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Role of Chromatin and *Xenopus laevis* Heat Shock Transcription Factor in Regulation of Transcription from the *X. laevis hsp70* Promoter In Vivo

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***Xenopus laevis* oocytes activate transcription from the *Xenopus hsp70* promoter within a chromatin template in response to heat shock. Expression of exogenous *Xenopus* heat shock transcription factor 1 (XHSF1) causes the activation of the wild-type *hsp70* promoter within chromatin. XHSF1 activates transcription at normal growth temperatures (18°C), but heat shock (34°C) facilitates transcriptional activation. Titration of chromatin in vivo leads to constitutive transcription from the wild-type *hsp70* promoter. The Y box elements within the *hsp70* promoter facilitate transcription in the presence or absence of chromatin. The presence of the Y box elements prevents the assembly of canonical nucleosomal arrays over the promoter and facilitates transcription. In a mutant *hsp70* promoter lacking Y boxes, exogenous XHSF1 activates transcription from a chromatin template much more efficiently under heat shock conditions. Activation of transcription from the mutant promoter by exogenous XHSF1 correlates with the disappearance of a canonical nucleosomal array over the promoter. Chromatin structure on a mutant *hsp70* promoter lacking Y boxes can restrict XHSF1 access; however, on both mutant and wild-type promoters, chromatin assembly can also restrict the function of the basal transcriptional machinery. We suggest that chromatin assembly has a physiological role in establishing a transcriptionally repressed state on the *Xenopus hsp70* promoter in vivo.**

The developmental and heat-inducible regulation of *Xenopus laevis hsp70* gene expression has been the subject of controversy. Initial reports suggested that within *Xenopus* oocytes, the *Xenopus hsp70* promoter was constitutively active and that gene expression was regulated at the translational level (8–13). Subsequent experiments attributed the induction of *hsp70* protein synthesis upon the heat shock of *Xenopus* oocytes (12) to the transcriptional response of contaminating somatic cells within the follicle surrounding the oocyte (35, 38). Only low levels of *hsp70* mRNA were detected within *Xenopus* oocytes (35). Nevertheless, microinjection of the *Xenopus hsp70* promoter into oocyte nuclei resulted in constitutive transcription that was insensitive to heat shock (35). It was therefore proposed that the *Xenopus hsp70* gene within oocytes represented an exception to the apparent universality of the heat shock response (35, 38). Within early embryos, the *Xenopus hsp70* promoter is not transcribed prior to the mid-blastula transition and becomes heat inducible only following this developmental transition (8, 31, 32). Thus, a model in which the transcription of the *Xenopus hsp70* promoter appears to be differentially controlled during *Xenopus* development was established (10, 32).

Several contributory factors might influence both oocyte-constitutive and heat-inducible transcription of the *Xenopus hsp70* gene. These include the heat shock transcription factor (HSF) that associates with the heat shock response elements (HSEs) found within heat-inducible promoters (50, 54). We have recently cloned *Xenopus* HSF1 (XHSF1) (62) and wished to investigate the proposed role of this protein in the developmental role of *hsp70* promoter activity (10, 11, 46). Chromatin structure appears to have a role in the heat shock response (40). Neither human nor *Drosophila melanogaster* HSF can bind efficiently to chromatin templates in vitro (6, 64). Thus, nucleosome assembly could potentially be responsible for the repression of *hsp70* gene transcription observed in vivo within somatic cells; however, the prior association of TFIID with the *hsp70* promoter can facilitate the subsequent activation of chromatin templates by either human or *Drosophila* HSF in vitro (6, 7, 64). TFIID is bound constitutively to the *Drosophila hsp70* promoter in vivo, so it may always be available to aid the recruitment of HSF to the promoter (23). Moreover, other proteins, such as the GAGA factor, and ATP-dependent activities that might directly modify chromatin structure and facilitate access of HSF to recognition elements in vivo have been defined (66, 68, 69). Once it is bound to the chromatin template, *Drosophila* HSF initiates an unknown process that releases stalled RNA polymerase II from a block to transcriptional elongation, and transcription of the *Drosophila hsp70* gene ensues (23, 44, 52). The generality of this model for other metazoan systems remains to be established. However, in earlier work (39), we have suggested that the efficiency with which exogenous *hsp70* DNA is assembled into chromatin in various *Xenopus* expression systems might contribute to the variability in heat-inducible *hsp70* gene transcription previously observed during *Xenopus* development (8–11, 13, 31, 32).

Constitutive transcriptional activity of the *Xenopus hsp70* promoter in oocytes is dependent on a specialized CAAT box subsequently defined as a Y box (11, 70, 72). Promoters containing the Y box (CTGATTGGC/TC/TAA) are especially active in oocytes (11, 14, 26, 65). The known *Xenopus* genes that contain this element and that are active in oocytes are not constitutively active in somatic cells (11, 14). Such transcription factors as NF-Y that recognize the Y box selectively (17) have previously been shown to facilitate the formation of tissue-specific transcription complexes (42, 73). Thus, the Y box elements may also have a role in the developmental control of *hsp70* gene transcription.

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In this study, we have explored the roles of HSF, chromatin structure, and the Y box elements in the regulation of *Xenopus hsp70* promoter activity following microinjection into *Xenopus* oocyte nuclei. The *Xenopus hsp70* promoter exhibits heat-inducible transcription if basal transcriptional activity is repressed by the assembly of the template DNA into nucleosomes (39). We find that expression of exogenous XHSF1 will constitutively activate the *Xenopus hsp70* promoter even when it is assembled into chromatin. The activation of transcription by exogenous XHSF1 from a chromatin template is facilitated by heat shock conditions and the presence of Y box elements in the promoter. We find that the presence of Y box elements in the *hsp70* promoter prevents the assembly of a repressive chromatin structure and facilitates the association of both XHSF1 and the basal transcription machinery. We suggest that nucleosome assembly has a physiological role of restricting *Xenopus hsp70* transcription in vivo. The Y box elements contribute to the assembly of a preset chromatin state, including the recruitment of the elements of the basal transcription machinery. This preset chromatin state facilitates the recruitment of XHSF1 and transcriptional activation.

MATERIALS AND METHODS

Plasmid constructions. The *Xenopus hsp70* promoter cloned into the *Xba*I-*Hin*dIII site of pCAT basic (HSP-CAT; Promega) has been previously described (51). To generate Y box mutant HSP-CAT (-Y1/Y2), CCAAT1 and CCAAT2 were changed to CGGCC and TACTC, respectively, by PCRs performed on the HSP-CAT basic DNA. The human cytomegalovirus (CMV) clone 101 (kindly provided by Lother Hennighausen, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md.) contains the promoter from the immediate early gene 1 from human CMV. This promoter was used to drive transcription of either the *CAT* gene or the H1° transcription unit (CMV CAT or CMV H1°) (3, 4). The coding region of the XHSF1 cDNA (62) was cloned by PCR from the pT3E1 clone with the primers 5′GCCTTGAAGCTTATGGACCCCCACGG GACT3′ and 5′GCCTTGGTCGACTCAGACTACACACGTATA3′. The obtained fragment (1,368 bp) was subcloned into the *Hin*dIII and *Sal*I sites of pSP64PA (XHSF-pSP64; Promega). All the constructs were confirmed by DNA sequencing.

Oocyte microinjection. Defolliculated oocytes were prepared as described by Almouzni and Wolffe (3). The oocytes were then incubated overnight at 18°C. The following day, healthy oocytes were collected and injected into the nucleus with 30 nl of a DNA solution. The different DNA masses utilized are indicated in the text and figure legends. The oocytes were then allowed to recover at 18°C for 4 h, and then they were maintained at 18°C or heat shocked at 34°C for 2 h. This is an effective heat shock temperature for *X. laevis* (71). Following this treatment, the oocytes were collected and assayed for transcript levels, chromatin assembly, and DNA recovery. The exogenous HSF expression is obtained by injecting 30 nl of in vitro-synthesized HSF mRNA (200 μg/ml) into the oocyte cytoplasm. The oocytes are then incubated for 16 h at 18°C. Following this time, the oocytes are either injected with DNA solution or collected and analyzed for protein expression.

RNA and DNA analysis of oocytes. The injected oocytes 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 three or four oocytes was analyzed by primer extension as described by Toyoda and Wolffe (65). The primer utilized was a 30-mer 3′TACCTCTT TTTTTAGTGACCTATATGGTGG5′ complementary to the *CAT* gene. A 167-nucleotide extension product is obtained from the *hsp70* promoter. The CMV promoter gives rise to an extension product that is 137 nucleotides long.

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% sodium dodecyl sulfate (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 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 made use of a 1% agarose gel and was carried out in the presence of chloroquine. For the first dimension, the chloroquine concentration was 0.8 μg/ml, and for the second dimension, it was 1.8 μg/ml. DNA topoisomers were analyzed by Southern blotting as described above.

In vitro transcription and translation. Full-length capped RNA was made from the cDNA for XHSF1 by SP6 RNA polymerase transcription of the linearized template plasmid with the mMessage mMachine kit (Ambion).

The resultant XHSF1 mRNA was then translated in a rabbit reticulocyte lysate in vitro translation system (Bio-Rad Laboratories). Each 30-μl reaction mixture contained 10 μl of lysate, 50 μM amino acids, 80 mM potassium acetate, and 0.6 μg of RNA. The reaction mixtures were incubated at 30°C for 1 h.

Protein analysis and Western blotting (immunoblotting). Whole-cell extracts were prepared by homogenizing the collected oocytes with buffer E (70 mM KCl, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 1 mM dithiothreitol, 5% sucrose) at 10 μl per oocyte. After the homogenate was centrifuged for 5 min, the supernatant was collected. The nuclei were manually isolated as described by Evans and Kay (18). The nuclear proteins and the proteins from two unlabeled oocytes, utilized as carriers, were extracted by the same procedure described above. Protein extract (10 μl) was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The protein gel was electroblotted on Hybond enhanced chemiluminescence nitrocellulose paper (Amersham) and stained with India ink. The immunodetection was achieved with a polyclonal serum directed against human HSF1 (kindly provided by Carl Wu, National Cancer Institute, Bethesda, Md.). The Amersham enhanced chemiluminescence kit was used for staining.

