Heat shock-regulated transcription *in vitro* from a reconstituted chromatin template

(nucleosome assembly/general transcription factor IID/heat shock factor)

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ABSTRACT **To investigate the mechanisms of transcriptional regulation of *Drosophila* heat shock genes we studied the activity of a heat shock promoter *in vitro* after reconstitution into chromatin. Increasing the duration of nucleosome assembly progressively inactivated a plasmid template when it was transcribed with extracts of either unshocked or heat-shocked *Drosophila* embryos, despite induction of the transcriptional activator heat shock factor. Addition of the general transcription factor IID (TFIID) before nucleosome assembly did not significantly relieve nucleosomal inhibition, but TFIID potentiated the promoter to be responsive to activation by heat shock factor in the heat shock transcription extract. The potentiation by TFIID could be related to the nucleosome-free, hypersensitive state of heat shock promoters previously observed *in vivo* before heat shock induction and may be necessitated by the need to expedite activation of heat shock genes in response to environmental stress.**

In eukaryotes, heat stress leads to a decrease in general transcriptional activity and to the specific induction of genes coding for heat shock proteins (1, 2). The transcriptional stimulation of heat shock genes depends on a key activator protein, termed heat shock factor (HSF) (3, 4). Upon heat shock HSF is converted from a preexisting, inactive form to an active species that binds to conserved heat shock elements (HSEs) (5–7) present in multiple copies upstream of all heat shock genes. We are interested in the mechanisms underlying the transcriptional activation of heat shock genes and in the maintenance of the inert state under nonshock conditions. For example, the *Drosophila* hsp70 gene is inactive under normal conditions *in vivo*, but in an *in vitro* transcription assay a naked hsp70 template can be transcribed efficiently with extracts prepared from unshocked cells (refs. 8 and 9; this report). The inactivity of the hsp70 promoter *in vivo* may thus depend on the negative influence of chromatin structure.

Previous nuclease digestion studies of hsp70 gene chromatin in intact nuclei show that under normal conditions the promoter elements are located within a broad region [≈300 base pairs (bp)] of DNase I hypersensitivity embedded in nucleosomal DNA (10, 11). The DNase I-hypersensitive promoter appears devoid of nucleosomes, as no histones can be crosslinked to the DNA under conditions where histones can be detected on the coding portion of the gene (12). Detailed analyses of nonhistone protein binding within the hypersensitive promoter region have revealed the binding of a protein, presumably the general transcription factor IID (TFIID), to the TATA box under nonshock conditions (13). In addition, the presence of a transcriptionally engaged, but blocked, polymerase on the heat shock promoter in the absence of heat shock has been demonstrated (14, 15). The uninduced hsp70 promoter is thus characterized by an open configuration with bound TFIID and poised RNA polymerase II. Upon heat shock induction the additional binding of HSF to the HSEs just upstream of the TATA box leads to the transcription of the hsp70 gene, presumably by a direct or indirect activation of the blocked polymerase. These basic features of hsp70 gene regulation are also applicable to other members of the hsp gene family (16, 17).

We have initiated experiments aimed at elucidating the mechanisms underlying the transcriptional activation of heat shock promoters in chromatin by studying the activity of the hsp70 promoter *in vitro* after the reconstitution of nucleosomes on the template. We find that for a chromatin template to be efficiently transcribed two requirements have to be met. (*i*) TFIID has to be present at the onset of nucleosome assembly (potentiation) and (*ii*) the transcription extract must contain an activated HSF during subsequent transcription of the reconstituted template (activation).

MATERIALS AND METHODS

Transcription Templates, Primers, and Competitor Plasmids. The p(−50) HSE maxigene contains hsp70 (locus 87A) gene sequences from −90 to +296 cloned into pBluescript (Stratagene). Sequences between −90 and −50 were replaced by the synthetic sequence 5′-*Apa* I–CTATTCTCGAAGCTTCGGGATCCCGCTTCTCGAATGTTCG*Nru* I-3′ to optimize the two HSEs (6, 7) and to weaken potential binding sites for a GAGA factor (18). The p(−50)HSE minigene is a derivative of the p(−50)HSE maxigene and harbors a deletion of an *Alu* I (A) fragment between +41 and +71 (see also Fig. 3). The fushi tarazu (*ftz*) template contains sequences from −950 to +151 of the *ftz* gene (19) inserted in pBluescript; the *ftz* primer is complementary to RNA sequences between +87 and +110. The hsp70 primer is complementary to sequences between +149 and +177. For HSE competition a pUC derivative was used into which 14 idealized HSEs (6, 7) were inserted in tandem orientation. For competitions identical amounts of pUC or the HSE-containing plasmid were compared.

