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Transient paralysis by heat shock of hormonal regulation of gene expression

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We have investigated the effect of heat shock on primary cultures of male and female *Xenopus laevis* hepatocytes as a function of estrogen-induced vitellogenin gene expression. Coincident with the induction of heat-shock protein (hsp) synthesis, thermal stress abolishes the estrogen activated transcription and accumulation of vitellogenin mRNA, at the same time causing the destabilization of vitellogenin mRNA accumulated by prior treatment with the hormone. Exposure of the cells to estrogen before heat shock allows an immediate resumption of vitellogenin gene transcription on return to 26°C. Heat shock applied to cells from hormonally naive male *Xenopus* extends the lag period preceding vitellogenin gene transcription upon return to normal temperatures. This transient and reversible paralysis of estrogen responsiveness is paralleled by reversible changes in the amount of nuclear estrogen receptor in the hepatocytes. Heat shock therefore offers a novel approach in the manipulation and analysis of the early stages of steroid hormonal regulation of gene expression.

Key words: estrogen receptors/heat-shock proteins/hepatocyte cultures/vitellogenin genes/*Xenopus*

Introduction

The phenomenon of heat shock, first described in *Drosophila* gene puffs (Ritossa, 1962; Ashburner, 1970), encompasses multiple effects on a variety of eukaryotic cellular activities (for reviews, see Schlesinger *et al.*, 1982; Tanguay, 1983). Induction of heat-shock proteins (hsps) in all organisms is associated with the rapid modification of cellular protein synthesis and gene transcription. Similar proteins have also been detected in response to other external stresses such as toxic agents, pH change and nutritional deprivation, while they may also be synthesized during the course of normal development (Schlesinger *et al.*, 1982).

Our laboratory has recently been analyzing the activation by estrogen of the genes of the vitellogenin multigene family in primary cultures of hepatocytes from male and female *Xenopus* (Tata, 1982; Wolffe and Tata, 1983; Ng *et al.*, 1984). We wanted to be able to inhibit transiently their transcription and accumulation of the induced mRNA. Our preliminary attempts at using the synthetic anti-estrogen Tamoxifen for this purpose were discouraging because of the agonist action of this compound, also encountered in other estrogen-induced systems (Sutherland and Murphy, 1982). We therefore decided to investigate the possibility of exploiting the heat-shock phenomenon to temporarily arrest the transcription of vitellogenin genes in cultured *Xenopus* hepatocytes as a tool to dissect the early events associated with activation by estrogen of this gene family. Here we report the conditions for the induction of hsps in primary cultures of *Xenopus* hepatocytes and describe for the first time the transient paralysis in normal, differentiated cells of transcription of hormone activated genes and the relatively rapid degradation of induced mRNA during heat shock. We also present evidence that the reversible arrest of estrogen-induced transcription of vitellogenin genes and accumulation of vitellogenin mRNA may result from the loss, upon heat shock, of functional hormone receptor in cultured hepatocyte nuclei. Heat shock therefore can be a valuable tool in analyzing the early molecular and cellular events underlying hormonal regulation of gene expression.

Results

Induction of heat-shock proteins

Figure 1 shows the electrophoretic pattern of ^{35}S-labeled proteins synthesized by *Xenopus* hepatocytes at temperatures ranging from 26 to 40°C. At temperatures >31°C, the synthesis of two prominent proteins of mol. wt. 85 000 and 70 000 was induced; these we refer to as hsps 85 and 70, respectively. Following the return to normal temperature, the time taken for resumption of normal protein synthesis was dependent on the temperature of heat shock (data not shown). The hepatocytes remained viable after long periods of heat shock, as shown by the recovery of normal protein synthesis and the exclusion of 0.5% trypan blue after 10 min

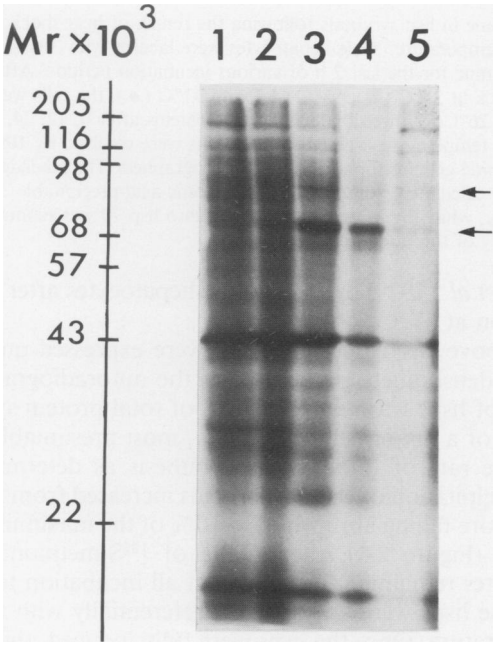

Fig. 1. Hsp synthesis in *Xenopus* hepatocyte cultures at different temperatures. Male hepatocytes were labeled with 30 μCi of [^{35}S]methionine for the last 2 h of incubation out of a total of 4 h. **Lanes 1-5** of the fluorogram show the pattern of proteins synthesized (equal amount of protein being loaded) in cells incubated at 26, 31, 34, 37 and 40°C, respectively. Arrows indicate the position of major hsps.

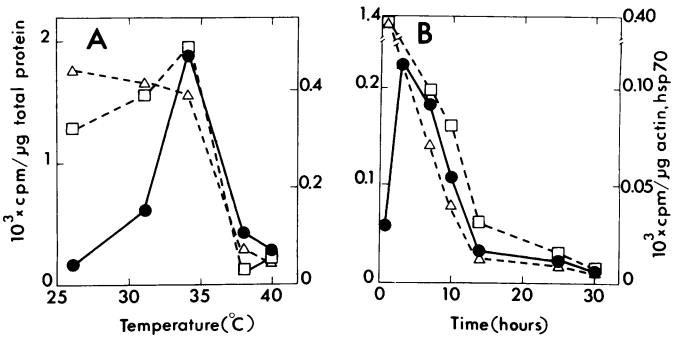

Fig. 2. Synthesis of hsp 70, actin and total protein **(A)** at different temperatures and **(B)** after varying periods of heat shock at 37°C. Male hepatocytes were labeled with [^{35}S]methionine as in Figure 1. The labeling of total protein (□) was based on 10% trichloroacetic acid-precipitable radioactivity, while the incorporation of ^{35}S into actin (△) and hsp 70 (●) was estimated by densitometry of fluorograms after the proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis.

