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Estrogen Dependent Expression of Heat Shock Transcription Factor: Implications for Uterine Synthesis of Heat Shock Proteins

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Transcriptional induction of heat shock protein genes is generally mediated by binding of heat shock transcription factor(s) to the heat shock element present in the promoters of heat shock genes. Although the steady-state levels of heat shock factor mRNAs vary among different tissues, at present virtually nothing is known regarding the cellular signals responsible for their synthesis and hence the observed variations. In this report we demonstrate that the heat shock transcription factor (HSTF or HSF) is under positive regulation by estrogen. The effect of estrogen was observed with both types of heat shock factors (HSF-1 and HSF-2) and occurred at both the mRNA and protein level. Immunolocalization studies emphasized the potential biological importance of these observations whereby the increase in uterine HSF-1 and HSF-2 due to estrogen was found to be associated with the endometrium, the primary tissue component which is targeted for estrogen action. This is the first demonstration of a cellular factor which can regulate HSF-1 and HSF-2 gene expression. The implications of these findings to uterine heat shock protein gene expression are discussed.

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INTRODUCTION

The hallmark of the highly conserved heat shock response is the rapid synthesis of heat shock mRNAs and proteins. The mechanism by which eukaryotic cells respond to heat shock is by transcriptional induction of heat shock genes mediated by the binding of a transcriptional activator, heat shock transcription factor (HSTF or HSF) to a short highly conserved DNA sequence known as the heat shock element (HSE) present in the promoters of heat shock genes [1–3]. Multiple genes have been shown to encode HSF in a variety of species [4–7]. In mouse, two genes HSF-1 and HSF-2 have been isolated and characterized [7]. It has also been shown that the levels of HSF mRNAs differ among various murine tissues [7].

Our previous studies on the mouse uterine 90 kDa heat shock protein (hsp90) demonstrated that the steady-state levels of hsp90 could be regulated by the steroid hormone, estradiol (E_2), in a target tissue and steroid specific manner [8]. The kinetics of the observed induction of hsp90 mRNAs and protein suggested that E_2 dependent expression of hsp90 might occur primarily at the level of transcription [9], implicating a potential role for HSF in this phenomenon. Therefore, as a first step towards examining the relative importance of HSF in mediating E_2 dependent expression of HSP90, we decided to analyze for the steady-state levels of uterine HSF-1 and HSF-2. This was particularly important since it appeared that HSF was present at a much lower level in the uterus as compared to some other tissues such that there was no detectable level of HSF mRNA in the total RNA of uterus [7]. During the course of these studies it became apparent that in addition to hsp90, the expression of the *hsf*-1 and *hsf*-2 genes were also regulated by E_2. This is the first demonstration of a cellular factor which can stimulate *hsf* gene expression. This finding has major and broad significance since while the levels HSF mRNAs have been shown to vary among various tissues [7], at present virtually nothing is known regarding the signals responsible for HSF-1 and HSF-2 synthesis and hence the observed variations.

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MATERIALS AND METHODS

Tissues and steroids

Tissues were obtained from 2–5 month-old nulliparous BALB/c mice and, where indicated, ovariectomy had been performed 7 days prior to tissue removal. 17β-Estradiol and other steroids were administered s.c. as a solution in 1% ethanol in saline. Tissue homogenates were prepared in TED buffer (10 mM Tris, 1.5 mM EDTA, and 1 mM dithiothreitol, pH 7.4) with protease inhibitors (77 μg/ml aprotinin, 0.1 mM leupeptin, 100 μg/ml bacitracin, and 1 μg/ml pepstatin) and was centrifuged for 1 h at 105,000 *g*, to obtain soluble extracts. The extracts were either used immediately or after being frozen in liquid nitrogen and stored at −70°C.

Antibodies and cDNA probes

Plasmid DNAs carrying the cDNAs for murine HSF-1 and HSF-2, HSF-1 and HSF-2, specific antisera [10] were gifts from Dr R. Morimoto at Northwestern University. The cDNA probe encoding the murine glucocorticoid receptor was provided by Dr G. M. Ringold. The cDNA inserts from each clone were purified from vector sequences prior to use as radiolabeled probes.