Preparation of Transcription Extracts and Transcription Reactions. Transcription extracts were prepared from nonshock or heat-shocked 0- to 12-hr *Drosophila* embryos (Oregon R), according to established procedures (20, 21). For the heat shock extract 30–50 g of dechorionated embryos in 200 ml of phosphate-buffered saline (PBS) was incubated for 30 min at 37°C in a water bath with shaking. Transcription reactions were performed in 25 μl containing 12.5 mM Hepes (pH 7.6), 6.25 mM $MgCl_2$, 5% (vol/vol) glycerol, 0.05 mM EDTA, 1 mM dithiothreitol, 40 mM KCl, 4 mM creatine phosphate, 2 units of RNasin, 0.5 unit of creatine phosphokinase, 0.5 mM each NTP, 120 μg of embryo extract protein, and 15 fmol (45 ng) of each template. After incubation for 30 min at 26°C the reaction was stopped with 100 μl of 20 mM

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Abbreviations: TFIID, transcription factor IID; HSE, heat shock element; HSF, heat shock factor; yTFIID, yeast TFIID.

EDTA/200 mM NaCl/yeast RNA at 250 μl/ml. RNA was purified by extractions with phenol/chloroform and chloroform/isoamylalcohol (24:1) and then precipitated and analyzed by primer extension. The extension products were separated on a 6% polyacrylamide gel. The dried gel was exposed to film at −80°C for 10–60 min. The start sites of transcription *in vitro* are identical to the *in vivo* start sites. All transcription *in vitro* was abolished with α-amanitin at 0.5 μg/ml, indicating transcription by RNA polymerase II. For quantitation of transcription, radioactive bands were cut from the dried gel, incubated in 1 ml of 30% (vol/vol) H_2O_2 overnight at 60°C, and counted in a liquid scintillation counter after addition of scintillation cocktail.

Preparation of Assembly Extract, Nucleosome Assembly, and Analysis of Assembled Plasmids. Preparation of the *Xenopus laevis* oocyte S-150 extract, supercoiling analysis of the assembled template, and digestion with micrococcal nuclease were as described (22, 23). All incubations were at 26°C with preequilibrated components. The number of supercoils introduced in the plasmid was determined by comparison with plasmid standards prepared according to Keller (24) containing defined numbers of superhelical turns on chloroquine gels (24, 25).

TFIID. Yeast TFIID (yTFIID) fractions were a gift of R. Kambadur and D. Hamer (National Cancer Institute). The plasmid pASY2D (26) was expressed in *Escherichia coli* and purified by chromatography over DEAE-cellulose (26). A control fraction was prepared identically from *E. coli* lacking plasmid pASY2D. In general, the DEAE flow-through fraction was used; more purified fractions gave similar results.

Coupled Assembly/Transcription Reactions. Prebinding reactions contained in 8 μl: 45 fmol of minigene (≈100 ng), 10 mM Hepes (pH 7.6), 50 mM KCl, ±1.5 μl of yTFIID. For nucleosome assembly 30 μl of *Xenopus* oocyte extract, 30 mM of creatine phosphate, 10 ng of creatine phosphokinase, 1 mM $MgCl_2$, and 3 mM ATP were added; the final volume was adjusted to 50 μl with extract buffer (23). After assembly for the indicated times, 40 μl of the reaction was assayed for supercoiling. For transcription assay a 5-μl aliquot of the chromatin assembly reaction (10 ng of template) was added to 750 ng of pUC in 3 μl of 10 mM Tris, pH 7.5/0.1 mM EDTA. Then 3 fmol of maxigene in 5.6 μl of HEMG [25 mM Hepes (pH 7.6)/0.1 mM EDTA/12.5 mM $MgCl_2$/10% (vol/vol) glycerol] (21) was added, followed by 11.4 μl of a nonshock or a heat shock transcription mixture containing components as described.