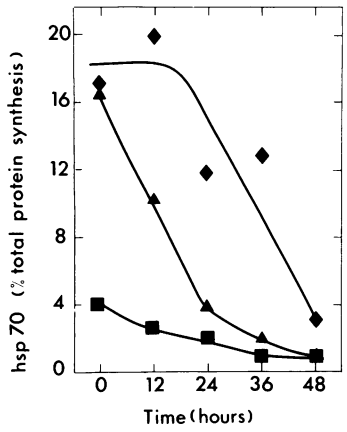

Fig. 3. Decline in hsp synthesis following the return of heat-shocked cells to normal temperature. Male hepatocytes were labeled with 30 µCi of [^{35}S]methioinine for the last 2 h of various incubation periods. After 12 h of heat shock at 31°C (■), 34°C (▲), and 37°C (♦), the cells were returned to 26°C followed by labeling the proteins after 0, 12, 24, 36 and 48 h at this temperature. The labeled proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and fluorographed. The labeling of total protein was calculated from 10% trichloroacetic acid-precipitable radioactivity, while the incorporation of ^{35}S into hsp 70 was estimated by densitometry of the fluorogram.

(Wangh *et al.*, 1979) by 85% of the hepatocytes after 16 h of incubation at 37°C.

The above electrophoretic data were expressed quantitatively by densitometric scanning of the autoradiograms and labeling of hsps was related to that of total protein synthesis and that of a major cellular protein, most presumably actin. Thus, the rate of total protein synthesis as determined by TCA precipitation of cellular proteins increased from 26°C to 34°C before falling abruptly to ~10% of the maximum value to 37°C (Figure 2A), the uptake of [^{35}S]methionine into hepatocytes remaining unchanged at all incubation temperatures. The hsps were synthesized preferentially with increase in temperature. Once the hsps were fully induced, the rate of their synthesis (illustrated in Figure 2 for hsp 70 only) declined sharply in parallel with that of total protein synthesis and also that of actin, with either increase in incubation temperature (Figure 2A) or increase in duration of incubation (Figure 2B). Thus the factors that affect the efficiency of translation of normal mRNAs at high temperatures affect hsp mRNA translation to the same extent. Figure 3 shows that the rate at which the amount of newly synthesized hsp 70 declined, relative to total protein synthesis, was inversely related to the temperature to which the cells had been previously exposed. In all cases, normal levels and patterns of protein synthesis were seen after 48 h of recovery period.

Transient and reversible inhibition by heat shock of the accumulation of vitellogenin mRNA in response to estrogen

Earlier studies from our laboratory (Wolffe and Tata, 1983; Tenniswood *et al.*, 1983) established that, providing the hepatocyte cultures were frequently replenished with the hormone, estradiol produced a sustained accumulation of vitellogenin mRNA and withdrawal of the hormone led to a cessation of vitellogenin mRNA accumulation. Figure 4A and B shows that when hepatocytes actively accumulating vitellogenin mRNA at 26°C were transferred to 37°C in the continuous presence of estradiol and maintained at that temperature, the vitellogenin mRNA levels fell rapidly in both male (Figure 4) and female (results not shown) hepatocytes with $t_{1/2}$ ~6 h. The rate of vitellogenin mRNA decay during the period of heat shock was unaffected by the presence or absence of estradiol. Returning the heat-shocked hepatocytes from 37°C to 26°C led to the resumption of vitellogenin mRNA accumulation, indicating that the effect of heat shock was readily reversible. The cessation of accumulation and the rapid decay of vitellogenin mRNA, as compared with the normal kinetics of hormonal response (Wolffe and Tata, 1983; Tenniswood *et al.*, 1983), implied both an inhibition of transcription and major changes in post-transcriptional and mRNA stabilizing mechanisms.

Next, the effect of varying periods of heat shock on vitellogenin mRNA levels in estrogen-stimulated male hepatocytes was investigated. Male hepatocytes were incubated with estradiol for 12 h at 26°C, then heat-shocked at 37°C for varying periods of time, and transferred back to 26°C in the absence of estradiol. The decay of vitellogenin mRNA that had accumulated initially at 26°C was a function of the duration of the heat-shock period (Figure 4A). After release from the heat shock, the rate of mRNA accumulation on re-exposure to estradiol was unaffected by the duration of the heat-shock period, as can be seen from the immediate recovery of estrogen responsiveness. This phenomenon demonstrates the retention of the full competence of hormonal response following different regimes of thermal stress. To simplify interpretation of further experiments on vitellogenin gene transcription, we adopted a standardized heat shock period of 12 h.

It has been suggested that hsps in *Drosophila* control the stability of pre-existing mRNA (Lindquist, 1980). Therefore, we first exposed male *Xenopus* hepatocytes to estradiol for 12 h at 26°C, to allow the accumulation of substantial quantities of vitellogenin mRNA. The hepatocytes were then heat-shocked at 31, 34 or 37°C in the presence of hormone for 12 h. As seen in Figures 1 and 2A, varying amounts of heat-shock proteins are synthesized at these temperatures. Figure 4B shows that the rate of decay of vitellogenin mRNA is not enhanced any further by raising the incubation temperature from 34 to 37°C, while vitellogenin mRNA did not decay at all on transfer from 26 to 31°C. The critical temperature, therefore, for de-stabilization of vitellogenin mRNA, lies between 31 and 34°C, which coincides with the massive induction of the hsps. The temperature of heat shock had little effect on the kinetics of vitellogenin mRNA accumulation when the hepatocytes were returned to the normal tempera-