Micrococcal nuclease digestion. The injected oocytes were collected and homogenized in 25 μl of buffer E per oocyte. $CaCl_2$ was added to a final concentration of 3 mM. The oocyte homogenate was incubated at room temperature in the presence of MNase (2 U per oocyte) for the times indicated 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 500 μg of proteinase K per ml. The samples were phenol-chloroform extracted and ethanol precipitated. After RNase A treatment, the DNA was again phenol-chloroform extracted and ethanol precipitated. Finally, the digested DNA was loaded on a 1.5% agarose gel and analyzed by hybridization as previously described. The probe utilized for analyzing the nucleosomal ladder on the whole minichromosome is the portion of the clone that includes the *CAT* gene. The promoter-specific probe is a *Hin*dIII-*Xba*I fragment (348 bp) purified from the HSP-CAT construct. For indirect end labelling, the DNA was linearized with *Nco*I before resolution on the gel and hybridization with an *Eco*RI-*Nco*I DNA fragment purified from the pCAT basic vector (Promega). All the probes have been labelled by random priming.

DNase I footprinting. Stage VI oocytes were injected and incubated as previously described. Groups of 45 healthy oocytes were collected and homogenized with 725 μl of the following buffer: 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 5 mM $MgCl_2$. The lysate was divided into three tubes, and 50, 60, and 70 U of DNase I, respectively, were added. The reaction mixture was 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 samples were RNase A treated for 1 h at 37°C. The DNA was purified by proteinase K treatment, phenol-chloroform extractions, and isopropanol precipitations. The purified DNA was linearized by restriction enzyme digestion. The DNA was incubated for 10 min at 95°C in the presence of 0.1 M NaOH and then was neutralized with HCl. After purification by phenol-chloroform extraction and ethanol precipitation, the DNA was resuspended in 50 μl of a mixture containing 250 μM deoxynucleoside triphosphates (each), 1× *Taq* buffer, 5 mM $MgCl_2$, 0.8 pmol of a ^{32}P-labelled primer, and 2 U of *Taq* polymerase (Promega). A linear PCR was performed. DNA thermal cycles were 95°C for 2 min, 52°C for 2 min, and 70°C for 3 min. This process was repeated 15 times.

SDS was then added to a final concentration of 0.2%, and the DNA was ethanol precipitated. The primer utilized to footprint the HSEs was a 25-mer, 5′CCTTCATCGTCTTAGATTCGACTGT3′, complementary to the HSP-CAT construct. The primer for the TATA box footprint was the same 30-mer utilized in the primer extension assay.

For indirect end labelling, the purified DNA was restricted with *Nco*I before resolution on a nondenaturing 1.5% agarose gel, transfer to a filter, and hybridization with an *Eco*RI-*Nco*I DNA fragment that had been radiolabelled by random priming.

Potassium permanganate footprinting. For footprinting with potassium permanganate, we made use of a method suggested by S. Brown (Massachusetts General Hospital) that was modified from that of Giardina and colleagues (23). Stage VI oocytes were injected and incubated as previously described. Groups of 10 oocytes were collected and transferred to 200 μl of phosphate-buffered saline. A fresh 0.2 M potassium permanganate solution (22 μl) was added, and the reaction mixture was incubated for 5 min at 25°C. The reaction was stopped by the addition of 300 μl of a stop solution (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 25 mM EDTA, 0.5% SDS, and 1 M β-mercaptoethanol). DNA was purified by proteinase K treatment, phenol-chloroform extraction, and isopropanol precipitation. The purified DNA was linearized by restriction enzyme digestion. DNA was phenol-chloroform extracted, ethanol precipitated, and resuspended in 100 μl of 10% piperidine. After 30 min at 90°C, the reaction mixture was dried in a speed vacuum. DNA was resuspended in 100 μl of water and dried once again. DNA was then resuspended in 50 μl of a solution containing dNTPs (250 μM each) in Vent buffer (New England Biolabs), 2 mM $MgCl_2$, 0.8 pmol of a ^{32}P-labelled primer (as in the primer extension assay for mRNA; see above), and 2 U of Vent polymerase (New England Biolabs). A linear PCR was performed. DNA thermal cycles were 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min. This process was repeated 25 times. SDS was then added to a final concentration of 0.2%, and the DNA was precipitated with ethanol.

Run-on transcription experiments. The injected oocytes were placed in isolation buffer (25 mM Tris-HCl [pH 8.0], 10% glycerol, 5 mM $MgCl_2$, and 2 mM dithiothreitol), and the nuclei were isolated as described by Evans and Kay (18).

After 25 germinal vesicles were collected, they were centrifuged at 1,500 × *g* in a Microfuge for 3 min and resuspended in nuclear freezing buffer (40% glycerol, 50 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol). To each reaction mixture containing 210 μl of nuclei, 60 μl of 5× transcription buffer (25 mM Tris-HCl [pH 8.0], 12.5 mM $MgCl_2$, 375 mM KCl, and 1 mM [each] triphosphates of A, G, and C), 40 U of RNasin (Promega), and 150 μCi of [α-^{32}P]UTP (Amersham) (3,000 Ci/mmol) were added. For reactions performed in the presence of Sarkosyl, the detergent was added to the 5× transcription buffer to 3% (wt/vol). After 5 min of incubation 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 *hsp70* wild-type template or CMV H1° template (see the figure legends) were prepared with a Schleicher & Schuell slot blot apparatus (0.5 μg per slot). For the *hsp70* wild-type template, 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 +2501 to +2711. The 3′ probe extends from +2863 to +3052. For the CMV H1° template, the 5′ probe extends from −526 to +120 and the 3′ probe extends from +265 to +585. 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 (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Nucleotide sequence accession number. The sequence for XHSF1 has been entered into GenBank under accession number L36924.

RESULTS

The *Xenopus hsp70* promoter responds to heat shock under conditions that promote chromatin assembly. Early work suggested that the *Xenopus hsp70* promoter was constitutively active after it was microinjected into *Xenopus* oocyte nuclei (9, 11, 35). The transcriptional efficiency of templates microinjected into oocyte nuclei depends on the efficiency with which they are assembled into nucleosomes (3, 48). Thus, the constitutive activity of the *Xenopus hsp70* promoter might reflect an inefficient assembly of the template DNA into chromatin by the earlier protocols (39). We wished to directly examine the extent to which the transcriptional activity of the *Xenopus hsp70* promoter was dependent on assembly into chromatin. We microinjected a low mass (3 ng) of template containing the *Xenopus hsp70* promoter mixed with the CMV promoter (0.1 ng) as an internal control into oocyte nuclei, and this was followed by a 4-h incubation to allow the assembly of chromatin. We then incubated the oocytes for a further 2 h at 18°C or under heat shock conditions at 34°C. Heat shock resulted in an increase of *hsp70* promoter transcription relative to that of the CMV control (Fig. 1A; compare lanes 1 and 2); quantitation by densitometry revealed an eightfold stimulation. In contrast, microinjection of a high mass (15 ng) of the *Xenopus hsp70* template mixed with the CMV promoter (0.1 ng) by the same experimental protocol led to constitutive *hsp70* promoter activity that was not increased by heat shock (Fig. 1A; compare lanes 3 and 4). This latter result is consistent with earlier observations (9, 11, 35, 39). Note that in these experiments, transcription from the CMV promoter remains constant, whereas transcription from the *hsp70* promoter varies over a wide range (see also Fig. 2 and 5). This indicates that transcription factors shared between the CMV and *hsp70* promoters are not limiting and that the CMV promoter functions effectively in this type of chromatin environment, in contrast to the *hsp70* promoter (3). As a control, DNA was isolated from the microinjected oocytes and analyzed by Southern blotting, which indicated that the *hsp70* template was stable during heat shock and that comparable levels of template were present in the oocytes (Fig. 1B). Moreover, the elimination of heat-inducible transcription from the *hsp70* promoter upon injection of a high mass of DNA correlates with a reduction in overall DNA superhelicity, which is indicative of a reduction in the number of nucleosomes assembled on the template (Fig. 1B, C, and D). Quantitation of the DNA superhelicity of chromatin templates by two-dimensional agarose gel electrophoresis with chloroquine (Fig. 1C) indicates that there is an average of 25 nucleosomes assembled on the 4,685-bp-long template DNA molecule used at a low concentration (3 ng per oocyte) in these experiments. This indicates that one nucleosome as-

