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Activation of the heat shock transcription factor by hypoxia in mammalian cells

(gene regulation/stress response/ischemic heart disease)

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ABSTRACT **Members of the stress protein family such as HSP70 are induced in ischemic tissues and may contribute to the ability of cells to survive episodes of transient circulatory insufficiency. However, the biochemical events that lead to this induction, and their degree of similarity with pathways triggered by heat stress, have not been defined. In this study, we demonstrate that transient exposure of cultured C2C12 mouse myogenic cells to a hypoxic atmosphere stimulates DNA binding activity of the heat shock transcription factor through mechanisms that are independent of new protein synthesis. Activation of heat shock transcription factor in hypoxic cells is temporally associated with induction of endogenous HSP70 gene transcription and with induction of a heterologous reporter gene controlled by the human HSP70 promoter. Furthermore, induction of the human HSP70 promoter by hypoxia requires an intact heat shock element, indicating that other cis-acting transcriptional control elements contained within this complex promoter are not sufficient to transduce signals generated within hypoxic cells. These findings provide strong evidence that hypoxia and heat shock induce expression of the HSP70 gene by similar, if not identical, mechanisms.**

Myocardial ischemia resulting from coronary occlusion produces metabolic and functional abnormalities in cardiac myocytes that appear within seconds of the reduction in blood flow (1–3). If ischemia is transient (minutes), myocytes survive and recover without permanent sequelae. However, if the ischemia is prolonged (hours), cell necrosis and myocardial infarction occur (2). Efforts to understand mechanisms that determine survival or cell death during ischemia have major physiological and clinical importance. To this end, recent studies have focused on the potential role of stress proteins, such as HSP70, as a factor that may limit cell damage during myocardial ischemia (4, 5).

Transcription of the gene encoding HSP70 is induced by a variety of physical and metabolic stresses (6–11). During heat shock, transcriptional activation of the HSP70 gene results from activation of a nuclear transcription factor, heat shock transcription factor (HSTF), that binds to distinctive regulatory motifs, heat shock elements (HSEs), within the 5′ flanking region (12–16). However, other stresses such as glucose deprivation, growth factor stimulation, and viral infection activate transcription of HSP70 and other genes encoding stress proteins by different mechanisms that act through other cis-acting transcriptional control elements and do not involve HSTF (17–20).

Although myocardial ischemia is known to increase the abundance of HSP70 mRNA and protein, the biochemical events that result in activation of HSP70 gene transcription as a result of metabolic stresses encountered during ischemia are currently unknown. As a step toward identifying these events, the purpose of this study was to define the cis-acting control elements within the complex HSP70 gene promoter required for transcriptional activation during hypoxia and to define the effects of hypoxia on the DNA binding function of HSTF.

MATERIALS AND METHODS

Cell Culture Conditions. Cultures of C2 myogenic cells (1.0×10^6 cells) were established in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum and 5% (vol/vol) embryo extract in Corning dishes (100 mm) or T75 flasks in a humidified atmosphere of 95% air/5% CO_2 and were subjected to stress conditions 2 days later. To study the effects of hypoxia, the dishes were placed in an atmosphere of 95% N_2/5% CO_2 for 1–6 hr at 37°C in a modular incubator chamber. The time course of changes in the partial pressure of oxygen (see Fig. 1) was determined by a polarographic method (21) employing a glass microelectrode immersed in the culture medium. After exposure to the hypoxic atmosphere, cells were harvested immediately for analysis of HSTF binding or harvested after 1–4 hr of recovery in a normoxic atmosphere for analysis of HSP70 mRNA or reporter enzyme activity. To study the effects of heat shock, sealed flasks were immersed in a water bath at 42.5 ± 0.2°C and either harvested immediately or returned to 37°C for 1–4 hr. In experiments to assess the dependence of HSTF activation on new protein synthesis, parallel cultures were treated with cycloheximide (100 μg/ml).

More limited analyses were performed on primary cultures of skeletal myotubes derived from chicken embryos, as described (22).