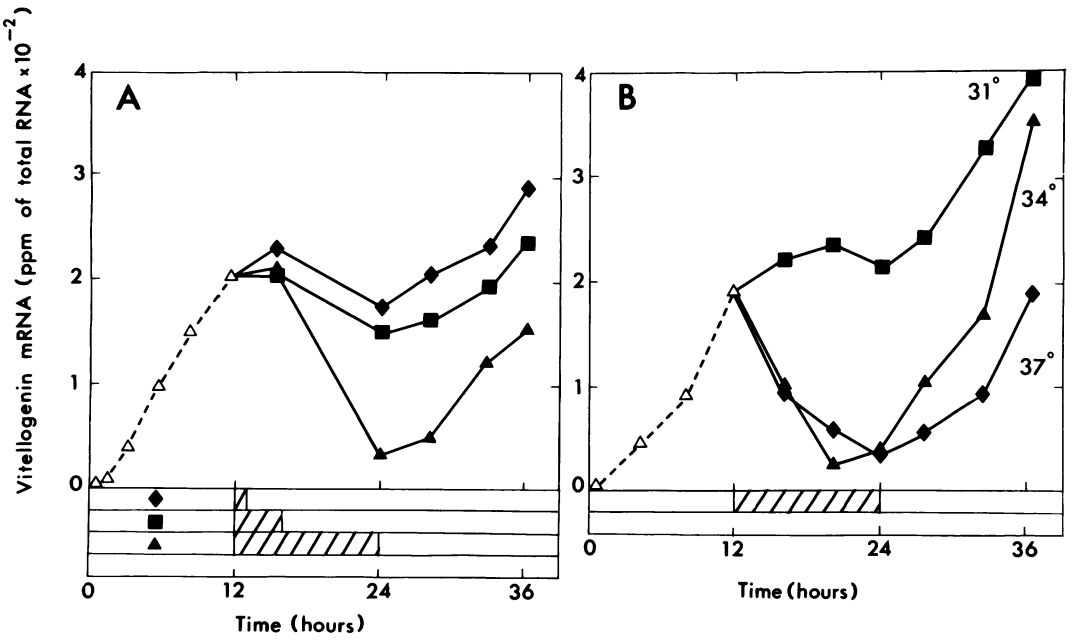

Fig. 4. Effect of estrogen-induced vitellogenin mRNA accumulation in hepatocyte cultures of **(A)** varying periods of heat shock at 37°C and **(B)** recovery from heat shock at different temperatures. Male *Xenopus* hepatocytes were treated with 10^{-6} M estradiol, replenished every 4 h throughout the experiment except where stated otherwise. Vitellogenin mRNA was quantitated at different times of incubation indicated. **(A)** After 12 h of incubation with the hormone at the normal incubation temperature of 26°C the cells were transferred to 37°C in the absence of the hormone. The heat shock period (▨) was maintained with equal batches of cells for 1 (♦), 4 (■), or 12 (▲) h. Twenty-four hours after the beginning of the experiment the cells were returned to 26°C in the presence of the hormone. **(B)** After 12 h at 26°C, equal batches of hepatocytes were incubated at 31°C (■), 34°C (▲), or 37°C (♦). Twelve hours later the cells were returned to 26°C in the presence of estradiol replenished every 4 h. Vitellogenin mRNA accumulation was measured at every 4 h interval.

ture of 26°C and maintained in the presence of estradiol.

In the same experiment as in Figure 4A, when proteins synthesized during the varying periods of heat shock and after the hepatocytes had been returned to 26°C and re-stimulated with estradiol were labeled with [^{35}S]methionine, the electrophoretic pattern shown in Figure 5 was obtained. Densitometric scanning of the subsequent fluorograph (data not shown) indicated that the synthesis of the hsps continues for some time after return to 26°C. Interestingly, therefore, the reinduction of vitellogenin mRNA accumulation is not inhibited by the continued presence of the hsps.

The duration of hormonal stimulation during the primary response of male *Xenopus* hepatocytes to estradiol *in vitro* affects the rate of vitellogenin mRNA accumulation during the secondary response to estradiol stimulation (Wolffe and Tata, 1983). Since the rate of accumulation of vitellogenin mRNA during the recovery period from heat shock was comparable to that observed prior to heat shock (Figure 4), we varied the duration of exposure to estradiol of male hepatocytes at 26°C, before subjecting them to heat shock at 37°C for 12 h. As Figure 6 shows, the rates of vitellogenin mRNA accumulation increased with longer periods of prior hormonal stimulation. However, if the cells were not exposed to hormone prior to heat shock for 12 h at 37°C and were then hormonally stimulated *de novo* at 26°C, they did not accumulate vitellogenin during 12 h of subsequent exposure to 10^{-6}M estradiol. Hepatocytes exposed to estradiol for at least 8 h at 26°C prior to heat shock accumulated vitellogenin mRNA at a rate equal to that seen immediately prior to heat shock in these cells. In other experiments (results not shown), the rate of vitellogenin mRNA accumulation was found to be unaffected by the presence or absence of estradiol during the heat-shock period.

In the converse experiments, male hepatocytes not pre-

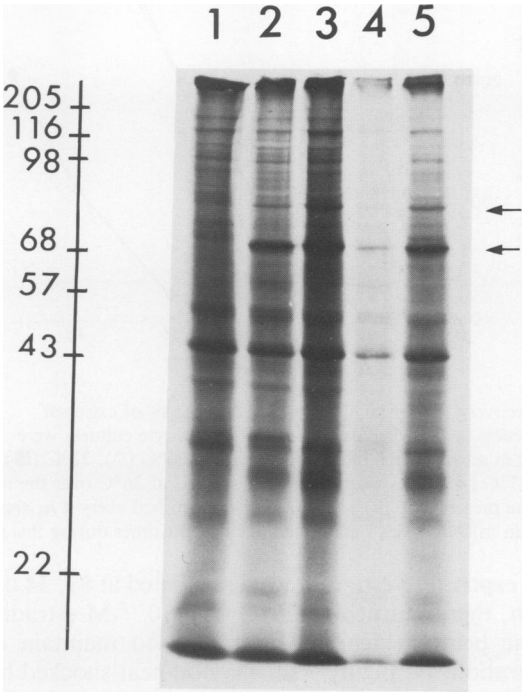

Fig. 5. Effect of varying periods of heat shock on estrogen-stimulated male hepatocytes. Male *Xenopus* hepatocytes were labeled with [^{35}S]methionine for the final hour of the incubation period and the pattern of protein synthesis recorded by fluorography after SDS-polyacrylamide gel electrophoresis of equal amounts of protein. **Lane 1:** after 12 h of incubation with estradiol at 26°C; **lane 2:** hepatocytes heat-shocked for 1 h at 37°C; **lane 3:** hepatocytes heat-shocked for 1 h at 37°C and allowed to recover at 26°C for 11 h; **lane 4:** hepatocytes heat-shocked for 4 h at 37°C; **lane 5:** hepatocytes heat-shocked for 4 h at 37°C and allowed to recover at 26°C for 8 h.