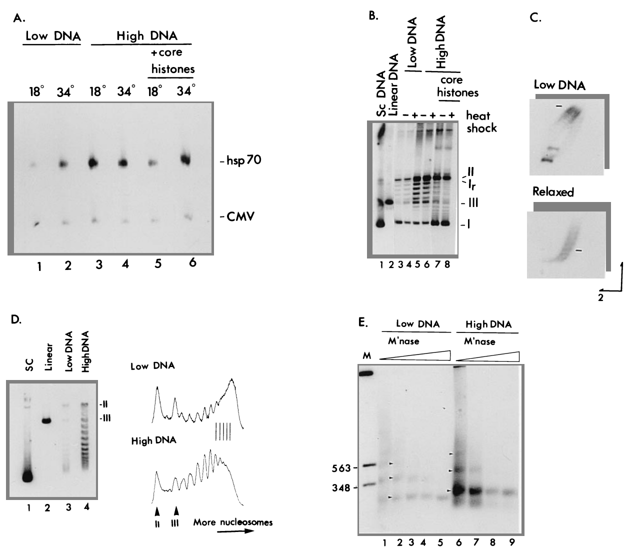

FIG. 1. A role for chromatin in the regulation of the *Xenopus hsp70* gene. *Xenopus* oocyte nuclei were injected with either a low mass of DNA (*hsp70* template, 3 ng, and CMV template, 0.1 ng) or a high mass of DNA (*hsp70* template, 15 ng, and CMV template, 0.1 ng) or with a high mass of DNA after being injected with 15 ng of a mixture of all four core histones. After 4 h of incubation at 18°C, the oocytes either were maintained for a further 2 h at 18°C or were heat shocked at 34°C for 2 h. (A) RNA was extracted from these oocytes and was analyzed by primer extension. The positions of correctly initiated transcripts from the *hsp70* promoter (hsp70) and the CMV promoter (CMV) resolved on a 6% polyacrylamide gel containing 7 M urea are indicated. (B) DNA was extracted from these oocytes and was analyzed by electrophoresis on a 1% agarose gel before transfer to a membrane and hybridization with radiolabelled *hsp70* template DNA. Markers of supercoiled (Sc) (lane 1) and linear (lane 2) *hsp70* template DNA were also resolved. The positions of supercoiled (I), nicked closed circular (II), relaxed closed circle (I_r), and linear (III) DNA are indicated. (C) DNA from oocytes injected with a low mass (3 ng) of DNA and incubated for 4 h at 18°C was extracted. The purified DNA (Low DNA) was resolved on a two-dimensional agarose gel containing chloroquine (see Materials and Methods). As a control, the same plasmid template previously relaxed with topoisomerase I (Relaxed) was also resolved. The arrows at the lower right indicate the two dimensions of electrophoresis. In each panel, the horizontal bar indicates the center of topoisomer distribution. (D) DNA supercoiling of *hsp70* template DNA at low and high concentrations following incubation at 18°C for 4 h. DNA was purified and resolved on a 1% agarose gel containing chloroquine (1 μg/ml). Lane 1, supercoiled (SC) marker DNA; lane 2, linear marker DNA, lane 3, topoisomers of a low mass (3 ng) of injected DNA; lane 4, topoisomers of a high mass (15 ng) of injected DNA. The positions of nicked closed circular (II) and linear (III) DNA are indicated. Densitometric scans of the autoradiograph are shown to its right. The vertical bars indicate the positions of the topoisomers, which facilitates a comparison of the superhelicities of the two DNA samples injected at low and high concentrations. (E) As in panel D, except that at the end of the incubation period, minichromosomes were digested with micrococcal nuclease for increasing times (2, 4, 8, 16, and 45 min, as indicated by the open triangles above the lanes) before deproteinization and resolution on a 1.5% agarose gel. The gels were transferred to nitrocellulose and hybridized with random-primed *hsp70* template DNA. Micrococcal nuclease (M'nase) digests of minichromosomes assembled on low and high masses of injected DNA are shown. Markers (M) are labeled in base pairs. The arrowheads indicate the positions of DNA fragments of mononucleosome, dinucleosome, and trinucleosome length.

sembles for every 188 bp of DNA, which is a physiological nucleosome density for *X. laevis* (74). One-dimensional chloroquine gels (Fig. 1D) indicate that there is an average of five to six fewer nucleosomes assembled on the DNA injected at a higher concentration (15 ng per oocyte) compared with the average on DNA injected at a lower concentration (3 ng per oocyte). This means that the spacing of the nucleosomes increases (Fig. 1E) from one nucleosome for every ~190 bp of DNA at a low DNA mass to one nucleosome for every ~240 bp at a high mass (see also references 23, 24, 56, and 57). Note that the apparent sizes of the mononucleosomes also differ on the basis of the DNA mass (Fig. 1E; compare lanes 5 and 9). This is probably due to different efficiencies of digestion that might reflect differences in the incorporation of linker histones or high-mobility group proteins into nucleosomes that are differently spaced. There is no detectable change in the rate or efficiency of chromatin assembly between 18°C and 34°C (Fig. 1B; compare lane 3 with lane 4 and lane 5 with lane 6) (data not shown). Thus, a small reduction in nucleosome density can have a major impact on the level of heat shock induction of transcription.

We next examined whether the readdition of purified *Xenopus* core histones to *Xenopus* oocyte nuclei when a high mass of DNA was microinjected would restore heat-inducible regulation of the *hsp70* promoter by restoring chromatin assembly (4, 20). Microinjection of core histone (15 ng) before the injection of a mixture of templates containing *hsp70* (15 ng) and CMV (0.1 ng) promoters restores significant heat-inducible *hsp70* promoter activity (Fig. 1A; compare lanes 5 and 6 [quantitation reveals a fivefold induction]). Control experiments indicate that equivalent quantities of template were recovered from control and heat-shocked oocytes (Fig. 1B; compare lanes 7 and 8). Moreover, these Southern blots indicate that the addition of the additional core histones restored negative supercoiling and hence chromatin assembly on the DNA microinjected at a high mass (Fig. 1B; compare lanes 5 and 6 with lanes 7 and 8). The microinjected templates are initially relaxed by endogenous topoisomerase activities following microinjection into oocyte nuclei and then are resupercoiled as nucleosomes are assembled (2, 3, 21, 53). These results are consistent with the hypothesis that chromatin assembly exerts a repressive role in the regulation of *hsp70* transcription (see also reference 39). The response of the *hsp70* gene to heat shock of exogenous DNA in the oocyte is less than might be anticipated from experiments with other organisms (28, 54); this may reflect a high constitutive level of activity in the absence of heat shock (8, 39) or the intrinsic stress of oocyte isolation and microinjection. It is not possible to assess the significance of this level of induction to that of the endogenous genes within the oocyte chromosome, since oocytes are relatively few in number and only tetraploid. Thus, the amounts of cells and genes are too small to rigorously assess transcriptional response by conventional PCR methodologies.

Expression of exogenous XHSF1 and titration of chromatin assembly cause constitutive transcription from the *Xenopus hsp70* promoter. In our next experiments, we wished to further examine the regulation of *Xenopus hsp70* gene expression by directly testing the hypothesis that the heat-inducible accumulation of transcripts from the *hsp70* promoter was a direct consequence of increased transcription rates. The use of run-on assays would also potentially provide insight into the potential mechanism of transcriptional regulation (52). We also examined the consequences for *hsp70* promoter activity of the expression of exogenous XHSF1 and of the titration of chromatin assembly.

We cloned and characterized XHSF1 (62). The 451-amino-acid protein has significant overall identity (>40%) to human HSF1 and >90% identity to it over the N-terminal DNA binding and trimerization domains (49, 55, 62). When it is synthesized in vitro in a rabbit reticulocyte lysate by translation of synthetic mRNA encoding XHSF1, the 67-kDa XHSF1 protein is recognized by antibodies against human HSF1 (the kind gift of C. Wu) (Fig. 2A, lane 2). Microinjection of synthetic mRNA encoding XHSF1 into the cytoplasm of *Xenopus* oocytes leads to the synthesis of XHSF1, which accumulates in the oocyte germinal vesicle (Fig. 2A, lanes 4 and 5 [arrow]). We did not detect any endogenous HSF in the oocyte by this immunoblotting method. Heat-inducible DNA-binding proteins that recognize HSEs are present in *Xenopus* eggs (46); thus, it is possible that endogenous oocyte HSF is present in too low an abundance to be detected or is posttranslationally modified so that it is not detectable by immunoblotting. Alternatively, the *Xenopus* HSF detected by Ovsenek and Heikkila (46) might be translated from stored mRNA activated after oocyte maturation (35, 63). The uptake of exogenous XHSF1 into the nucleus in the absence of heat shock is similar to that of mouse HSF1 or HSF2 that is overexpressed in mouse tissue culture cells; these proteins can demonstrate constitutive trimerization, nuclear localization, and DNA binding in the absence of stress (54). Nuclear localization occurred with equivalent efficiencies at 18 and 34°C (data not shown). Note that because of differences in the volumes of nuclei and cytoplasm, equivalent amounts of XHSF1 protein within nuclei and cytoplasm amount to more than 10-fold preferential localization to nuclei (16).

Our next experiments examined whether XHSF1 might have a role in the transcriptional regulation of the *Xenopus hsp70* promoter microinjected into *Xenopus* oocyte nuclei. We again microinjected mixtures of templates containing either the *hsp70* (2 ng) or CMV (0.2 ng) promoters. We reduced the DNA mass to promote the assembly of template DNA into chromatin; in all other respects, the experimental protocol remained unchanged. Control experiments demonstrated a significant increase in *hsp70* promoter activity relative to CMV transcription under heat shock conditions (Fig. 2B; compare lanes 1 and 2). When XHSF1 is expressed in *Xenopus* oocytes following microinjection of XHSF1 mRNA (Fig. 2A), the *hsp70* promoter is active at both 18 and 34°C (Fig. 2B; compare lanes 3 and 4). Control experiments demonstrated that the injection of a comparable mass (6 ng) of nonspecific RNA into oocyte cytoplasm had no effect on *hsp70* transcription (data not shown). Increased expression of exogenous XHSF1 causes activation of *hsp70* transcription in the absence of heat shock. Similar results have been obtained with mouse tissue culture cells following overexpression of mouse HSF1 or HSF2 at non-heat shock temperatures for these cells (54). Compared with that of endogenous HSF under heat shock conditions (Fig. 2B, lane 2), the expression of exogenous XHSF1 significantly augments *Xenopus hsp70* promoter activity relative to that of the CMV promoter at both 18°C (a 5-fold increase in efficiency) and 34°C (a 10-fold increase) (Fig. 2B; compare lanes 2, 3, and 4). We suggest that following the microinjection of 2 ng of template DNA containing the *Xenopus hsp70* promoter, the endogenous *Xenopus* HSF is limiting for transcriptional activation of this DNA in the oocyte (see below).

Our next experiments examined whether the presence of exogenous XHSF1 or titration of chromatin by injection of high masses of DNA would stimulate transcription directly. We confirmed our earlier observation (Fig. 1A) that microinjection of a high-mass mixture of *hsp70* (15 ng) and CMV (0.2 ng) templates eliminated heat-inducible transcription of the *hsp70* promoter and led to constitutive promoter activity (Fig.