Analysis of HSTF Binding Activity. For each analysis, cells were harvested from two or three dishes or flasks and pooled, and whole-cell extracts were prepared as described by Dignam *et al.* (23) with modifications as noted below. The cells were washed twice with ice-cold 1× isotonic phosphate-buffered saline (PBS), scraped with a rubber policeman, and collected in 2 ml of PBS. After centrifugation at 1200 rpm (Beckman J2-21, JS13.1 rotor) for 7 min, cell pellets were frozen in a dry ice/ethanol bath and resuspended in 1.5 vol of lysis buffer (10 mM Hepes, pH 7.9/10 mM KCl/1.5 mM $MgCl_2$/0.4 mM sodium orthovanadate/0.4 mM NaF/0.5 mM phenylmethylsulfonyl fluoride/1.0 mM dithiothreitol). The samples were allowed to stand on ice for 20 min before adding 1.6 vol of extraction buffer [20 mM Hepes, pH 7.9/0.2 mM EDTA/20% (vol/vol) glycerol/1.66 M KCl/0.4 mM NaF/0.4 mM sodium orthovanadate/0.1 mM phenylmethylsulfonyl fluoride/1.0 mM dithiothreitol] with constant shaking at 4°C for 1 hr. The samples were centrifuged at 55,000 × *g* for 1 hr

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Abbreviations: HSTF, heat shock transcription factor; HSE, heat shock element; CAT, chloramphenicol acetyltransferase.

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and the supernatant was dialyzed for 4 hr in 20 mM Hepes, pH 7.9/50 mM KCl/0.1 mM EDTA/10% glycerol/0.4 mM NaF/0.4 mM sodium orthovanadate/0.1 mM phenylmethylsulfonyl fluoride/1.0 mM dithiothreitol. Samples were frozen in liquid nitrogen and stored at −70°C. Protein concentrations were determined in the Bio-Rad reagent assay.

Cell extracts were analyzed for HSE binding activity by gel-retention assays. The binding reaction mixture contained 10 μg of protein extract and 1.0 μg of poly(dI-dC) in a final volume of 30 μl of binding buffer (20 mM Hepes, pH 7.9/40 mM KCl/1.0 mM $MgCl_2$/0.1 mM EGTA/0.5 mM dithiothreitol). Each reaction mixture contained 20,000 cpm of a double-stranded HSE oligonucleotide (AATTCGAAACCCCTGGAATATTCCCGACCTGGCAGC and its complementary strand) labeled with ^{32}P by filling-in 5′ overhangs with the Klenow fragment of DNA polymerase I. The reaction mixture was allowed to stand for 25 min at room temperature. After addition of 5 μl of 20% (vol/vol) Ficoll, samples (8 μl) were loaded onto a 4% polyacrylamide gel and electrophoresed for 2 hr at 200 V. The gel was dried under vacuum and exposed overnight to Kodak X-Omat film at −70°C.

Endogenous HSP70 Gene Expression. Total RNA was extracted from cells after heat shock or hypoxia as described (24). Briefly, cells were washed with PBS and extracted in guanidinium isothiocyanate buffer. RNA was sedimented through a 5.7 M cesium chloride cushion for 16 hr at 35,000 rpm (Sorvall TH-641 rotor). Pellets were dissolved in TES (10 mM Tris Cl, pH 7.9/5 mM EDTA/1% SDS), precipitated twice in ethanol, and redissolved in water. RNA concentrations were estimated from A_{260} and the samples were stored at −70°C.