Western blot analysis

The tissue extract was made up in SDS sample buffer to a final concentration of 135 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.05% bromophenol blue, and the sample was heated in a boiling water bath for 5 min. An aliquot of the sample was separated using a discontinuous polyacrylamide gel containing 7.5% acrylamide–0.075% bis in the resolving gel and 3% acrylamide–0.08% bis in the stacking gel. Extracts containing equivalent amounts of proteins were used for analysis of HSF-1 and similarly equivalent amounts were used for analysis of HSF-2; however, the blots probed for HSF-2 contained three times the amount of protein (300 μg) present in blots probed for HSF-1 (100 μg). The buffer system used was Laemmli's [11]. After electrophoresis, the gel was electroblotted onto nitrocellulose using Tris–glycine buffer, blocked and incubated with either HSF-1 or HSF-2 antiserum [10]. The antigen–antibody complexes were detected using the ECL system (Amersham) following standard protocols.

Immunofluorescence

Tissue specimens, immediately upon excision, were embedded in OCT compound, rapidly frozen in liquid nitrogen and stored at −70°C until sectioned. Tissue blocks were sectioned at 4–5 μm with a cryostat at −36°C and fixed with methanol:acetone (1:1) at −20°C for 5–7 min. All staining procedures were carried out at room temperature. The frozen sections were brought to room temperature and treated with phosphate buffered saline (PBS) containing goat serum prior to incubation with either HSF-1 or HSF-2 antiserum in a humidified chamber. Subsequently, the sections were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG. They were next washed three times with PBS, mounted in glycerol and photographed with an epifluorescent microscope.

Northern blot analysis

Total mRNA was prepared by the acid guanidinium thiocyanate-phenol–chloroform method [12] and enriched for poly A^+ RNA by oligo dT-cellulose chromatography [13]. Northern blots were run in formaldehyde-containing buffers according to standard procedures. The blots were hybridized at 65°C with random primer-labeled HSF-1 and HSF-2 cDNA probes (0.5–1 × 10^9 cpm/μg DNA) and sequentially washed at 25°C, and 42°C in 1 × SSC/0.1% SDS and then at 65°C in 0.2 × SSC/0.1% SDS (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0).

RESULTS AND DISCUSSION

Immunoblot analysis

To analyze for the presence of HSF-1 and HSF-2 in uterine extracts, we initially performed immunoblot analyses. Both HSF-1 and HSF-2 were present in the uterus as revealed by the presence of bands specific for each antisera [Fig. 1(A); compare lanes 1 and 3 with lane 5] which migrated at positions corresponding to those reported by Sarge *et al.* [7]. Both HSF-1 and HSF-2 also had the characteristic double banding pattern observed by Sarge *et al.* [7]. Figure 1(A) also shows that ovariectomy reduces both the level of HSF-1 and HSF-2 in the uterus (compare lane 2 with 1 and lane 4 with 3). A reduction in both HSF-1 and HSF-2 due to ovariectomy was also observed with other E_2 target tissues, such as vagina and mammary glands (data not shown).

To determine whether E_2 was responsible for maintaining the elevated levels of HSF-1 and HSF-2 in the uterus of non-ovariectomized animals, ovariectomized animals were administered with either saline or E_2 and the extracts from E_2 treated tissues were compared with saline treated control extracts. As shown in Fig. 1(B), there was an E_2 dependent increase in both HSF-1 and HSF-2 (compare lane 2 with 1 and lane 4 with lane 3); the increase in HSF-1 and HSF-2 due to E_2 was approx. 3-fold and 5-fold, respectively, over the saline treated controls. The steroid specificity of the observed E_2 dependent increase in HSF-1 and HSF-2 is shown in Fig. 1(C). Administration of promegestone (R5020; a synthetic progestin) had a marginal effect on HSF-1 and HSF-2. (Compare lanes 3 and 7 with lanes 1 and

5) while testosterone (an androgen), has no effect (compare lanes 4 and 8 with lanes 1 and 5); in contrast, similar to E_2, diethylstilbestrol (a synthetic estrogen) caused an increase in both HSF-1 and HSF-2 (compare lanes 2 and 6 with lanes 1 and 5).