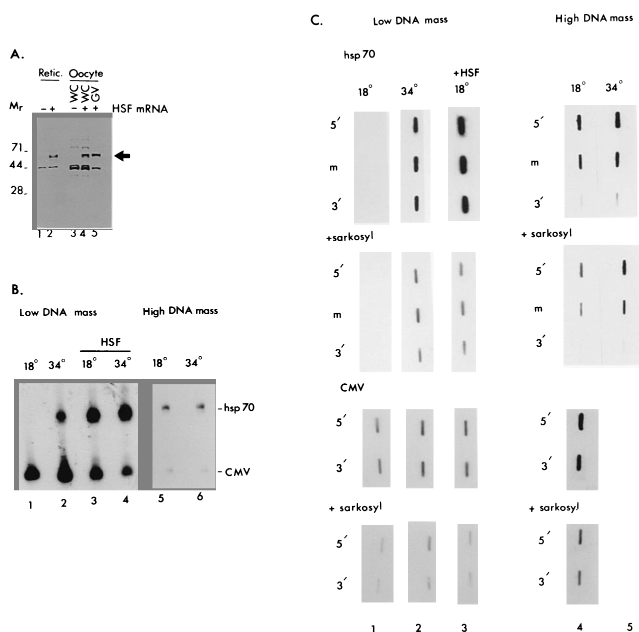

FIG. 2. Expression of exogenous XHSF1 or titration of chromatin assembly activates transcription from the *Xenopus hsp70* promoter. (A) Synthesis of XHSF1 by translation of XHSF1 mRNA in vitro or in vivo. An immunoblot of proteins resolved by SDS–10% PAGE following translation of XHSF1 mRNA in a rabbit reticulocyte lysate (Retic.) (lanes 1 and 2) or following microinjection into *Xenopus* oocytes (lanes 3 to 5) is shown. Control translations in the absence of XHSF1 mRNA (lanes 1 and 3) together with those containing XHSF1 mRNA (lanes 2, 4, and 5) are shown. The stage VI oocytes were injected with 6 ng of the HSF mRNA. After the oocytes were incubated for 16 h at 18°C, batches of 10 oocytes were dissected into germinal vesicles (GV), nuclei, and cytoplasm. In lanes 3 and 4, a whole-cell extract (WC [1 oocyte equivalent]) from uninjected or injected oocytes, respectively, was loaded. Lane 5 contains a nuclear extract from an equivalent number of injected oocytes (1 oocyte equivalent). The polyacrylamide gel was immunoblotted against polyclonal antibodies against human HSF1 (the kind gift of C. Wu). The arrow indicates XHSF1. Markers (M_r) are in kilodaltons. (B) The heat shock response of the *Xenopus hsp70* promoter in the absence or presence of exogenous XHSF1 and in the presence or absence of efficient chromatin assembly. XHSF1 mRNA was not injected (lanes 1, 2, 5, and 6) or was injected (lanes 3 and 4) into stage VI oocytes. After the oocytes were incubated for 16 h at 18°C, a mixture of templates containing *hsp70* (2 ng) and CMV (0.2 ng) immediate early promoters was injected (lanes 1 to 4). Alternatively, a mixture of templates containing *hsp70* (15 ng) and CMV (0.2 ng) was injected (lanes 5 and 6). The oocytes were incubated for 4 h at 18°C, divided, and incubated a further 2 h at either 18°C (lanes 1, 3, and 5) or 34°C (lanes 2, 4, and 6). After this time, RNA was extracted and analyzed by primer extension. The positions of accurately initiated transcripts from the *X. laevis hsp70* promoter (hsp70) and the CMV immediate early promoter (CMV) are indicated. (C) Template-engaged RNA polymerase on the *Xenopus hsp70* promoter under different transcription conditions in vivo. Radiolabelled RNA was hybridized to DNA probes immobilized on a filter (see Materials and Methods). In the *hsp70* panels, probes correspond to DNA sequences at the 5′ end, middle (m), and 3′ end of the *CAT* gene downstream of the *hsp70* promoter. In the CMV panels (control), probes correspond to DNA sequences at the 5′ and 3′ regions of the H1° transcription unit downstream of the CMV promoter (see Materials and Methods). Transcripts generated under the various conditions indicated were obtained from isolated nuclei in the absence or presence (+sarkosyl) of 0.6% Sarkosyl (see Materials and Methods). Run-on transcription after injection of *hsp70* template (2 ng) and CMV-H1° template (0.2 ng) was followed by incubation at 18°C in the absence (lane 1) or presence (lane 3) of exogenous XHSF1 in vivo or at 34°C in the absence (lane 2) of exogenous XHSF1 (as in panel B). Run-on transcription after injection of *hsp70* template (15 ng) and CMV-H1° template (0.2 ng) was followed by incubation at 18°C (lane 4) or 34°C (lane 5) in vivo (as in panel B). The numbers of labelled uridine residues in the sequences complementary to each probe are 25 for the *hsp70* 5′ probe, 59 for the middle (m) probe, 65 for the *hsp70* 3′ probe, 17 for the CMV 5′ probe, and 38 for the CMV 3′ probe.

2B; compare lanes 5 and 6). We repeated these expression experiments, making use of a run-on transcription protocol. Radiolabelled transcripts were hybridized to regions at the 5′ end, middle, and 3′ end of the transcribed region. We found that transcript accumulation (Fig. 2B) correlated very well with transcription as assayed by a run-on protocol (Fig. 2C [upper panels]). We conclude that the changes in RNA accumulation are primarily the consequence of changes in transcriptional efficiency.

Lis and colleagues have convincingly demonstrated the presence of a transcriptionally engaged, paused RNA polymerase II molecule at the 5′ end of an uninduced *D. melanogaster hsp70* gene (23–25, 44, 52). Rougvie and Lis have proposed that chromatin or associated proteins might impede the progression of RNA polymerase (52). The addition of Sarkosyl (0.6%) to transcriptionally active chromatin strips nontranscribing RNA polymerases and the vast majority of other chromatin-associated proteins, including histones, from the DNA template (22, 27). It is, however, possible that Sarkosyl-resistant proteins might also contribute to the generation of a paused RNA polymerase II molecule. Transcriptionally engaged RNA polymerases remain on the template and, in the presence of RNA precursors, can continue to elongate along genes. Thus, initiation of new transcription does not occur under these conditions, but transcriptional elongation proceeds. Incubation of DNA templates in which RNA polymerase is engaged at the 5′ end of the gene but is blocked by chromatin in Sarkosyl might allow the progression of the polymerase along the gene. Run-on experiments with the *Xenopus hsp70* promoter in which hybridization to regions at the 5′ end, middle, and 3′ end of the transcribed region takes place show that incubation in Sarkosyl does not facilitate transcription at 18 or 34°C when a high mass of DNA is injected into the oocyte (Fig. 2C, lanes 4 and 5 of the *hsp70* and Sarkosyl panels; note in particular the lack of enhancement at the 3′ end of the transcribed region). Nor does Sarkosyl facilitate transcription when a low mass of DNA is injected and then incubation at either 18 or 34°C takes place (Fig. 2C, lanes 1 to 3 of the *hsp70* and Sarkosyl panels). It is, however, clear that more RNA polymerase is recruited to the *hsp70* gene and is transcriptionally engaged at 34 than at 18°C; a comparable recruitment occurs when exogenous XHSF1 is overexpressed at 18°C. Control run-on experiments indicate that transcription of the CMV promoter-driven transcription unit did occur in the microinjected oocytes at 18°C in the absence of exogenous HSF (Fig. 2C, CMV panels). In a result consistent with the RNA accumulation data (Fig. 2B), CMV transcription was not significantly influenced by heat shock or expression of exogenous HSF. The apparent lack of transcription from the Sarkosyl-treated *hsp70* transcription unit at 18°C in the absence of exogenous HSF (Fig. 2C, lane 1) opens the possibility that template-engaged RNA polymerase II is not present in significant levels on the wild-type *Xenopus hsp70* promoter when the promoter is inactive (see Discussion). However, to prove the absence of RNA polymerase II would require the demonstration that our run-on experiments are capable of detecting transcripts produced by one RNA polymerase molecule per active template. We do detect active transcription of the CMV promoter-driven transcription unit under conditions in which the *hsp70* template is inactive at 18°C without exogenous HSF. This indicates that transcription does occur in the injected nuclei. However, we have not demonstrated in our experiments that each template has equivalent maximal transcriptional activities following heat shock or the addition of exogenous HSF (see Fig. 4). Thus, we do not know if each template has the potential to be transcribed. Moreover, we cannot ex-

clude the possibility that multiple polymerases (that may or may not be paused) are associated with a template, even after Sarkosyl treatment, when transcriptional reinitiation does not occur. Thus, we cannot definitively conclude that the regulation of the *Xenopus* genes differs from that of *Drosophila* genes (see Discussion).

An interesting feature of transcription with high masses of DNA is that RNA polymerase clearly prefers to associate with the 5′ end of the *Xenopus hsp70* transcription unit (Fig. 2C, lanes 4 and 5 of the *hsp70* panel; the number of uridine residues is actually greater at the 3′ end than at the 5′ end of the transcription unit [see the legend to Fig. 2C]). This result is specific for the *hsp70* gene, since RNA polymerase shows a much more uniform distribution on the CMV-driven transcription unit (Fig. 2C, lane 4 of the CMV panel). It is possible that the preferential association of RNA polymerase at the 5′ end of the *hsp70* transcription unit at a high mass of DNA indicates that the polymerase prefers to pause close to the transcription start site (see Discussion). However, the preferential association of RNA polymerase with the 5′ end of the *hsp70* transcription unit is not relieved by the addition of Sarkosyl (Fig. 2C, lanes 4 and 5 of the *hsp70* and Sarkosyl panels). This result and the absence of preferential 5′ association at low concentrations of DNA make it unlikely that histone proteins contribute to any pausing of polymerase. However, other Sarkosyl-resistant proteins either in the transcription complex or downstream of the transcription start site might contribute to polymerase pausing.