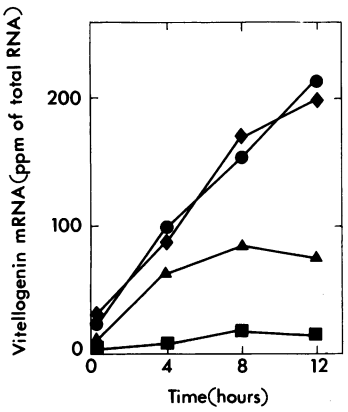

Fig. 6. Influence of varying periods of estradiol stimulation prior to heat shock on subsequent recovery from heat shock. 10^{-6} M estradiol was added every 4 h for varying periods of time to male hepatocyte cultures. The cells were then heat-shocked for 12 h at 37°C, returned to 26°C, with the replacement of the hormone and the measurement of vitellogenin mRNA being carried out at intervals of 4 h thereafter. The amounts of vitellogenin mRNA in hepatocytes not exposed to estradiol (■) or exposed to the hormone for 4 (▲), 8 (●), or 12(◆) h prior to heat shock are shown.

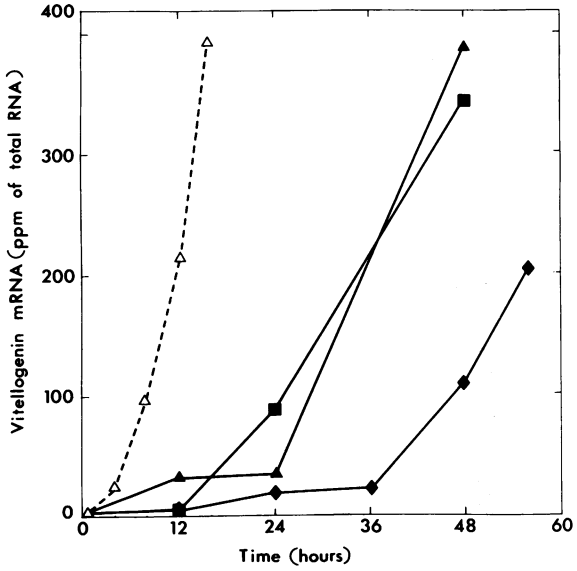

Fig. 7. Recovery from heat shock-induced paralysis of estrogen responsiveness in male hepatocytes. Male hepatocyte cultures were incubated in estrogen-free medium for 12 h at 26°C (△), 31°C (■), 34°C (▲), or 37°C (◆). They were then all incubated at 26°C over the next 56 h in the presence of 10^{-6} M estradiol, replenished every 4 h, and the vitellogenin mRNA levels quantitated at different times during this period.

viously exposed to estradiol were incubated at 31, 34 or 37°C for 12 h, then returned to 26°C with 10^{-6} M estradiol, the hormone being replenished every 4 h to maintain a high concentration. As Figure 7 shows, non-heat-shocked hepatocytes respond to the hormone by accumulating vitellogenin mRNA rapidly from zero time. Heat-shocked cells exhibited a paralysis of response to estrogen for 12–48 h upon returning them to the normal incubation temperature of 26°C. The lower the temperature of prior heat shock, the more rapid was the recovery of estrogen responsiveness in terms of vitellogenin mRNA accumulation. Once the cells re-acquired estrogen responsiveness, vitellogenin mRNA accumulated with kinetics equal to those of primary hormonal induction. Varying the duration of heat shock between 4 and 12 h was without effect on the time of recovery of responsiveness

Table I. Relative and absolute rates of transcription of vitellogenin genes in *Xenopus* hepatocyte cultures as a function of time after heat shock in the presence of estrogen

Time after transfer to 26°C or 34°C (h)	Relative transcription rate at 26°C (p.p.m.)	Absolute transcription rate at 26°C (molecules/cell/h)	Relative transcription rate at 34°C (p.p.m.)	Absolute transcription rate at 34°C (molecules/cell/h)
0	7000	1070	7000	1070
2	–	–	3000	430
4	6000	920	1600	250
8	–	–	700	23
12	6000	1047	200	14

10^{-6} M Estradiol was added every 4 h to female *Xenopus* hepatocyte cultures at 26°C over a period of 12 h, after which the cells were either incubated at 34°C or maintained at 26°C with continued exposure to estradiol. At the indicated times, the cells were pulsed with [^{3}H]uridine, total RNA extracted and the radioactive vitellogenin mRNA quantitated by hybridization to vitellogenin cDNA, both as parts per million (p.p.m.) of total radioactivity incorporated into total RNA and as absolute transcription rates, as described in Materials and methods.

(results not shown). The shortening of the temporal lag in recovery may be due to the more rapid resumption of normal protein synthesis in hepatocytes heat-shocked at lower temperatures (see Figure 3).