RESULTS

Transcription of a Heat Shock Promoter in Extracts of Normal and Heat-Shocked *Drosophila* Embryos. As a prelude to our investigation on the role of chromatin structure on transcriptional regulation, we first characterized the transcription potential of naked plasmid templates carrying a minimal heat shock promoter [p(−50)HSE maxi- or minigene (see below)] in extracts prepared from either unshocked or heat-shocked *Drosophila* embryos (these extracts are denoted nonshock and heat shock extract, respectively). We found that p(−50)HSE was transcribed efficiently in both extracts (Fig. 1, lanes 3 and 4). By comparison, the *ftz* promoter (or the alcohol dehydrogenase [*adh*] promoter; data not shown) present in the same reaction was efficiently transcribed in the nonshock extract but not in the heat shock extract. The poor transcription of the *ftz* and *adh* genes in heat shock extracts is reminiscent of the general inhibition of transcription during heat shock and is probably due to a stress-induced deficiency of RNA polymerase II or general transcription factors. Although the levels of transcription from the heat shock promoter are similar in both nonshock and heat shock extracts, they are qualitatively different in

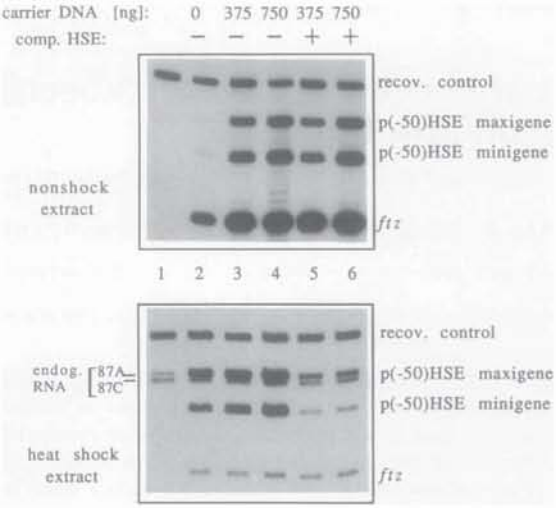

FIG. 1. *In vitro* transcription in nonshock (*Upper*) and heat shock (*Lower*) extracts, as analyzed by primer extension. Lanes: 2–6, cotranscription of templates p(−50)HSE maxigene, p(−50)HSE minigene, and the fushi tarazu gene. The reactions also contained carrier DNA (pUC) or HSE competitor (comp.), as indicated. Lanes 1, plasmid template was omitted, thus revealing the endogenous hsp70 RNAs present as background in the heat shock extract. As internal control for RNA recovery and efficiency of primer extension, a defined amount of RNA synthesized from a T7 promoter located 43 nucleotides upstream of the hsp70 insert in pBluescript was added at the end of the transcription reaction (recov. control).

their dependence on transcription factors. Transcription in the nonshock extract is driven apparently only by general transcriptional components, whereas it is critically dependent on HSF in the heat shock extract. Titration of HSF by the introduction of competing HSEs resulted in a significant reduction of p(−50)HSE transcription with the heat shock extract but did not affect transcription with the nonshock extract (Fig. 1, lanes 5 and 6). Hence, HSF appears capable of specifically counteracting the deficiency in general transcription components after heat shock. Interestingly, when the carrier DNA was omitted from the nonshock reaction, transcription of p(−50)HSE decreased significantly (Fig. 1, lane 2). This decrease, which is somewhat variable between different extract preparations and is less pronounced in the heat shock extract, is probably due to the presence of inhibitors, such as nonspecific DNA-binding proteins in crude transcription extracts (27, 28).

***In Vitro* Transcription of Reconstituted Chromatin Templates.** To learn how heat shock promoters are repressed under normal conditions *in vivo*, we analyzed the transcription potential of p(−50)HSE reconstituted into chromatin *in vitro*, using a nucleosome assembly system derived from *Xenopus* oocytes (S-150 extract, refs. 22 and 23). Because previous studies of protein binding at the uninduced heat shock promoter had indicated occupancy of the TATA box, presumably by TFIID (10, 13), we performed the chromatin assembly in the presence or absence of recombinant yTFIID (26, 29–34). Recombinant yTFIID binds specifically to the TATA boxes of many eukaryotic promoters (26, 32, 33), including the *Drosophila* hsp70 TATA box (data not shown) and can substitute for the natural human and *Drosophila* TFIID in an *in vitro* transcription assay (24, 29–33).