We wished to determine the local organization of chromatin over the *hsp70* promoter on the basis of the concentration of DNA by nuclease cleavage and indirect end labelling. Our initial analysis of nucleosome density on the minichromosomes indicates that an increase in average nucleosome spacing will occur following the injection of a high mass (15 ng) of DNA (Fig. 1). More importantly, indirect end labelling reveals marked differences in the micrococcal nuclease and DNase I cleavage patterns of the *hsp70* promoter that are dependent on the mass of DNA injected (Fig. 3). When a low mass of DNA is assembled into chromatin, there is a clear micrococcal nuclease-hypersensitive site close to the TATA homology (Fig. 3A [large arrowhead]; compare lanes 1 and 2 with lanes 3 and 4). When a high mass of DNA is injected, no such hypersensitive site is apparent (Fig. 3A; compare lanes 5 and 6 with lanes 7 and 8). A comparable DNase I-hypersensitive site is seen within chromatin when a low mass of DNA is injected (Fig. 3B [arrowhead], lanes 3 and 4). The hypersensitive site at a low mass of DNA might reflect the constitutive association of transcription factors with the *hsp70* promoter, which confers accessibility to nuclease and is flanked by nucleosomes that restrict cleavage (see below). At a high mass of DNA, the reduction in the density of nucleosomes (Fig. 1) might reduce these differences in nuclease accessibility. For conditions with both low and high masses of DNA, certain similarities in micrococcal nuclease cleavage were also revealed (Fig. 3A). Regions of protection from micrococcal nuclease can be found to either side of the promoter in the assembled minichromosomes. These protected regions (Fig. 3A [vertical lines]) are punctuated by micrococcal nuclease cleavage sites (Fig. 3A [small arrowheads]), and their spacing reflects a nucleosomal organization. Thus, while the overall spacing of nucleosomes changes on the basis of the mass of the injected DNA and while there is a distinct nucleoprotein organization of the *hsp70* promoter, the chromatin organization flanking the *hsp70* promoter is similar at high and low concentrations of DNA.

We next examined whether the association of proteins with the *hsp70* promoter on the microinjected templates varied under the different transcription conditions that were established at a low mass (2 ng) of DNA, conditions under which chromatin would also be efficiently assembled (Fig. 2). We digested the minichromosomes with DNase I (3). The *Xenopus hsp70* promoter contains three HSEs, two Y boxes, and a TATA box (8) (see Fig. 5A). We initially examined protein binding to the three HSEs (Fig. 4). When XHSF1 is overexpressed, footprints are visible on all three HSEs at 18 and 34°C (Fig. 4, lanes 4 and 5). Without exogenous XHSF1, only weak footprinting is visible at 34°C (Fig. 4; compare lanes 2 and 3). We conclude that under the conditions in which exogenous XHSF1 is expressed, XHSF1 stably associates with all three HSEs at 18 and 34°C. As we will discuss later, under all of these experimental conditions, footprinting around the TATA box itself was obtained with the wild-type *hsp70* promoter (see Fig. 7) (data not shown). There is an approximate correlation between the efficiency of *hsp70* transcription in *Xenopus* oocytes under these conditions (Fig. 2B) and the efficiency of the DNase I footprinting of the HSE elements (Fig. 4). This observation is consistent with the hypothesis that endogenous HSF is limiting for transcription of the microinjected *hsp70* templates. However, the limitation of endogenous HSF indicates that there will be template heterogeneity under conditions in which HSF mRNA is not injected. Since the expression of high levels of HSF circumvents normal heat-inducible transcription (Fig. 2B and 3), there might also be an alteration in the normal order of the assembly of transcription complexes under these conditions. Both of these observations could also explain our failure to detect paused RNA polymerase on the *hsp70* promoter (Fig. 2C) (see Fig. 7B). Nevertheless, our in vivo footprinting experiment (Fig. 4) demonstrated that the overexpression of XHSF1 allows XHSF1 to associate with the HSEs within the wild-type *hsp70* promoter on the vast majority of templates, which thereby allows the more effective transcriptional activa-

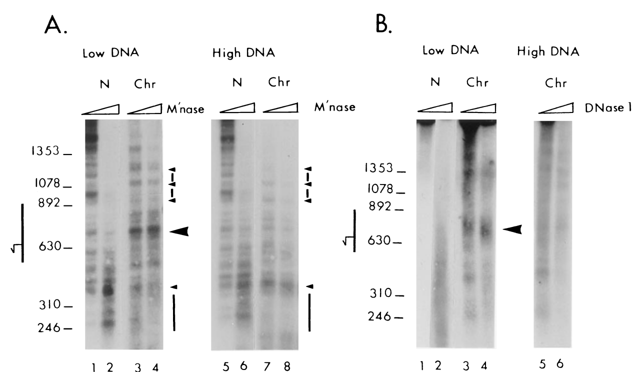

FIG. 3. Micrococcal nuclease and DNase I cleavage of the *hsp70* template assessed by indirect end labelling. Stage VI oocytes were injected with a low mass (2 ng) of DNA (lanes 1 to 4) and a high mass (15 ng) of DNA (lanes 5 to 8) of wild-type *hsp70* template and incubated for 4 h at 18°C. After this time, batches of 10 injected oocytes were digested with micrococcal nuclease or DNase I as indicated. (A) Micrococcal nuclease (M'nase). Control naked DNA (30 ng) was digested with 0.5 (lanes 1 and 5) and 1 (lanes 2 and 6) U of micrococcal nuclease. Minichromosomes were digested with 25 U (lanes 3 and 7) and 50 U (lanes 4 and 8) of micrococcal nuclease. DNA was purified and digested to completion with *Nco*I, and half of the DNA from each sample was electrophoresed on a 1.5% agarose gel. The gel was transferred to nitrocellulose and hybridized with a radiolabelled *Nco*I-*Eco*RI fragment. Distances in base pairs relative to the *Nco*I site are indicated at the left. Regions of protection from micrococcal nuclease cleavage in the minichromosomes are indicated by the vertical lines; small arrowheads indicate the sites of cleavage between protected regions. A micrococcal nuclease-hypersensitive site is indicated by the large arrowhead. The vertical line to the left of the panel indicates the *hsp70* promoter sequence, and the hooked arrow indicates the start site of transcription. N, naked DNA; Chr, chromatin. (B) DNase I. All conditions and procedures were the same as those in panel A, except that DNase I was used in the place of micrococcal nuclease.

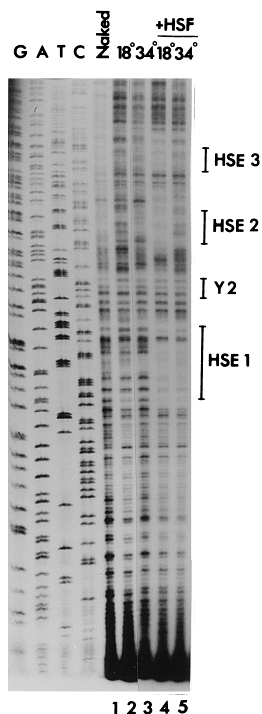

FIG. 4. In vivo footprinting of the *Xenopus hsp70* promoter microinjected into oocyte nuclei in the absence and presence of heat shock and with and without exogenous XHSF1. XHSF1 mRNA was not injected (lanes 2 and 3) or was injected (lanes 4 and 5) into stage VI oocytes. After the oocytes were incubated for 16 h at 18°C, a DNA solution containing *hsp70* (2 ng) was injected. The oocytes were incubated for 4 h at 18°C, divided, and incubated a further 2 h either at 18°C (lanes 2 and 4) or at 34°C (lanes 3 and 5). After this time, DNase I footprinting was carried out (see Materials and Methods). After denaturation in formamide, the DNA samples were resolved on a denaturing 6% polyacrylamide gel containing 7 M urea. Lane 1 shows the cleavage of naked DNA. Sequencing reactions are shown as markers (lanes G, A, T, and C), and the positions of the three HSEs and Y2 are also shown.