Transcription and stability of vitellogenin mRNA during heat shock and subsequent recovery

Although the cessation of vitellogenin mRNA accumulation implied an inhibition of transcription of vitellogenin genes, we decided to measure directly the effect of heat shock on transcription and stability of vitellogenin mRNA. Female *Xenopus* hepatocytes were used since they accumulate vitellogenin mRNA at rates 6–8 times those of male hepatocytes in culture (Wolffe and Tata, 1983). Female *Xenopus* hepatocytes were continuously stimulated with 10^{-6} M estradiol for 12 h at 26°C, after which some batches of cells were transferred to 34°C, while others were maintained at 26°C, with estradiol present throughout. At the times indicated in Table I, cell cultures were pulse-labeled with [^{3}H]-uridine for 1 h and the radioactive RNA hybridized to cloned vitellogenin cDNAs. There was a sharp decline in absolute transcription rate in cells transferred to 34°C (similar results were also obtained by heat-shocking cells at 37°C), with a half-time of decline of ~2 h, whereas cells maintained at 26°C continued to transcribe vitellogenin genes at a constant rate for 12–24 h in the presence of the hormone (similar decreases in transcription rate were also observed in male cells). The measurements of absolute transcription rates of vitellogenin genes validated the results of steady-state levels of mRNA expressed as p.p.m. Although heat shock is also known to inhibit rRNA synthesis, the direct estimation of transcription rates of a specified gene obviates the necessity of correcting for its effect on transcription of other genes.

Vitellogenin mRNA in cultured male *Xenopus* hepatocytes has a relatively short half-life of 10–16 h in the absence of estradiol (Wolffe and Tata, 1983; Tenniswood *et al.*, 1983), as compared with $t_{1/2}$ ~3 weeks in organ cultures in the presence of the hormone (Brock and Shapiro, 1983). The relatively rapid loss of vitellogenin mRNA upon heat shock in spite of continued, albeit reduced, transcription (see Table I) led us to investigate the effect of heat shock on the decay of labeled vitellogenin mRNA. A single dose of 10^{-6} M estra-

Table II. Recovery of estrogen-induced transcription of vitellogenin genes in *Xenopus* hepatocytes following heat shock

Sequence of incubation					
1 12 h with estradiol	2 12 h heat shock (34°C) with estradiol	3 1 h recovery at 26°C with estradiol	4 Further 24 h at 26°C with estradiol	Relative transcription rate (p.p.m.)	Absolute transcription rate (molecules/cell/h)
−	−	−	−	0	0
+	−	−	−	1450	118
+	+	−	−	0	0
+	+	+	−	1490	122
−	+	+	−	0	0
−	+	+	+	2140	176
−	−	−	+	6180	506

Male hepatocytes were exposed to various pre-treatments, with or without 10^{-6} M estradiol before labeling with [^{3}H]uridine for 1 h, the relative and absolute rates of transcription of the vitellogenin genes then determined.

diol, known to be adequate to maintain vitellogenin mRNA accumulation for 12 h, was added to male hepatocytes along with [^{3}H]uridine. Twelve hours later the hepatocytes were transferred to fresh culture medium (containing 10^{-6} M estradiol replenished every 4 h) at either 26 or 34°C. Control hepatocytes were transferred to fresh culture medium at 26°C in the absence of estradiol. Another hour later, and at the times indicated subsequently, total RNA was extracted and the amount of radioactive vitellogenin mRNA per unit mass of total RNA was determined by hybridization to cloned vitellogenin cDNAs. As shown in Figure 8, vitellogenin mRNA appears to be completely stable during the duration of this experiment in the continued presence of estradiol at 26°C. In the absence of estradiol at 26°C, vitellogenin mRNA decayed with a $t_{1/2}$ ~15 h, while at 34°C in the presence or absence of estradiol it disappeared with $t_{1/2}$ ~3 h. The loss of radioactive vitellogenin mRNA is an accurate measure of the rate of mRNA degradation since we have previously shown that vitellogenin genes are no longer transcribed if 12 h have elapsed after a single addition of 10^{-6} M estradiol in our hepatocyte culture system (Wolffe and Tata, 1983). Similar results were obtained either using female hepatocytes or a heat-shock temperature of 37°C (results not shown). Thus the drastic destabilization of vitellogenin mRNA produced by the transfer of hepatocytes from 26°C to 34°C partly accounts for the rather abrupt cessation of vitellogenin mRNA accumulation and its subsequent loss upon heat shock.

Recovery of responsiveness after heat shock is controlled at the transcriptional level

The dissociation of the paralysis of response to estrogen following heat shock at 31°C from the decay of vitellogenin mRNA levels (see Figure 4B) pointed to transcriptional control being a major and separate mechanism underlying the effect of heat shock. Male hepatocytes were therefore subjected to various pre-treatments before pulse-labeling for 1 h with [^{3}H]uridine in the presence of 10^{-6} M estradiol to investigate the effect of heat shock on transcription. As shown in Table II, heat shock leads to a sharp drop in vitellogenin gene transcription rate from 118 molecules/cell/h to almost nil within 24 h. However, if hormonally pre-stimulated cells are allowed 1 h to recover from heat shock the transcription rate returns to that seen before heat shock. If the hepatocytes had not been exposed to estradiol before heat shock, recovery is significantly slower, taking 24 h to reach a

Table III. Effect of heat shock on total and nuclear estrogen receptor levels in the presence and absence of estradiol in cultured male *Xenopus* hepatocytes

Treatment	Receptors/cell	Receptors in nuclei
None	1200	420
Estradiol alone	1200	1200
Heat shock alone	0	0
Heat shock with estradiol	1200	500
Estradiol for 1 h, then heat shock with estradiol	900	680
Heat shock alone, then recovery at 26°C for 4 h	200	120
Heat shock alone, then recovery at 26°C for 20 h	600	480

After 3 days in estrogen-free medium, male hepatocyte cultures were incubated for 6 h with 10^{-6} M estradiol. The cells were transferred to hormone-free medium and allowed to incubate for 12 h to enable the cells to metabolize completely any remaining estradiol. The cells were then incubated in estrogen-free or 10^{-6} M estrogen-supplemented medium at normal temperatures of 26°C or at the heat-shock temperature of 34°C, as indicated, for 12 h. Some batches of naive male hepatocytes were allowed to recover for varying periods of time at 26°C following a 12 h heat shock at 34°C. At the end of each regime of treatment the total number of receptors per hepatocyte as well as the tightly bound fraction in the nucleus were determined.

level of transcription seen in control cells after 12 h exposure to estradiol. Control hepatocytes exposed to estradiol continuously for 24 h acquired rates of transcription of vitellogenin genes three times those seen in heat-shocked naive cells after a comparable time. Thus, the paralysis of hormonal responsiveness resides at the level of transcriptional inhibition which is overcome by 24 h after heat shock in hormonally naive male *Xenopus* cells. Prior exposure of hepatocytes to estradiol at normal incubation temperatures protects them against this paralysis, but the presence of estradiol during the heat-shock period does not confer any protection from paralysis of responsiveness of naive cells.