We constructed a p(−50)HSE minigene for chromatin reconstitution to distinguish transcription of the assembled and free DNA templates. The p(−50)HSE minigene has a 30-bp deletion in the hsp70 coding sequence (Fig. 2, *Inset*). Transcription of the p(−50)HSE minigene results in a shortened RNA distinguishable from the wild-type hsp70 (maxigene) transcript and from endogenous hsp70 mRNAs that

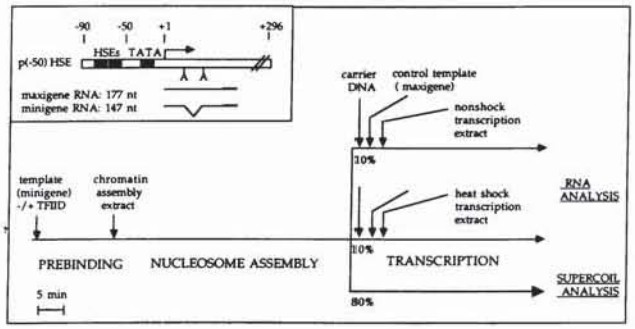

FIG. 2. Experimental outline of nucleosome assembly and transcription *in vitro*. (*Inset*) Schematic representation of heat shock templates used in this study. nt, Nucleotide.

originate from hsp70 genes at cytogenetic loci 87A and 87C and are present in heat shock transcription extracts (Fig. 1, lane 2).

As outlined in Fig. 2, we subjected the p(−50)HSE minigene to chromatin assembly *in vitro* after preincubation with or without yTFIID. After increasing times of assembly, an aliquot (80%) of the chromatin template was analyzed for DNA supercoiling as a measure of nucleosome reconstitution (35, 36). Remaining aliquots of the assembled template (10%) were analyzed in parallel for transcriptional activity in nonshock and heat shock extracts. We introduced carrier DNA at the junction between assembly and transcription to terminate nucleosome assembly and to permit optimal transcription by titrating residual assembly components and transcription inhibitors. The effectiveness of the carrier DNA was demonstrated by the activity of the internal control template, p(−50)HSE maxigene, termed "free template," which was added immediately after the carrier DNA.

Nucleosomes Inhibit Transcription from a Heat Shock Promoter. In the absence of yTFIID, increasing the duration of nucleosome assembly resulted in progressive transcriptional inhibition of the p(−50)HSE minigene in both nonshock and heat shock extracts, whereas no inhibition of the free template was seen (Fig. 3*A*, lanes 1–4). The degree of nucleosome assembly on the reconstituted template was estimated by the superhelical density of the deproteinized plasmid DNA (Fig. 3*B*) (35, 36). Upon incubation with the *Xenopus* S-150 extract, the supercoiled minigene plasmid is initially relaxed, followed by the introduction of increased numbers of superhelical turns. After 60-min assembly, 14–18 negative supercoils, corresponding to the same number of nucleosomes, are introduced by the reconstitution procedure (see Fig. 5*B* for quantitation). The extent of transcriptional inhibition of the minigene template correlates well with the extent of nucleosome reconstitution, suggesting that the inhibition is primarily caused by nucleosome formation. If all reaction components are mixed, but nucleosome assembly is not allowed to proceed (2 min assembly), no inhibition occurs.

To confirm that supercoiling under the conditions used was, indeed, from nucleosome assembly we digested the template after 1 hr of assembly with micrococcal nuclease, which cleaves DNA in the linker region between nucleosomes. The resulting DNA fragments, as analyzed on an agarose gel, revealed a characteristic ladder of fragments spaced at 180-bp intervals, in agreement with previous reports (23) (Fig. 3*C*; 1- to 4-min digest). A more extensive digest (8 min) produced mostly mono- and dinucleosomal fragments with the monomer fragments centered around 146 bp, corresponding to the nucleosome core particle. This result illustrates that nucleosomes are assembled efficiently and with regular spacing on the plasmids under our experimental conditions.