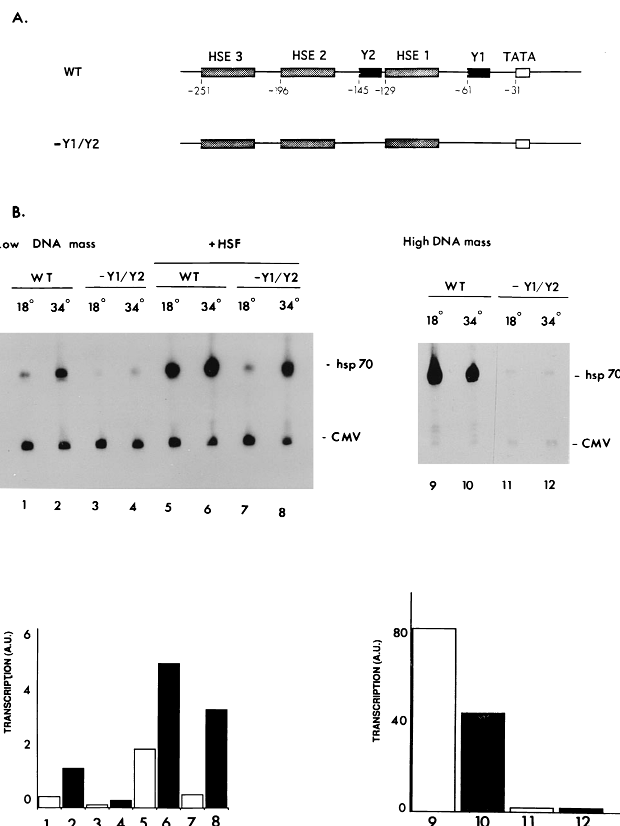

FIG. 5. Role of the Y box elements in *Xenopus hsp70* transcription at low and high masses of DNA. (A) Map of the *Xenopus hsp70* promoter (8, 9). Distances are indicated relative to the start site of transcription at position +1. See Materials and Methods for details of -Y1/Y2 mutant construction. WT, wild type. (B) Exogenous XHSF1 overcomes the requirement for the Y box elements to activate transcription at 34°C but does not do so effectively at 18°C within a chromatin environment (lanes 1 to 8). XHSF1 mRNA was not injected (lanes 1 to 4) or was injected (lanes 5 to 8) into stage VI oocytes. After the oocytes were incubated for 16 h at 18°C, a mixture of templates containing a low mass of DNA and wild-type (WT) or Y box mutant *hsp70* promoters (2 ng) and the CMV immediate early promoter (0.2 ng) was injected into the *Xenopus* oocytes. After incubating for 4 h at 18°C, the oocytes were divided and incubated a further 2 h at either 18°C (lanes 1, 3, 5, and 7) or 34°C (lanes 2, 4, 6, and 8). After this time, RNA was extracted and analyzed by primer extension. The positions of accurately initiated transcripts from the *X. laevis hsp70* promoter (hsp70) and the CMV immediate early promoter (CMV) are indicated. The Y box elements contribute to high levels of constitutive basal transcriptional activity in the absence of efficient chromatin assembly (lanes 9 to 12). A mixture of templates containing a high mass of DNA and wild-type (WT) or mutant *hsp70* promoters (15 ng) and the CMV immediate early promoter (0.5 ng) was injected. After incubating for 4 h at 18°C, the oocytes were divided and incubated a further 2 h at either 18°C (lanes 9 and 11) or 34°C (lanes 10 and 12). After this time, RNA was extracted and analyzed by primer extension. The positions of accurately initiated transcripts from the *X. laevis hsp70* promoter (hsp70) and the CMV immediate early promoter (CMV) are indicated. The transcription signals were quantitated with a PhosphorImager, and the levels of *hsp70* transcription are indicated in the bar graphs. A.U., arbitrary units.

tion of the *hsp70* promoter within chromatin. This allows us to further examine the potential role of chromatin structure in regulating *Xenopus hsp70* gene transcription.

Role of Y box elements in transcriptional control of the *Xenopus hsp70* promoter. Bienz (11) has demonstrated that under conditions of chromatin titration, the Y box elements within the *Xenopus hsp70* promoter (Fig. 5A, [WT]) have an important role in determining the efficiency of constitutive transcription (see also reference 39). Our next experiments compared the roles of the Y box elements in *hsp70* transcription under conditions of efficient chromatin assembly and chromatin titration. This was accomplished by introducing point mutations into both of the Y box elements. These mutations severely compromise any influence of the Y box on transcription within oocytes or somatic cells (26, 51) (Fig. 5A [-Y1/Y2]). We find that a low mass of injected DNA (*hsp70*, 2 ng, and CMV, 0.2 ng) allows a significant induction (greater than eightfold) of *hsp70* promoter activity relative to that of the CMV promoter following heat shock (Fig. 5B, lanes 1 and 2). Mutation of the Y box elements (Y1 and Y2) leads to a significant reduction (~70%) in the magnitude of transcription under these conditions at both 18 and 34°C (Fig. 5B; compare lanes 1 and 2 with lanes 3 and 4 and see the quantitation of *hsp70* promoter activity in the lower panel). However, a low level of heat-responsive transcription remains (Fig. 5B, lanes 3 and 4 of the lower panel). Elimination of all three HSEs (data not shown) is necessary to prevent heat-inducible transcription. When a high mass of DNA is injected into oocyte nuclei, the heat shock response of the *hsp70* promoter relative to that of the CMV promoter is eliminated (Fig. 5B, lanes 9 and 10 of the upper panel). Under these conditions, mutation of the Y box elements leads to a major reduction (>95%) in constitutive transcriptional activity (Fig. 5B; compare lanes 9 and 10 with 11 and 12 [upper panel]), which is in agreement with the results of Bienz (11). The Y box elements clearly influence basal transcriptional activity, especially when a high mass of DNA is injected into the oocyte nucleus.

We next examined whether the expression of exogenous XHSF1 at 18 and 34°C would alleviate any requirement that Y box elements be present for *hsp70* transcription. We found that the expression of exogenous XHSF1 at 18°C stimulates transcription from the *hsp70* promoter lacking Y boxes (Fig. 5B; compare lanes 3 and 7 of the upper panel); however, transcriptional efficiency is substantially reduced compared with that from the wild-type promoter (by >70%) (Fig. 5B; compare lanes 5 and 7 of the upper panel). Nevertheless, at 34°C, exogenous XHSF1 activates transcription to comparable extents for both the wild-type and the Y box mutant promoter (Fig. 5B; compare lanes 6 and 8 of the upper panel). We suggest that the properties of exogenous HSF change between 18 and 34°C so that transcription activation is more effective at the higher temperature (see below). With respect to the role of the Y box elements, our results suggest that they have an important role in the functionality of the *hsp70* promoter under conditions of both chromatin assembly (Fig. 5B, lanes 1 to 4 of the upper panel) and chromatin titration (Fig. 5B, lanes 9 to 12 of the upper panel). Moreover, the Y box elements clearly facilitate the activation of the *hsp70* promoter by exogenous XHSF1 at 18°C (Fig. 5B; compare lanes 5 and 7 of the upper panel) and by endogenous XHSF1 at 34°C (Fig. 5B, compare lanes 2 and 4 of the upper panel). Our next experiments examined how this might be accomplished.

There is excellent experimental precedent for a regulatory role for chromatin in the in vitro transcription of the *Drosophila hsp70* gene (6, 7). Recent observations have suggested that the repressive character of chromatin (6, 7, 64) can be alleviated by such proteins as the GAGA factor and by ATP-dependent activities (66, 68, 69). We therefore examined the chromatin organization of the *Xenopus hsp70* promoter under conditions of efficient chromatin assembly and the role of the Y box elements, heat shock, and exogenous XHSF1 in determining this organization. We initially employed a low-resolution approach in which the incorporation of the *hsp70* promoter region into a nucleosomal array was compared with that of the *CAT* gene within the same vector (66). In our experiments, we carried out micrococcal nuclease digestion of the chromatin templates under the different conditions for transcription and then deproteinized the DNA and resolved the fragments on an agarose gel before Southern blotting to a filter. We then sequentially hybridized the same filter with different probes, one of which was specific for the promoter. In all cases, the bulk of the DNA, including the *CAT* gene, was incorporated into a clear nucleosomal repeat (Fig. 6A, C, and E [Control]). Our micrococcal nuclease digestions were designed to allow a substantial amount of digested material of mononucleosomal size to accumulate (Fig. 6A, C, and E [arrowheads]). Hybridization with a promoter-specific probe revealed that a substantial disruption of the nucleosomal array occurred over the wild-type promoter under all conditions (at both 18 and 34°C and with or without expression of exogenous XHSF1). In particular, a substantial amount of DNA fragments (Fig. 6 [dots]) smaller than those within mononucleosomes (Fig. 6 [arrowheads]) accumulates. We conclude that proteins that disrupt the assembly of a canonical nucleosomal array are associating with the wild-type promoter.

We next examined the consequences of mutating the Y box elements for chromatin disruption. We found that mutation of the Y box elements restores a regular nucleosomal array over the promoter (Fig. 6D and F). In the wild-type *hsp70* promoter, nuclear proteins interacting with the Y box element presumably disrupt chromatin. This could account for how the Y box elements facilitate the activation of *hsp70* transcription by exogenous XHSF1 at 18°C or by endogenous HSF at 34°C.

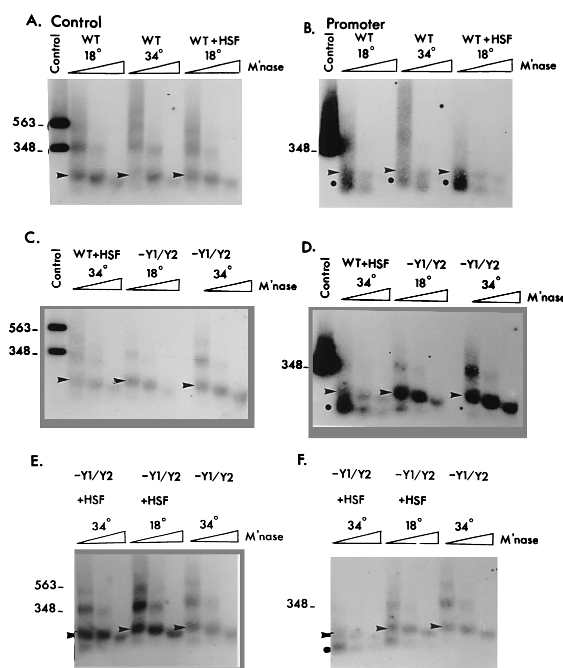

FIG. 6. The nucleosomal organization of the *hsp70* promoter in the presence and absence of heat shock, with and without exogenous XHSF1, and with and without intact Y box elements. XHSF1 mRNA was (+HSF) or was not injected into stage VI oocytes. After the oocytes were incubated for 16 h at 18°C, the *hsp70* template (2 ng) was injected. The oocytes were incubated 4 h at 18°C, divided, and incubated a further 2 h at either 18 or 34°C as indicated. After this time, minichromosomes were digested with micrococcal nuclease (M'nase) for 3, 10, and 45 min (as indicated by the open triangles) before they were processed as described in the legend to Fig. 1E. Sequential hybridization of the same filters was done with probes specific for either a portion of the *CAT* gene (Control; panels A, C, and E) or the *hsp70* promoter (Promoter; panels B, D, and F [see Materials and Methods]). The markers contain a promoter-specific DNA fragment (348 bp) and a vector DNA fragment (563 bp) as controls for hybridization specificity (Control lanes). DNA fragments corresponding to mononucleosomes (arrowheads) and subnucleosomal particles (dots) are indicated. WT, wild type.

The Y box elements might promote the association of XHSF1 by disrupting repressive histone-DNA interactions (6, 7, 66). We next investigated the consequences of expressing XHSF1 for nucleosome organization over the -Y1/Y2 mutant *hsp70* promoter. We have suggested that both exogenous XHSF1 and endogenous *Xenopus* HSF become more competent to activate transcription at 34 than at 18°C. Interestingly, expression of exogenous XHSF1 at 18°C does not lead to a clear disruption of the canonical nucleosomal array, but expression at 34°C does (Fig. 6F [HSF at 34°C]). Exogenous XHSF1 at 34°C leads to a decrease in mononucleosome length DNA (Fig. 6 [arrowheads]) and an increase in subnucleosomal material (Fig. 6 [dots]). These observations are consistent with an increased competence of XHSF1 at 34°C to gain access to recognition sites and to prevent the assembly of a repressive chromatin structure compared with that at 18°C.