Effect of heat shock on nuclear estrogen receptor levels

According to current ideas of steroid hormone action (O'Malley *et al.*, 1977), the number of occupied nuclear receptors (Palmiter *et al.*, 1976) would be the important determinant in regulating the transcription of vitellogenin genes. It was therefore important to establish whether or not the marked inhibition of transcription of vitellogenin genes would be correlated with an effect of heat shock on the levels of estrogen receptor in cultured *Xenopus* hepatocyte nuclei. Heat-shock experiments were performed under conditions similar to those described above for the accumulation and transcription of vitellogenin mRNA in which the amount of tightly bound nuclear estrogen receptor was determined. After 3 days in estrogen-free medium, the male hepatocytes used in these experiments were incubated for 6 h with 10^{-6} M estradiol. The cells were then transferred to hormone-free medium and allowed to incubate for 12 h. This estradiol pre-treatment was carried out in order to increase the number of estrogen receptors in the hepatocytes (Hayward *et al.*, 1980, 1982; Westley and Knowland, 1979), while the withdrawal period would enable the cells to metabolize completely any remaining estradiol and therefore to become inactive with respect to vitellogenin gene transcription (Wolffe and Tata, 1983; Tenniswood *et al.*, 1983). The results summarized in Table III reveal that, at normal temperature in the absence of

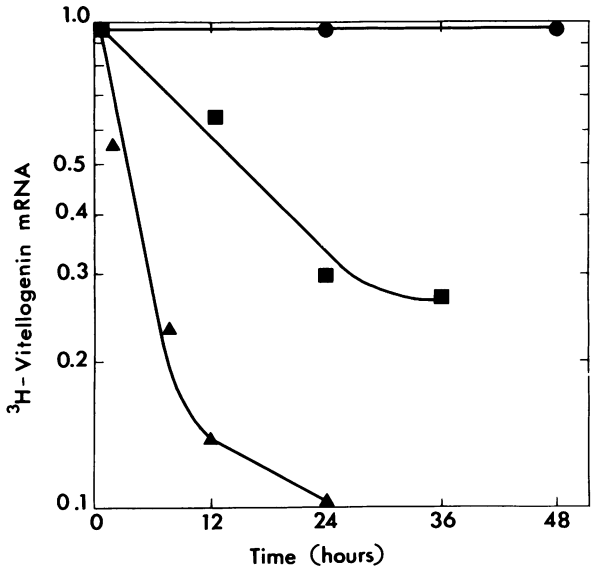

Fig. 8. Rate of disappearance of estrogen-induced vitellogenin mRNA during heat shock. Male hepatocytes were incubated for 12 h at 26°C with a single addition of 10^{-6} M estradiol in 2 ml of culture medium containing [^{3}H]uridine. The cultures were then transferred to fresh medium containing 10^{-6} M estradiol, replenished every 4 h, and incubated at either 26°C (●), or 34°C (▲). Another batch of hepatocytes (■) was transferred to fresh estradiol-free culture medium and incubated at 26°C. One hour later, total RNA was extracted at the times indicated and the amount of radioactive vitellogenin mRNA present per unit mass determined by hybridization to cloned vitellogenin cDNA. The results are expressed relative to the zero time value of 6000 d.p.m./μg RNA.

estradiol, 35% of total receptors were recovered in the tightly bound nuclear fraction of cultured male *Xenopus* hepatocytes. Following exposure to estrogen at normal temperature 1200 receptors were recovered in the tightly bound nuclear fraction 12 h later. In contrast, heat shock at 37°C for 12 h in the absence of estradiol caused a total loss or inactivation of the receptor. Concurrent exposure of cells to estradiol during heat shock allows for 500 receptors to be recovered in the nucleus and also be protected from inactivation. Furthermore, treatment of the cells with estradiol 1 h before heat shock seemed to protect receptor from heat shock inactivation and allowed 75% of the receptors to be retained in the nuclear fraction.

In a related experiment, the recovery of nuclear estrogen receptor following heat shock of naive male *Xenopus* hepatocytes was determined in the absence of estrogen. Cells were pre-treated with 10^{-6} M estradiol for 6 h to elevate the levels of estrogen receptor prior to withdrawal from hormone for 12 h. At the end of this period the levels of total and tightly bound nuclear estrogen receptor were found to be identical to those seen previously (i.e., 1200 total; 420 tightly bound). When the cells were heat-shocked at 34°C for 12 h at the end of this period, high affinity estrogen binding was found to have totally disappeared (Table III). If the hepatocytes were then maintained for varying periods of time at 26°C and estrogen receptor measured in the absence of added estrogen, the time of recovery of estrogen responsiveness correlates well with the recovery of high levels of total and tightly bound nuclear estrogen receptors (compare Table III and Figure 7).