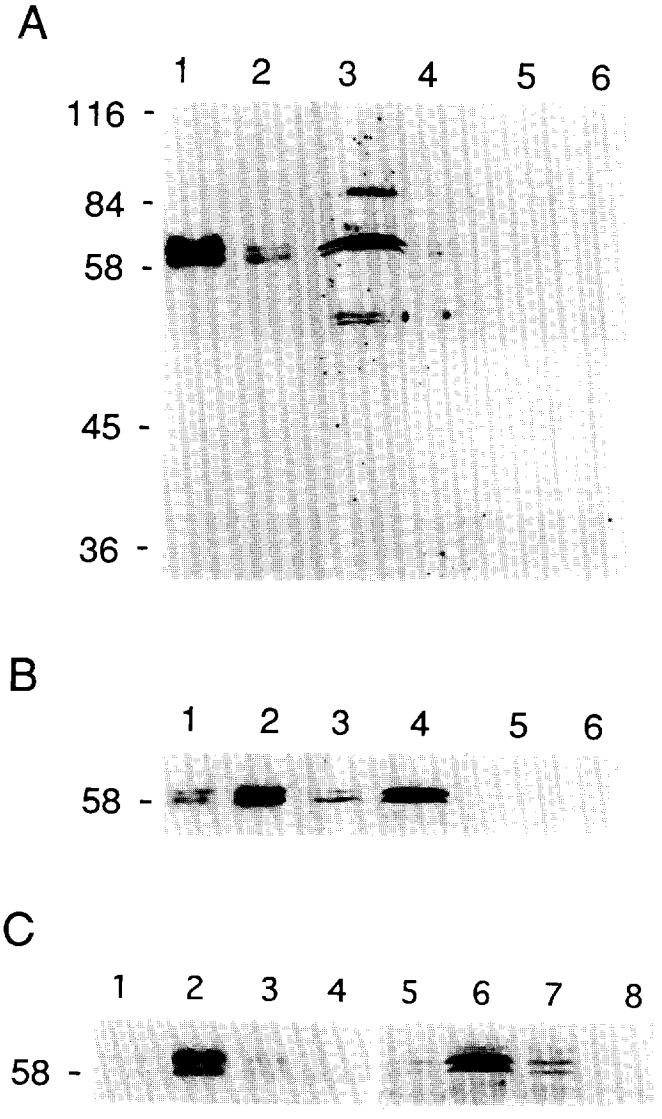

Fig. 1. Immunoblot analysis of uterine HSF-1 and HSF-2. Uterine extracts were subjected to immunoblot analysis for HSF-1 and HSF-2 as described in text. Lanes 1 and 2 (Panels A and B), lanes 1, 2, 3 and 4 (Panel C) were probed for HSF-1; Lanes 3 and 4 (Panels A and B) and lanes 5, 6, 7, 8 (Panel C) were probed for HSF-2; Lanes 5 and 6 (Panel A and B) did not receive primary antibody. The position of the molecular weight standards is indicated on the left ($\times 10^{-3}$). (Panel A) Lanes 1, 3 and 5 were loaded with extracts from non-ovariectomized animals while lanes 2, 4 and 6 were loaded with extracts from ovariectomized animals. (Panel B) Lanes 1, 3 and 5 were loaded with extracts from ovariectomized animals treated with saline for 18 h (controls) while 2, 4 and 6 were loaded with extracts from ovariectomized animals injected with 1 μg of E_2 for 18 h. (Panel C) Extracts from ovariectomized animals injected for 18 h with either saline (lanes 1 and 5) or diethylstilbestrol (land 2 and 6) or promegestone (lanes 3 and 7) or testosterone (lane 4 and 8) were analyzed for HSF-1 and HSF-2 described in text.

Immunocytochemical analysis

It is well known that when E_2 is administered *in vivo*, there is an influx of foreign cells such as eosinophils into the rodent uterus [14]. Since the heat shock response has been observed in all species and organisms tested so far, as a mediator of this response HSF may be expected to be present in almost all cells. Therefore, prior to assessing the biological importance of the modulation in the levels of uterine HSF-1 and HSF-2 it was necessary to verify that the estrogen dependent increase was intrinsic to the tissue. In particular, it was of importance to examine whether E_2 dependent expression of HSF was associated with the endometrium; we have previously observed that E_2 dependent expression of uterine hsp90 is predominantly associated with the endometrium [15] and our main purpose for initiating the studies on uterine HSF was to assess the relative roles of HSF-1 and HSF-2 in mediating E_2 dependent hsp90 gene expression. Accordingly, an *in situ* analysis for HSF-1 and HSF-2 was performed and the results of these experiments are shown in Fig. 2. Both HSF-1 and HSF-2 were present in the endometrium of the non-ovariectomized uterus (panels A and E) and the immunoreactivity was associated with both the luminal and glandular epithelium. With ovariectomy, there was a dramatic decrease in both HSF-1 and HSF-2 (Panels B and F) which, however, reappeared with E_2 treatment (Panels C and G). Once again, with both HSF-1 and HSF-2, the increase due to E_2 was associated with both the luminal and glandular epithelium.