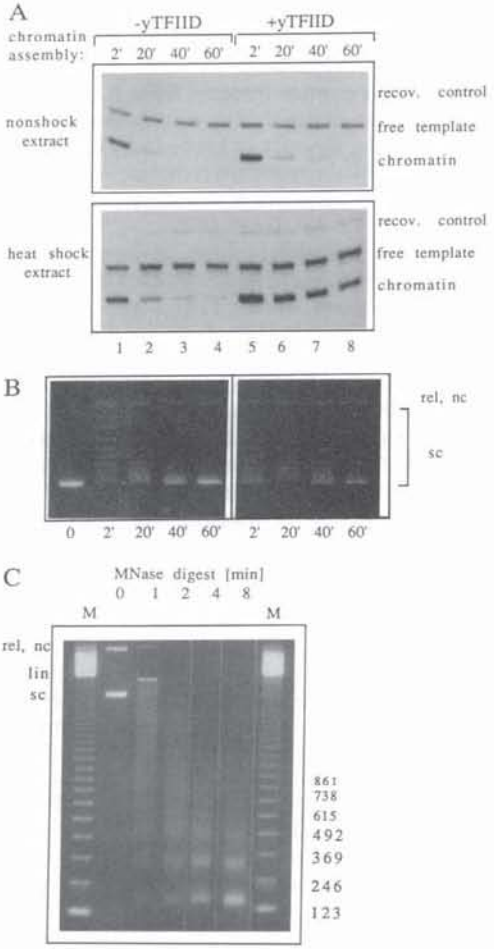

FIG. 3. (*A*) Transcription of p(−50)HSE maxigene (free) and p(−50)HSE minigene (assembled in chromatin) in nonshock and heat shock extracts. Before chromatin assembly for the indicated times, templates were incubated without yTFIID (lanes 1–4) or with yTFIID (lanes 5–8) for the indicated minutes. (*B*) DNA supercoils introduced by nucleosome assembly. An aliquot (80%) of the assembled minigene template was deproteinized, electrophoresed on an agarose gel, and stained with ethidium bromide. The presence of yTFIID does not influence the bulk nucleosome assembly. rel, Relaxed, closed plasmids; nc, nicked plasmids; sc, supercoiled plasmids. Lane 0, supercoiled p(−50)HSE minigene before incubation with assembly extract. (*C*) Nucleosomes are regularly spaced on *in vitro*-assembled chromatin templates. p(−50)HSE minigene (650 fmol) was assembled by using 300 μl of oocyte S-150 extract in a volume of 500 μl for 1 hr at 26°C. To the reaction 3 mM $CaCl_2$ was added. One hundred-microliter aliquots of the assembly reaction were then treated with 4 units of micrococcal nuclease (Boehringer Mannheim) for the indicated times at 37°C. DNA was purified and electrophoresed on a 1.5% agarose gel as described along with size markers (123 bp ladder, BRL). rel, Relaxed plasmids; nc, nicked closed plasmids; lin, linear plasmids; and sc, supercoiled plasmids.

To Overcome Nucleosomal Inhibition Requires Both TFIID and HSF. Inhibition of RNA polymerase II initiation by nucleosomes has been reported for a variety of eukaryotic templates (37–43). Furthermore, it has been suggested that binding of a TFIID fraction alone to the adenovirus 2 major late promoter before chromatin reconstitution is sufficient to alleviate this inhibition (37). We found that incubation of the p(−50)HSE minigene with a yTFIID fraction alone before chromatin assembly only modestly alleviated transcriptional repression (1.5-fold) when assayed in a nonshock extract (Fig. 3*A*, *Upper*, lanes 5–8; also Fig. 5, lanes 1–4). However, a significant relief of inhibition (≈10-fold, on average) was

seen when the chromatin template prebound with yTFIID was transcribed in a heat shock extract (Fig. 3*A*, *Lower*, lanes 5–8). No relief of nucleosome-mediated repression was observed by using a control fraction from *E. coli* lacking the yTFIID expression plasmid (data not shown). It should also be noted that in the absence of prebound yTFIID, transcription of the assembled chromatin template was also inhibited in the heat shock transcription extract, despite the presence of HSF (Fig. 3*A*, *Lower*, lanes 1–4).

The relief of nucleosomal inhibition depends on the order of addition of TFIID. Increased transcription of the chromatin template was seen when yTFIID was added before or along with the *Xenopus* S-150 extract but was not seen when yTFIID was introduced after nucleosome assembly (Fig. 4). In addition, the relief of inhibition critically depends on activated HSF because the effect was abolished by deletion of the HSE from p(−50)HSE (data not shown) and by titration of HSF with competing HSE in the transcription reaction (Fig. 5*A*, compare lanes 5–8 with lanes 9–12). In this experiment, the number of negative supercoils introduced by chromatin assembly was quantitated by direct comparison with standards containing defined amounts of supercoils (Fig. 5*B*) (24). Assuming the introduction of one supercoil per assembled nucleosome (35, 36) in the 3370-bp p(−50)HSE minigene, we infer an average nucleosome density of one nucleosome per 305 bp, 225 bp, and 210 bp (±15%) for the 15-min, 30-min, and 60-min assemblies, respectively. Transcriptional inductions of the chromatin template in the heat shock extract were also quantitated by normalizing the transcription levels of the assembled templates to the activity of the template before assembly (2-min incubation). For each time of assembly the normalized transcription levels in nonshock and heat shock extracts were compared. The relief of inhibition in the heat shock extract translates into 7-fold, 14-fold, and 9-fold increases in transcription for the chromatin templates assembled for 15 min, 30 min, and 60 min, respectively.

