oocytes or matured oocytes were collected and assayed for transcript levels, chromatin assembly, DNA recovery and protein expression. For the expression of exogenous transcriptional activators, full-length capped RNAs were synthesized by SP6 RNA polymerase transcription of linearized templates with the mMessage mMachine kit (Ambion). Unless indicated otherwise in the figure legends, 30 nl of *in vitro*-synthesized mRNA (200 µg/ml) were injected into the oocyte cytoplasm. The oocytes were then incubated for 16 h at 18°C. Following this time, the oocytes were either incubated as described above or injected with DNA and incubated as previously described.

RNA and DNA analysis of injected oocytes

The injected oocytes (20 per sample) were collected and homogenized with 20 mM Tris–HCl (pH 8.0) (10 µl per oocyte). The homogenate was processed for both DNA and RNA analysis. From half of the sample, RNA was extracted by the RNazol TM method (TM Cinna Scientific). RNA equivalent to five oocytes was analyzed by primer extension as described by Toyoda and Wolffe (1992). The primer utilized (complementary to the CAT gene) and the extension product have been described in Landsberger and Wolffe (1995). Every transcriptional assay has been confirmed by three independent sets of injections.

For DNA analysis, the samples were incubated for 1 h at 37°C in 15 mM EDTA, 20 mM Tris (pH 8.0), 0.5% SDS and 500 µg of proteinase K per ml. The DNA was extracted twice with phenol–chloroform (1:1) and ethanol precipitated. After RNase A treatment, the samples corresponding to two oocyte equivalents were subjected to 1% agarose gel electrophoresis, transferred on a Hybond N membrane (Amersham), and hybridized against a random-primed HSP-CAT plasmid with the Rapid-hyb buffer (Amersham).

Two-dimensional gel electrophoresis was carried out on a 1% agarose gel in the presence of chloroquine. In the first dimension chloroquine concentration was 1.8 µg/ml, and in the second dimension it was 2.8 µg/ml. DNA topoisomers were analyzed by Southern blotting as described above.

Electrophoretic mobility shift assay

Extracts were prepared by homogenizing the injected oocytes in buffer E. After centrifugation for 10 min the supernatant was collected. The assay was performed in a mixture of 15 µl containing the specific DNA binding fragment, 2 mg of poly(dI:dC), 10 mg BSA, in a buffer containing 10 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 5% glycerol. The different amounts of protein extracts utilized are indicated in the figure legend. The reaction was incubated for 20 min at room temperature and then directly loaded on a 1% agarose gel in 0.5× TBE. For the XHSF1 binding assay a double-stranded oligonucleotide (5'-GAATTCATGGAAGCCTCGGGAACTTCGGG-3') containing the HSE1 of the *Xenopus* hsp70 promoter was used. For the Gal4-VP16 binding reaction a 172 bp *Hind*III restriction fragment, containing five Gal4 binding sites, was isolated from pG5-HSPCAT. Labeling was by filling in the ends of the restriction fragment in the presence of [α -³²P]dCTP.

Micrococcal nuclease digestion

Groups of 35 injected oocytes were collected and homogenized in buffer E (25 µl per oocyte). CaCl_2 was added to a final concentration of 3 mM. The oocyte homogenate was incubated for 5 min at room temperature in the presence of MNase as described in the figure legends. The reaction was stopped by adding SDS and EDTA to final concentrations of 0.5% and 15 mM respectively, and the mixture was incubated for 1 h at 37°C with proteinase K (500 µg/ml). Purification and concentration of DNA samples was as before. The digested DNA was loaded on a 1.5% agarose gel, transferred on a Hybond N⁺ membrane (Amersham) and analyzed by hybridization as previously described.

DNase I footprinting

Stage VI oocytes were injected and incubated as previously described. Groups of 25 healthy oocytes were collected and homogenized in 560 µl of buffer E. MgCl_2 was added to a final concentration of 5 mM. The oocyte homogenate was divided into four aliquots, 5, 10, 15 and 20 U of DNase I, respectively, were added and the reaction incubated for 5 min at room temperature. The digestion was stopped by adding an equal volume of stop solution containing 0.5% SDS and 20 mM EDTA. The DNA was purified by proteinase K treatment, phenol-chloroform extractions and isopropanol precipitation. The purified DNA was linearized with *NcoI* and Rnase A digested. After purification by phenol-chloroform extraction and ethanol precipitation, the DNA was resolved on a 1.5% agarose gel. A Southern blot analysis was performed as before. The hybridization probe was an *EcoRI-NcoI* DNA fragment purified from the pCAT basic vector (Promega).

Run-on transcription assay

Groups of 20 healthy oocytes were collected and homogenized in 200 µl of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl_2 , 0.1 mM EDTA, 0.5 mM DTT, 65 mM KCl and 10% glycerol. The homogenate was spun on a G-Sephadex 50 column to clear the endogenous nucleotides. 60 µl of 5× transcription buffer [25 mM Tris-HCl (pH 8.0), 12.5 mM MgCl_2 , 75 mM KCl and 1 mM (each) triphosphates of A, G and C], 40 U of RNasin (Promega) and 150 mCi of a [α -³²P]UTP (3000 Ci/mmol, Amersham) were added to each sample.

For reactions performed in the presence of Sarkosyl, the detergent was added to the 5× transcription buffer to 3% (w/v). After 5 min at room temperature, the reactions were stopped by the addition of SDS and EDTA to final concentrations of 1% and 5 mM, respectively. RNA was extracted by the RNazol TM method (TM Cinna Scientific). The resuspended pellets were digested with 1 U of RNase-free DNase (RQ1 DNase; Promega) for 10 min at 37°C. After ethanol precipitation, the pellets were resuspended in water.

Hybond N⁺ filters containing PCR-amplified DNA from the 5' end, middle and 3' end of the transcribed region of the HSPCAT template were prepared with a Schleicher & Schuell Slot blot apparatus (0.5 µg per slot). The 5' probe extends from position -258 (relative to the start site of transcription, which is +1) to position +122. The middle probe extends from +323 to +533 and the 3' probe from +685 to +914.

Each result has been confirmed by three independent sets of injections. The filters were prehybridized at 42°C for 3 h in Hybrisol I (Oncor). The filters were then hybridized to the run-on products in the same solution for 36 h at 42°C. After hybridization, the filters were washed twice for 30 min at 55°C in 0.1% SDS, 0.1× SSC.

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