Taken together, the results of our studies of the assembly of the *hsp70* promoter into chromatin suggest that while a disrupted nucleosomal array does not correlate with transcriptional activity per se, the presence of a regular nucleosomal array over the *hsp70* promoter does correlate with transcriptional inactivity (Fig. 5 and 6). Our next experiments attempted to obtain a higher-resolution analysis of protein association with the *Xenopus hsp70* promoter under these various conditions. We examined the association of proteins with the TATA box (Fig. 7A and B) and with the HSEs (Fig. 7C).

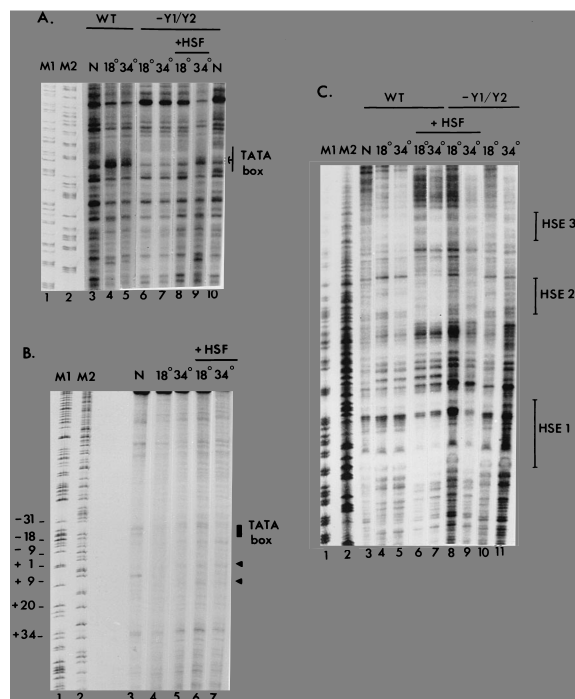

FIG. 7. In vivo footprinting of the wild-type and -Y1/Y2 mutant *Xenopus hsp70* promoters in the absence and presence of heat shock and with and without exogenous XHSF1. (A) DNase I footprinting of the TATA box. XHSF1 mRNA was not (lanes 4 to 7) or was (lanes 8 and 9) injected into stage VI oocytes. After the oocytes were incubated for 16 h at 18°C, a DNA solution containing either the wild type (WT) (lanes 4 and 5) or the -Y1/Y2 mutant *hsp70* (lanes 6 to 9) (2 ng) was injected. The oocytes were incubated for 4 h at 18°C, divided, and incubated a further 2 h at either 18°C (lanes 4, 6, and 8) or 34°C (lanes 5, 7, and 9). After this time, DNase I footprinting was carried out (see Materials and Methods). After denaturation in formamide, the DNA samples were resolved on a denaturing 6% polyacrylamide gel containing 7 M urea. Lane 3 shows cleavage of naked (N) wild-type *hsp70* DNA, and lane 10 shows cleavage of naked -Y1/Y2 *hsp70* DNA. Sequencing markers (M1 and M2) are shown. The position of the TATA box is indicated. The asterisk indicates a site of enhanced DNase I cleavage around the TATA box where TFIID is presumably bound. (B) Potassium permanganate footprinting of the transcription start site. XHSF1 mRNA was (lanes 6 and 7) or was not (lanes 4 and 5) injected into stage VI oocytes. After the oocytes were incubated for 16 h at 18°C, a DNA solution containing the wild-type *hsp70* template (2 ng) was injected into the oocyte nuclei. The oocytes were incubated for 4 h at 18°C, divided, and incubated a further 2 h at 18°C (lanes 4 and 6) or 34°C (lanes 5 and 7). After this time, potassium permanganate footprinting was carried out (see Materials and Methods). After denaturation in formamide, the DNA samples were resolved on a denaturing 6% polyacrylamide gel containing 7 M urea. Lane 3 shows the cleavage of naked (N) DNA. Sequencing markers (M1 and M2) are shown. The numbers indicate base pair positions relative to the start site of transcription (+1). The position of the TATA box is indicated. Arrowheads indicate the sites of cleavage that are enhanced in vivo relative to those of naked DNA. (C) DNase I footprinting of the HSEs. XHSF1 mRNA was not (lanes 3 to 5, 10, and 11) or was (lanes 6 to 9) injected into stage VI oocytes. After the oocytes were incubated for 16 h, a DNA solution containing either the wild type (WT) (lanes 4 to 7) or the -Y1/Y2 mutant *hsp70* promoter (lanes 8 to 11) (2 ng) was injected. The oocytes were incubated for 4 h at 18°C, divided, and incubated a further 2 h at either 18°C (lanes 4, 6, 8, and 10) or 34°C (lanes 5, 7, 9, and 11). After this time, DNase I footprinting was carried out (see Materials and Methods). After denaturation in formamide, the DNA samples were resolved on a denaturing 6% polyacrylamide gel containing 7 M urea. Lane 3 shows cleavage of naked (N) wild-type *hsp70* DNA. Sequencing markers (M1 and M2) are shown. The positions of the three HSEs are indicated (Fig. 4).

We found footprinting around the TATA box of the wild-type *Xenopus hsp70* promoter at 18 and 34°C in the absence of exogenous XHSF1 (Fig. 7A; compare lanes 3, 4, and 5). This footprinting is significantly reduced by mutation of the Y box elements (Fig. 7A; compare lanes 4 and 5 with lanes 6 and 7). Nevertheless, a chromatin-specific cleavage pattern can still be seen, even in the absence of exogenous HSF (Fig. 3). Importantly, the appearance of a footprint over the TATA box correlates with basal transcriptional activity (Fig. 5 and 7) and the appearance of a disrupted nucleosomal array (Fig. 6). Expression of exogenous XHSF1 leads to footprinting of the -Y1/Y2 mutant *hsp70* promoter over the TATA box at 34 but not at 18°C (Fig. 7A; compare lanes 8, 9, and 10). Thus, for the -Y1/Y2 construct, footprinting of the TATA box (Fig. 7A) corresponds with activated transcription (Fig. 4) and disruption of chromatin structure over the *hsp70* promoter (Fig. 6).

Potassium permanganate detects the single-stranded DNA associated with promoter melting directed by RNA polymerase and reveals the protection of the T residues in the TATA box by TFIID complexes (23). We found that the wild-type *Xenopus hsp70* promoter shows no sites that are strongly hypersensitive to potassium permanganate cleavage at 18 or 34°C in the absence or presence of exogenous HSF (Fig. 7B; compare lanes 3 and 4). This result suggests that RNA polymerase II is not paused at the transcription start site in the majority of *hsp70* promoters under these conditions. As we have previously discussed, this could reflect a small number of potentially active templates. Some protection of the T residues in the TATA box following minichromosome assembly is apparent under all conditions (Fig. 7B; compare lane 3 with lanes 4 to 7). Thus, our results (Fig. 7A and B) suggest that protein associates with the TATA box following the microinjection of the wild-type *hsp70* promoter. The appearance of such protection is consistent with the nuclease hypersensitivity of the promoter in chromatin at 18°C in the absence of exogenous HSF when a small mass (2 ng) of DNA is microinjected (Fig. 3). Following the activation of transcription at 34°C or with the expression of exogenous XHSF1, the cleavage of DNA by permanganate increases, particularly downstream of the transcription start site (Fig. 7B, lanes 6 and 7 [arrowheads]). This might reflect the increased recruitment of RNA polymerase (Fig. 2C).

We next examined the footprinting of the HSEs in the wild-type and -Y1/Y2 mutant *hsp70* promoters. Clear protection of HSEs 1, 2, and 3 is obtained with the wild-type promoter at 18 and 34°C following the expression of XHSF1 (Fig. 7C, lanes 6 and 7). In contrast, protection of all three HSEs is much weaker for the mutant promoter under these conditions (Fig. 7C, lanes 8 and 9). This result leads to the suggestion that the presence of intact Y box elements facilitates HSF access to their recognition elements. Protection of HSEs 1 and 2 from DNase I cleavage with the mutant -Y1/Y2 promoter in the presence of exogenous XHSF1 appears more pronounced at 34 than at 18°C (Fig. 7C; compare lanes 8 and 9). This correlates with the increased transcriptional activity at 34°C (Fig. 5), chromatin disruption (Fig. 6), and the recruitment of the basal transcriptional machinery (Fig. 7A). The absence of a complete footprint under these conditions suggests either that transient association of XHSF1 can occur to direct assembly of a functional preinitiation complex at 34°C or that a capacitation of smaller amounts of bound HSF at 34°C to activate transcription occurs (33, 36, 37, 47, 59, 61). Either of these events might also contribute to final *hsp70* promoter activity.

DISCUSSION

The major conclusion from this work is that the transcriptional activity of the *Xenopus hsp70* promoter is dependent not only on the efficiency of chromatin assembly (Fig. 1) but also on the abundance of HSF (Fig. 2), the association of HSF with HSEs (Fig. 4), the presence of the Y box elements (Fig. 5), and also on heat shock itself (Fig. 5). All of these parameters

influence chromatin structure and the recruitment and activity of the basal transcriptional machinery (Fig. 3, 6, and 7).