DISCUSSION

In this report we have studied the activity of a reconstituted hsp70 chromatin template by a coupled *in vitro* assembly-transcription assay. We have found that for the hsp70 promoter in chromatin to be transcriptionally active, two requirements have to be met. TFIID has to be present at the onset of nucleosome assembly and HSF during the subsequent transcription reaction. We refer to these two distinct

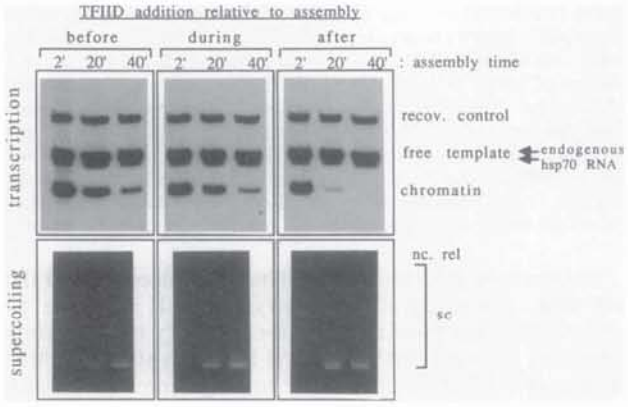

FIG. 4. Order of addition of yTFIID. (*Upper*) yTFIID was added to minigene template for 15 min before assembly (before), along with assembly reaction (during), or after assembly (after) in a coupled assembly/transcription reaction as in Fig. 3*A* and described in text. (*Lower*) Supercoil analysis of the assembled templates analogous to Fig. 3*B*. nc. rel, Nicked closed, relaxed plasmids; sc, supercoiled plasmids.

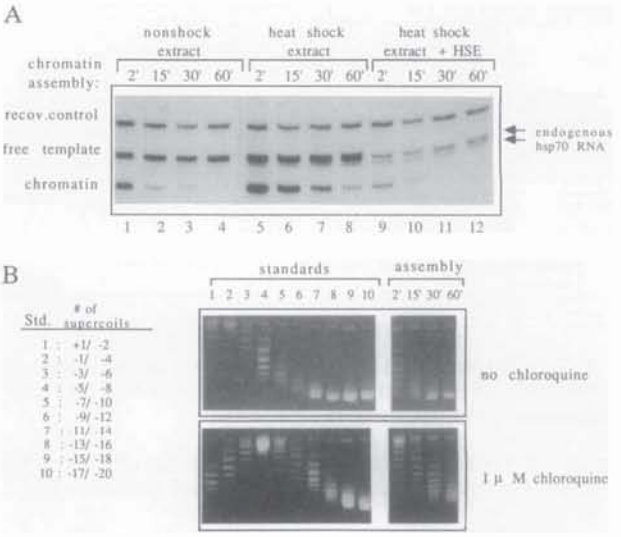

FIG. 5. (*A*) Transcription of assembled chromatin template depends on HSF. p(−50)HSE minigene preincubated with yTFIID before nucleosome assembly was transcribed in a nonshock extract (lanes 1–4), in a heat shock extract (lanes 5–8), and in a heat shock extract with HSE competitor (lanes 9–12). recov., Recovery. (*B*) Estimation of the average nucleosomal density of *in vitro*-assembled chromatin. Aliquots of the assembled minigenes analyzed for transcription in *A* were separated on agarose gels with or without 1 μM chloroquine along with the standards (Std.) of defined linking number at left. Comparison of topoisomer patterns of template plasmids and standards allows estimation of average numbers of supercoils introduced into template during assembly reaction, which corresponds to number of nucleosomes formed. Estimated nucleosomal densities are given in text.

stages in the transcriptional induction of hsp70 gene chromatin as "potentiation" and "activation." Initial studies of the *Drosophila* hsp70 chromatin *in vivo* showed that the promoter is organized in a nucleosome-free, hypersensitive region punctuated by protein binding at the TATA box before (and after) heat shock induction. These observations led to the hypothesis that binding of TFIID to the TATA box results in the formation of a nucleosome-free region that potentiates the promoter to be activatable by HSF (13). The two requirements we have demonstrated here for *in vitro* transcription from a reconstituted chromatin template are fully consistent with and provide strong functional support for this hypothesis.