Analysis for HSF-1 and HSF-2 mRNA

In an earlier report Sarge *et al.* [7] demonstrated that the expression of HSF-1 varied among different murine tissues and also that there was no detectable level of HSF-1 mRNA in the total RNA of uterus. Simultaneous analysis of HSF-2 mRNA in these studies also failed to detect HSF-2 in any of the murine tissues tested indicating that, overall, HSF-2 mRNA was less abundant than HSF-1 mRNA. Therefore, to examine if the increase in HSF-1 and HSF-2 levels in response to E_2 occurs at the level of mRNA, we performed Northern blot analysis using poly(A^+) mRNA prepared from the uteri of E_2- and saline-treated ovariectomized animals. As shown in Fig. 3, with E_2 there was an increase in both HSF-1 and HSF-2 mRNA (compare lane 2 with lane 1 and lane 4 with lane 3), which migrated at the same relative positions corresponding to approx. 2.3 kb as observed by Sarge *et al.* [7]. To normalize for mRNA present in each lane, the same blot was reprobed with murine glucocorticoid receptor (GR) cDNA, since GR, a transcription factor present in a variety of tissues and cells, is not regulated by E_2 [16]. As shown in lanes 5 and 6, in contrast to HSF-1 and HSF-2 there was no significant difference in the levels of GR mRNA between