Discussion

Among the wide variety of cells and organisms in which the phenomenon has been recorded (Schlesinger *et al.*, 1982 ; Tanguay, 1983), the induction by thermal stress of hsps in *Xenopus* cells is of special interest since two quite distinct mechanisms seem to operate in different cell types. *Xenopus* oocytes store substantial amounts of hsp mRNAs and the synthesis of hsps in response to heat shock is accounted for by translational control, whereas in somatic cells of *Xenopus* the induction is *via* transcriptional mechanisms (Bienz and Gurdon, 1982; Bienz, 1982). The fact that the major hsps were not synthesized in response to thermal shock in the presence of actinomycin D (data not shown) establishes that in primary cultures of *Xenopus* hepatocytes hsp synthesis is transcriptionally regulated. At the same time, and in common with other systems (see Schlesinger *et al.*, 1982), the rapid cessation of all normal protein synthesis in cultured hepatocytes followed by its recovery (Figures 1, 2, 3 and 5) suggests that the inhibition of hsp synthesis may also involve translational arrest. These and other studies from our laboratory (A.P.Wolffe, J.F.Glover, S.C.Martin, M.P.R.Tenniswood, J.L.Williams and J.R.Tata, unpublished) have also shown that the application of thermal shock, or the presence of large amounts of hsps, has a marked effect on the expression of vitellogenin genes as well as of genes expressed in *Xenopus* hepatocytes but not induced by estrogen. In particular, both the absolute rate of transcription and the steady-state levels of albumin mRNA were found to be also similarly sensitive to heat shock.

As regards the action of thermal stress on hormonally regulated gene expression, an inhibition by heat shock of both the translational and transcriptional processes is evident from our finding of a sudden and not gradual cessation of estrogen-induced transcription and accumulation of *Xenopus* vitellogenin mRNA at elevated temperatures (Figures 4, 6 and 7; Tables I and II). The paralysis of response to the hormone is analogous to the inhibition by heat shock of normal differentiative processes (Lindquist, 1981). That mild heat shock may inhibit transcription in the absence of a high degree of mRNA destabilization is suggested by the maintenance of vitellogenin mRNA levels at 31°C (Figure 4B). There is much evidence correlating the appearance of hsps with the accelerated breakdown of pre-existing messenger in a variety of systems (see Schlesinger *et al.*, 1982). The destabilization of vitellogenin mRNA observed at 34 or 37°C (Figure 8) could be related to the finding in *Drosophila* that raising the temperature higher than that necessary to produce the maximum amount of hsps may cause a rapid breakdown of pre-existing mRNA (Lindquist, 1980). It is interesting to note that hsp synthesis continued at a low rate when the accumulation of vitellogenin mRNA had resumed upon returning the hepatocytes to their normal temperature in the presence of estradiol. This suggests that heat shock itself, and not necessarily the presence and continued synthesis of hsps, may be responsible for mediating these effects, or that a minimum threshold level of hsps may be necessary for hsps to exert their action on transcriptional processes. While the mechanism remains unknown, it is relevant to note that in naive male hepatocytes heat-shocked at different temperatures (Figure 7) the bulk of hsp synthesis has ceased and normal protein synthesis resumed by the time estrogen responsiveness is re-established. It is also relevant that the refractoriness to estrogen of freshly prepared primary cultures of *Xenopus* hepatocytes was associated with the appearance of hsp-like stress proteins induced by procedures of cell isolation (Wolffe *et al.*, 1984).

As regulation of gene transcription by steroid hormones is dependent upon the accumulation of hormone receptor in the

nucleus (O'Malley *et al.*, 1977; Palmiter *et al.*, 1976), we investigated the fate of nuclear estrogen receptor before and after heat shock, in the presence or absence of estrogen. As a result of pre-treatment of hepatocytes with estradiol followed by withdrawal for 12 h, all the cells had accumulated a total of 1200 receptor copies per cell. The increase in receptors recovered in nuclei from 420 to 1200 per cell of hepatocytes cultured with estradiol at normal temperature is in accord with the findings of other workers in intact male *Xenopus* liver pre-treated with the hormone (Westley and Knowland, 1979; Hayward *et al.*, 1982). Other studies from our laboratory (unpublished data) have established a tight quantitative correlation between the accumulation of estrogen receptor in the nucleus and the absolute rate of transcription of vitellogenin genes in estradiol-stimulated male *Xenopus* hepatocytes. The total disappearance of receptor after heat shock would explain both the failure of estradiol to activate transcription of vitellogenin genes during the heat-shock period (Figure 4, Tables I and II) and the delayed hormonal response in hepatocytes that had been heat-shocked just prior to the addition of the hormone (Figure 7). Recovery of hormonal responsiveness was found to be associated with recovery of nuclear estrogen receptor levels (Table III). Probably, the binding of estrogen to the receptor prevents its inactivation and allows for increased nuclear accumulation, thus also explaining the protection of receptor against heat shock by exposure of the cells to estradiol prior to their transfer to a higher temperature (Figure 6). We do not know whether the loss of receptor upon heat shock is due to loss of hormone-binding sites by partial proteolysis or a more direct consequence of thermal stress. It is interesting that recovery of hormonal responsiveness correlates with that of high affinity estrogen binding, but we do not know whether or not the phenomenon is due to *de novo* receptor synthesis. Our findings may also be related to the loss of binding of ecdysterone to its receptor in heat-shocked *Drosophila* cells (Schaltman and Pongs, 1982). However, it is worth noting that in our studies, unlike ecdysterone (Ireland *et al.*, 1982), estradiol on its own did not induce hsps in *Xenopus* hepatocytes.

Our studies represent the first evidence that, in normal, fully differentiated cells, heat shock can transiently and reversibly paralyze the hormonally regulated expression of specific genes essential for development. They also provide the first demonstration of a complete but reversible inactivation by heat shock of a nuclear steroid hormone receptor, suggesting novel experiments in assessing its role in the early events determining hormonally responsive gene activation. Whether or not our observations are of general significance and applicable to other tissues, genes and hormones remains to be seen. In any event, the demonstration that by heat shock of primary cell cultures it is possible to manipulate the level of steroid receptor and the transcription, translation and stability of a steriod induced messenger RNA offers new means to analyze hormonal regulation of gene expression.

Materials and methods

Cell culture

The preparation of purified parenchymal cells from *X. laevis* liver was as described by Searle and Tata (1981). The cells were cultured in 33 mm diameter plastic Petri dishes in Wolf and Quimby's medium at a density of 2.3 x 10^5 cells/cm^2, a density found optimal for studying the induction of vitellogenin mRNA accumulation *in vitro* by estradiol (Tenniswood *et al.*, 1983). Purified parenchymal cells were routinely prepared and cultured for 3 days in estradiol-free medium at 26°C before the experiments were begun to allow the cells sufficient time to recover from the isolation procedures and to adhere to the culture dishes (Wolffe and Tata, 1983). The hormone was added to the cell cultures by replacing the steroid-free medium with 2 ml Wolf and Quimby's medium supplemented with 10^{-6} M estradiol-17β.