Although our study implicates TFIID as a crucial component in the potentiation of the heat shock promoter, it does not demonstrate that recombinant yTFIID functions by itself. yTFIID might well act in association with other factor(s) present in the *Xenopus* S-150 nucleosome-assembly extract, or in the crude *Drosophila* transcription extract. Binding of TFIID (in concert with other factors) could directly exclude nucleosome formation over the heat shock promoter or alter subsequent nucleosome binding such that it is transparent to the interactions of general and specific transcription factors and RNA polymerase II with DNA.

In a previous study, prebinding of a TFIID fraction to the adenovirus 2 major late promoter was found sufficient in alleviating nucleosomal repression (37). TFIID alone in direct competition with nucleosomes during reconstitution was ineffective for the relief of repression and required the additional presence of upstream activators (the IE protein or USF) (38, 39). In a study published during the preparation of this manuscript, prebinding of recombinant yTFIID to the adenovirus 2 major late promoter was found to prevent nucleosomal inhibition (44). In contrast to the observations on the adenovirus 2 major late promoter, recombinant yT-

FIID prebinding at the hsp70 promoter does not result in a significantly increased transcription, even when the chromatin template was incubated with a vigorous (nonshock) transcription extract. The difference between the two studies could be related to somewhat different procedures used for nucleosome assembly. Alternatively, the difference could also be related to the specific nature of the DNA sequences surrounding the TATA box of the two promoters. Heat shock genes contain regions of sequence similarity immediately downstream of the TATA box (RCMGGCGC where M = C or A) and between −1 and +30 relative to the start site of transcription (CAgTT-AAat-aAA-Aa-C-AAg-Ga-AACA) (45, 46). Deletion of these sequences immediately downstream of the start site leads to a significant decrease of transcription *in vivo* (46). Perhaps heat shock promoter sequences have evolved specifically to trap TFIID and RNA polymerase (15, 17) in a potentiated complex.

A number of studies on the properties of recombinant yTFIID have concluded that while the protein was able to functionally substitute for a native factor to promote basal transcription from a free DNA template, it was not capable of mediating the effect of upstream transcriptional activator proteins (44, 47, 48). Whether the stimulation of transcription by HSF on the hsp70 chromatin template directly depends on recombinant yTFIID or whether the recombinant yTFIID is replaced by the natural *Drosophila* factor present in our crude transcription extract requires further analysis. In any event, the demonstration of an upstream activator protein to stimulate transcription from a preassembled chromatin template is, to our knowledge, unprecedented, and signifies that there may be mechanisms other than facilitating TFIID binding by which upstream activators exert their effects on chromatin.

What could be the advantage of potentiating a promoter with bound TFIID under nonshock conditions? We suggest that prebound TFIID would preempt the requirement for template commitment, the rate-limiting step in promoter activation, leaving only the requirement to convert inactive HSF to a form that binds to HSEs (<1 min) (49). Promoter potentiation by TFIID may thus be a hallmark of genes that need to respond expeditiously to cellular, developmental, and environmental signals. Our reconstitution experiments do not address whether the potentiation by TFIID binding results in the assembly of a preinitiation complex or the assembly of an initiated, but arrested, transcription complex, as observed *in vivo* (14, 15, 17).

How might the upstream activator HSF act on such a potentiated promoter? Binding of HSF could facilitate completion of an initiation complex by direct interactions with general transcription factors or by antagonizing the negative effect of neighboring nucleosomes on the completion of an initiation or elongation complex. In a different, but not mutually exclusive, scenario HSF could act by releasing an arrested transcription complex (15). Although the present reconstitutions with heterologous TFIID and nucleosomes (lacking histone H1) cannot be expected to fully mimic conditions *in vivo*, the ability to elicit regulated transcription from such a chromatin template *in vitro* provides opportunities to study interactions between the transcriptional apparatus, nucleosomes, and an upstream activator protein.

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