Heat-inducible and constitutive *Xenopus hsp70* transcription in *Xenopus* oocytes. Previous studies have reported a lack of heat-inducible transcription in *Xenopus* oocytes (8, 9, 11, 35). We have found that the heat shock response is dependent on the mass of DNA injected into *Xenopus* oocyte nuclei (Fig. 1 and 2). Templates with fewer, more widely spaced nucleosomes display constitutive *hsp70* promoter activity (Fig. 1 and 2). In a finding that is in agreement with the conclusions of previous work, this constitutive activity depends in part on the integrity of the Y boxes (Fig. 5) (11, 39). Transcription experiments under conditions of efficient chromatin assembly also indicate that the Y box elements have a role in facilitating basal transcription within chromatin (Fig. 5). Moreover, under these conditions, intact Y box elements increase the degree to which endogenous HSF activates *hsp70* transcription at 34°C and to which exogenous XHSF1 activates *hsp70* transcription at 18°C (Fig. 5). The findings of nuclease accessibility studies are consistent with the positing of multiple interrelated roles for the Y box elements: the disruption of canonical nucleosomal arrays over the *hsp70* promoter (Fig. 6), an influence on the association of the basal transcriptional machinery with the TATA box (Fig. 7A and B), and the facilitation of HSF binding to HSEs in the *hsp70* promoter (Fig. 7C). On the basis of sequence specificity, the most probable protein to interact with the Y box element is the transcription factor NF-Y (17). NF-Y contains three protein subunits which are homologous to the *Saccharomyces cerevisiae* HAP2, HAP3, and HAP5 activators (41, 45, 58) and is found in all metazoan species and tissues examined (34). Although the exact biological role of NF-Y in the transcription process is unknown, it has been proposed to have an architectural role by virtue of the sequence similarity of two subunits and the core histones (5) and to promote the assembly of stable transcription complexes (42, 73). We did not observe strong DNase I footprinting of the Y box elements (Fig. 4 and 7). The DNase I analysis suggests that stable protein binding to the distal Y box element occurs only in the presence of exogenous HSF, whereas the micrococcal nuclease digestion analysis (Fig. 6) suggests that the Y box elements and their cognate proteins function to prevent the assembly of a repressive chromatin structure, even in the absence of exogenous HSF. There are several potential explanations for this apparent discrepancy. NF-Y does not give a strong DNase I footprint in vitro (17); this may reflect a histone-like interaction with DNA (see reference 30). Alternatively, the role of NF-Y might only be transient during nucleosome assembly; NF-Y might promote transcription complex assembly and then dissociate from DNA. Components of the basal transcriptional machinery might maintain a disrupted chromatin structure. Future work will explore the role of NF-Y in potentiating *hsp70* transcription within a chromatin environment.

Role of HSF in *Xenopus hsp70* transcription. The expression of exogenous XHSF1 at 18°C in oocytes following the microinjection of mRNA encoding the protein into the cytoplasm leads to the localization of XHSF1 in the nucleus (Fig. 2A). Exogenous XHSF1 associates with HSEs and activates transcription from the wild-type *hsp70* promoter at 18°C (Fig. 4 and 7). These properties are similar to those of mouse HSF1 and HSF2 when they are overexpressed in 3T3 cells (54). Our results are also similar to those of Gross and colleagues (28, 29) with *S. cerevisiae*. These workers observed that overexpression of yeast HSF can drive the disruption of a repressive chromatin structure on a mutant *HSP82* promoter. The association of yeast HSF with the *HSP82* promoter has also been proposed to be facilitated by constitutively bound TFIID (28, 29). There is an apparent difference between the properties of endogenous *Xenopus* HSF and those of the exogenous XHSF1 overexpressed following the microinjection of mRNA. The endogenous *Xenopus* HSF does not activate *hsp70* transcription at 18°C, presumably because it is functionally constrained. Sarge and colleagues have proposed that negative regulatory factors function to keep endogenous HSF in an inactive state at 18°C (54). In their model, excess XHSF1 overcomes this inhibitory influence and provides unconstrained XHSF1. This unconstrained protein will nevertheless have to trimerize in order to exhibit DNA binding (40, 43, 50, 60). It has been proposed that the constitutive binding of HSF overexpressed in *Escherichia coli* is due to improper folding of the protein or to the lack of a negative regulator that prevents trimerization (15, 50). These parameters could account for the activity of exogenous XHSF1. In any event, the exogenous XHSF1 expressed in *Xenopus* oocytes is competent to localize to the nucleus, bind to the HSEs within the wild-type *hsp70* promoter, and activate transcription at 18°C (Fig. 1, 2, and 4). Since the wild-type *hsp70* template is assembled into chromatin containing physiologically spaced nucleosomes (every ~190 bp) at 18°C, the exogenous XHSF1 has the capacity to compete against histones for assembly on the promoter.

Heat shock at 34°C leads to an increase in *hsp70* transcriptional activity directed by both endogenous HSF and exogenous XHSF1 (Fig. 2 and 5). There are several direct and indirect possibilities for how this might be accomplished. (i) Trimerization, nuclear localization, and DNA binding might be activated (50, 54). (ii) The capacity of HSF to activate transcription might be influenced by posttranslational modification and/or conformational changes altering the effectiveness of a transactivation domain (59, 61). (iii) The properties of chromatin might change in a temperature-dependent manner; for example, nucleosomes might become more mobile and hence more permissive to *trans*-acting factor access at 34°C compared with that at 18°C (67–69). The increase in *hsp70* transcriptional activity from the low levels obtained with endogenous protein at 18°C to the intermediate levels at 34°C to the high levels at 18°C and 34°C in the presence of exogenous XHSF1 roughly correlates with the efficiency of footprinting of the HSEs within the *hsp70* promoter in vivo (Fig. 4). This suggests that a component of increased transcription may follow from the increased access of XHSF1 to the promoter within chromatin. However, the efficiency of *hsp70* transcription relative to that of the CMV promoter in the presence of exogenous XHSF1 increases between 18 and 34°C (Fig. 2B, lanes 3 and 4, and Fig. 5B, lanes 5 and 6) without a proportional increase in footprinting (Fig. 4; compare lanes 4 and 5) (Fig. 7C). This may reflect the increased capacity of bound *Xenopus* HSF to activate transcription under heat shock conditions. This could be due to the removal of an inhibitor or a conformational change or modification of XHSF1 itself. There is excellent precedent for this temperature-dependent step from work on the activation of human *hsp70* by human HSF1 in response to different cellular stresses (37). In these experiments, HSF binding to DNA can be separated from transcriptional activation. Moreover, this appears to be the normal mechanism mediating *hsp70* transcriptional activation in *S. cerevisiae* (19, 59, 61). The lack of significant change in the footprinting of the HSEs in the -Y1/Y2 mutant promoter in the presence of exogenous XHSF1 between 18 and 34°C (Fig. 7C), in spite of a strong increase in *hsp70* transcription, is consistent with this hypothesis. The Y box elements promote the association of exogenous HSF with the *hsp70* promoter within chromatin at 18 and 34°C (Fig. 7B). In this respect, our results are similar to those from experiments that indicated that *Drosophila* and human HSF alone

could not gain access to preassembled chromatin templates (6, 7, 64). We suggest that the Y box elements associate with proteins that actively promote the association of XHSF1 with the promoter through direct protein-protein contacts, that actively destabilize histone-DNA interactions to facilitate HSF binding (see, for example reference 66), or that passively exclude nucleosomes or destabilize histone-DNA interactions, allowing HSF to bind (see, for example, reference 1). Both of these last two possibilities are consistent with the absence of a canonical nucleosomal array over the *hsp70* promoter in the presence of the Y boxes (Fig. 6).

Chromatin and the heat shock response. Several investigators have suggested that chromatin structure has a role in the heat shock response. Prior nucleosome assembly can restrict HSF access to DNA and repress *hsp70* gene transcription (6, 7, 28, 64). Nevertheless, under physiological conditions, HSF gains access to HSEs and activates the transcription process. In *D. melanogaster*, the facilitation of HSF binding appears to rely on a combination of transacting factors, nucleosome positioning, molecular machines that modify chromatin, and the basal transcriptional machinery (6, 7, 40, 66). We have established that in *X. laevis* the Y box elements have a key role in regulating XHSF1 accessibility (Fig. 6 and 7) within chromatin. In the absence of the Y box elements, XHSF1 does not bind the *hsp70* HSEs as efficiently as in their presence (Fig. 7C). Moreover, the association of components of the basal transcription machinery appears to be facilitated by intact Y box elements (Fig. 7A). Thus, chromatin assembly might impede recruitment of HSF and the basal transcriptional machinery to the *hsp70* promoter. Titration of chromatin assembly clearly facilitates basal transcription (Fig. 2 and 5), implying that restrictions on the recruitment of components of the basal transcriptional machinery by chromatin structures, including the core histones (Fig. 1), contribute to the repression of *hsp70* transcription at 18°C. Expression of XHSF1 in the presence of the Y box elements at 18°C or with the -Y1/Y2 mutant *hsp70* promoter at 34°C can clearly overcome these impediments. Since XHSF1 can potentiate transcription from the *hsp70* promoter within chromatin more effectively at 34°C than at 18°C (Fig. 5) in spite of approximately equivalent occupancies of the HSEs of the *hsp70* promoter (Fig. 4), it is likely that chromatin structure might impede transcriptional activation even after HSF is bound to DNA. It might be these restrictions on the transcription process that the heat-activated HSF relieves on the wild-type *hsp70* promoter. Rougvie and Lis have suggested that chromatin might present an impediment to the elongation of template-engaged RNA polymerase on the *hsp70* promoter (52). Our results do not exclude this possibility for the *Xenopus hsp70* promoter. However, we have not detected template-bound RNA polymerase (Fig. 2C and 7B). Our failure to detect any such polymerase could be due to a number of reasons, including the heterogeneity of our samples, the insensitivity of our run-on assay, and the potential failure of Sarkosyl to strip nonhistone proteins directing pausing from the *Xenopus hsp70* gene. Nevertheless, we also failed to detect any permanganate-induced cleavage of the *hsp70* promoter under conditions in which active transcription does not occur (non-heat shock conditions at low concentrations of DNA). Thus, we suggest that XHSF1 may function to regulate transcription of the *Xenopus hsp70* promoter by initiating a process that facilitates the recruitment of RNA polymerase and/or by elongation that is impeded by the assembly of chromatin. Future experiments will explore this possibility.

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