Extraction of RNA

RNA was extracted from cultured hepatocytes as previously described (Searle and Tata, 1981) and poly(A)-enriched RNA to be used as assay standard was also prepared from female *Xenopus* treated with estrogen *in vivo*, as described by Wolffe and Tata (1983).

Hybridization and quantification of vitellogenin messenger RNA levels

'Northern' blotting and disc hybridization assays for *Xenopus* vitellogenin mRNA were performed with aminothiophenol paper as described by Searle and Tata (1981), except that the hybridization probes used in these experiments were mixtures of all four plasmids pXlvc 10, pXlvc 18, pXlvc 19 and pXlvc 23 containing cDNA inserts corresponding to the four expressed *Xenopus* vitellogenin genes, linearized and labeled with ^{32}P by nick-translation with [α-^{32}P]dCTP and [α-^{32}P]TTP (2000–3000 Ci/mmol, Amersham International) to a specific activity of 5–50 x 10^7 c.p.m./μg. Standard curves were constructed for each disc hybridization assay with the vitellogenin mRNA standard, containing equal amounts of A and B group vitellogenin gene transcripts, diluted appropriately with total liver RNA from hormonally untreated male *Xenopus* lacking vitellogenin mRNA sequences. Hybridization and washing conditions were as previously described and the amount of vitellogenin mRNA was expressed as parts per million (p.p.m.) of total RNA (Wolffe and Tata, 1983).

Determination of the absolute rate of transcription of vitellogenin genes

Pulse-labeling of total cellular RNA with [5,6-^{3}H]uridine (52 Ci/mmol) and the determination of labeled uridine incorporated into vitellogenin mRNA was carried out as described previously (Wolffe and Tata, 1983). A mixture of 400 ng of each of the four plasmids containing vitellogenin cDNA inserts was immobilized on aminothiophenol paper discs. The specific activity of [^{3}H]-UTP pool and the absolute transcription rate were calculated as described by us earlier (Wolffe and Tata, 1983), based on the proportion of uridine residues in vitellogenin mRNA as described by Brock and Shapiro (1983).

Determination of the stability of vitellogenin mRNA

Xenopus hepatocytes were labeled with 250 μCi/ml of [^{3}H]uridine per dish for 12 h in the presence of 10^{-6} M estradiol added at time zero. At the end of this incubation period at 26°C the hepatocytes were washed with fresh Wolf and Quimby's medium and incubated at the temperature indicated with 2 ml of fresh culture medium in the presence or absence of 10^{-6} M estradiol; during this period the hormone was replenished every 4 h as described below. At different times after the removal of the [^{3}H]uridine, total cellular RNA was extracted and the [^{3}H]uridine incorporated into vitellogenin mRNA determined as described above with appropriate corrections for the different lengths of the cloned vitellogenin cDNA inserts.

Radioactive labeling of proteins synthesized in culture

To determine the pattern of protein synthesis, the culture medium was replaced with fresh medium containing 30 μCi of [^{35}S]methionine (1150–1300 Ci/mmol) for the final 2 h of incubation at the temperatures indicated. At the end of the labeling period the culture medium was removed, the cells rinsed in phosphate buffered saline and homogenized in 0.1% SDS. Incorporation of [^{35}S]methionine into hot tricholoracetic acid-insoluble material was determined on duplicate 10 μl samples of the cell homogenate and equal amounts of acid-insoluble radioactivity or protein were analysed by SDS-10% polyacrylamide electrophoresis on slab gels (Clemens *et al.*, 1975). Protein concentration was determined by the method of Bradford (1976). Gels were fluorographed according to Bonner and Laskey (1974), using Fuji RX X-ray film at −70°C. The density of the film image was determined using a Joyce-Loebl microdensitometer in order to determine the relative fraction of an individual protein band.

Subcellular fractionation and receptor assay

The method of Westley and Knowland (1978) was followed with minor modifications. All procedures were carried out at 0°C. Typically for each experimental point 8 x 10^6 cells (from two 50 mm culture dishes) were harvested in 300 μl cytosol buffer (50 mM Hepes pH 7.4, 0.25 M sucrose, 1 mM $MgCl_2$, 20 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 20 μg/ml benzamidine) and homogenized with 5–20 strokes in a 1 ml glass-glass homogenizer. The homogenate was centrifuged at 800 *g* for 10 min and cytosol was prepared from the supernatant by centrifugation at 100 000 *g* for 1 h in a Beckman SW 60 Ti rotor. This fraction would contain receptor that is loosely bound in the nucleus. The 800 *g* nuclear pellet was washed twice in cytosol buffer and the nuclei re-suspended in 300 μl nuclear extraction buffer (0.5 M KCl, 50 mM Hepes pH 7.4, 10% (v/v) glycerol, 1 mM dithiothreitol, 20 μg/ml PMSF and 20 μg/ml benzamidine). The mixture was incubated for 30 min with vortexing every 10 min and the extracted nuclear receptor was recovered as the supernatant after centrifugation at 100 000 *g* for 1 h. This

extract is referred to as 'tightly bound nuclear receptor'. Receptor assays were then promptly performed on 50 μl aliquots of cytosol or nuclear extract incubated in duplicate with various concentrations of [16α-^{125}I]iodoestradiol-17β (with or without a 500-fold excess of unlabeled diethylstilbesterol) at 16°C for 90 min. Unbound estradiol was then removed by Sephadex LH-20 column chromotography and non-specific binding was estimated from parallel incubations containing 500-fold excess diethylstilbesterol. Scatchard analysis of the data was performed to determine the number of estradiol binding sites and dissociatioin constant K_d. All the major characteristics of the estrogen receptor and its nuclear distribution in our cultured hepatocytes were identical to those described by other workers for *Xenopus* liver (Westley and Knowland, 1978; Hayward *et al.*, 1980).

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