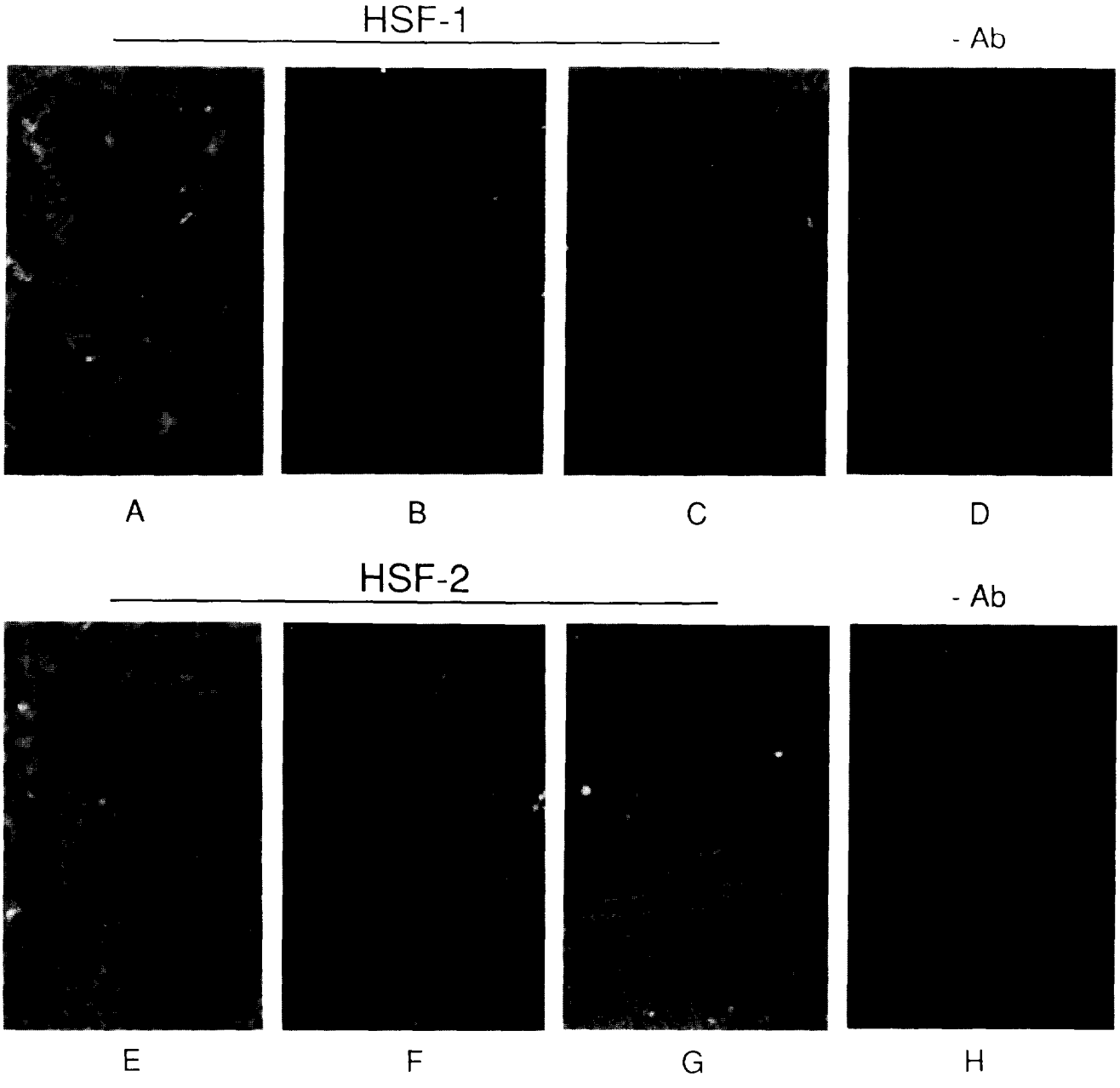

Fig. 2. Immunolocalization of HSF-1 and HSF-2. Uterus from non-ovariectomized (panels A, E and D), ovariectomized and treated with saline for 18 h (panels B and F), ovariectomized and treated with E_2 for 18 h (panels C, G and H) were examined for *in situ* localization of HSF-1 (panels A, B and C) or HSF-2 (panels E, F and G) as described in text. Panels D and H show the lack of immunoreactivity with the deletion of primary antibody. Original magnification 20×.

control and E_2 treated samples. Densitometric scanning analysis revealed that the magnitude of increase due to E_2 was not significantly different between HSF-1 and HSF-2 mRNA which was approx. 4-fold over the saline treated controls; this was in the range of increase observed for the protein level suggesting that overall the E_2 dependent expression of HSF may be primarily occurring at the level of transcription.

At present we can only speculate on the biological importance of E_2 dependent expression of HSF. Since (i) during each menstrual cycle, the uterine endometrium undergoes a cycle of proliferation, differentiation and apoptosis and (ii) the proliferation of the new endometrium at the beginning of each cycle occurs in response to E_2, an important biological significance of estrogen dependent expression of HSF may be to ensure the immediate presence of HSF in the new endometrial cells, as they are formed. This may be especially important to maintain cellular homeostasis since HSF, in particular HSF-1, is an essential cellular factor for mediating stress responses. Alternatively, it is well known that steroid hormones such as estrogens elicit a spectrum of biological responses and there is an evolving consensus that transcriptional regulation of

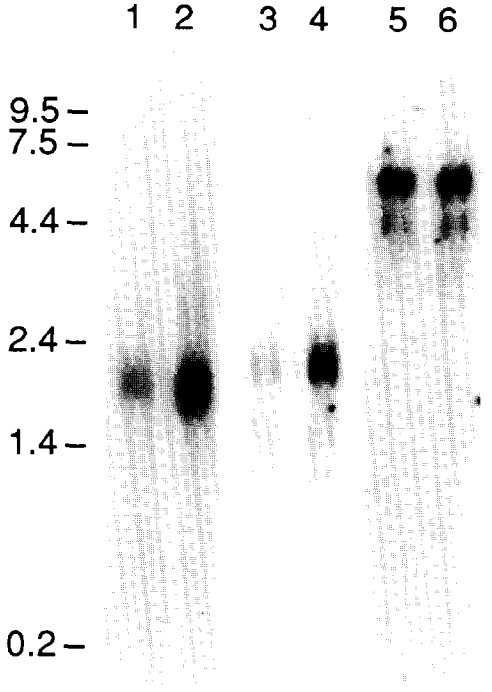

Fig. 3. Effect of E_2 on uterine HSF-1 and HSF-2 mRNA. Uterine Poly (A^+) RNA from ovariectomized animals treated either with saline for 6 h (Lane 1) or E_2 for 6 h (Lane 2) was electrophoresed, transferred to nitrocellulose and hybridized sequentially with ^{32}P-labeled HSF-1 cDNA, HSF-2 cDNA and glucocorticoid receptor cDNA (of equivalent specific activities) as described in text. Lanes 1 and 2 correspond to HSF-1; lanes 3 and 4 correspond to HSF-2; lanes 5 and 6 correspond to GR. The position of the molecular weight standards (kb) is indicated on the left.

most genes occurs through the binding of eukaryotic transcription factors from different gene families as multiprotein complexes to composite regulatory elements on the genome [17–19]. Therefore, it is also possible that the biological significance of estrogen dependent HSF synthesis lies in its potential to regulate a set of genes including HSPs which may contain HSE's but lack the consensus estrogen responsive element. Indeed, studies in Drosophila have revealed potential targets in chromatin for HSF, in addition to the classical heat shock gene loci [20], which have prompted the belief that in addition to the classical heat shock genes, HSF may also regulate the activity of "non-heat shock" genes and these may contain HSE [1]. Regardless, observations reported in these studies namely that a steroid hormone can regulate the expression of HSF have major implications for understanding both the overall regulation of HSP gene expression and those responsible for mediating steroid dependent gene expression.

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