Northern blot analysis was performed with 10 μg of cytoplasmic RNA after electrophoresis through 1.2% formaldehyde/agarose gels and capillary transfer to Nytran filters. The filters were baked under vacuum at 80°C for 2 hr and prehybridized in 50% (vol/vol) formamide/5× Denhardt's solution/2.5× SSPE/0.1% SDS/salmon sperm DNA (20 μg/ml) at 42°C for 4 hr. (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin; 1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA.) A double-stranded 800-base-pair *Bam*HI–*Eco*RI fragment from the protein-coding region of human HSP70 cDNA was labeled by the multiprime method (Pharmacia) to $>5 \times 10^8$ cpm/μg and added to the hybridization mixture. As a control for the equivalence of gel loading in each lane, the same filters also were probed with a cloned fragment of mouse mitochondrial DNA complimentary to 12S and 18S mitochondrial rRNA (25). After 18–24 hr of hybridization, the filters were washed twice in 1× SSC/0.1% SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at room temperature before continuous washing in a shaking incubator at 42°C for 1–2 hr. The filters were exposed to Kodak X-Omat film overnight at −70°C and the relative labeling was determined by scanning densitometry.

HSP Promoter Constructions and Reporter Gene Expression Assays. C2 myogenic cells were stably transformed with plasmid constructs (provided by R. Morimoto, Northwestern University, Evanston, IL) in which the choramphenicol acetyltransferase (CAT) gene was placed under the control of human HSP70 promoter sequences containing a native consensus HSE (5′ C--GAA--TTC--G) (Δ5N/-105) or a mutant HSE (LSPN) (10). These constructions also included a neomycin-resistance gene driven by a constitutive promoter to permit selection of stably transformed cells.

C2C12 cells (1×10^5) were transfected by liposome fusion (Lipofectin; Bethesda Research Laboratories). Cells were washed twice with Opti-MEM (GIBCO) and the medium was replaced with 6 ml of Opti-MEM. Plasmid DNA (10 μg) was combined with 30 μg of Lipofectin reagent in 1.5 ml of Opti-MEM in polystyrene tubes. After standing at room temperature for 25 min, the suspension was added dropwise to each plate. After 20 hr, cells were refed with growth medium containing 10% fetal bovine serum. Clonal selection in G418 (26) was carried out for 3 weeks before resistant clones were pooled and subjected to stress conditions.

In addition, a more limited series of transient expression assays were performed after transfection of primary cultures of skeletal myotubes derived from chicken embryos. Myoblast cultures were established and transfected by liposome fusion. Cells were subjected to stress conditions on the fifth day after transfection, when cultures consisted primarily of large multinucleated myotubes.

CAT enzyme activity was determined in whole-cell extracts (200 μg) as initial rates (linear over 4 hr) of partitioning of acetylation products of [^{14}C]chloramphenicol into toluene overlying the reaction mixture (27).

RESULTS

Oxygen tension in the cell culture medium fell rapidly [$Po_2 <$ 7 torr (1 torr = 133.3 Pa) within 15 min] when cultures were shifted from air to a nitrogen atmosphere (Fig. 1). This degree of hypoxia induced expression of endogenous HSP70 mRNA within 2 hr (Fig. 2). The human HSP70 cDNA probe used in these analyses hybridized strongly to two species of mRNA. The lower molecular weight species encodes an HSP70 isoform expressed in many cell types under nonstressed conditions (28), as we observed in C2C12 cells. The larger mRNA species encodes the major heat-inducible form of HSP70. The induction of HSP70 mRNA by heat stress was more rapid than that induced by hypoxia in this model. One hour of heat stress was sufficient to evoke a maximal response, while the inducible form of HSP70 mRNA was not detectable in hypoxic cells until 2 hr.

Activation of HSTF followed a time course similar to that of HSP70 mRNA during each stress condition (Fig. 3). During heat shock, HSTF binding activity was detectable within 20 min, and HSTF binding activity became evident only after 2 hr of hypoxia. Each of these stresses appears to exert its effect upon a preexistent but inactive pool of HSTF within the cell, since protein synthesis was not required for activation of HSTF by heat shock or hypoxia.

The DNA binding activity induced by hypoxia in these cells was specific for the cognate binding site of heat-inducible HSTF: Competition by unlabeled native HSE sequences eliminated protein binding to the labeled probe, whereas a mutant HSE or unrelated sequences failed to compete (Fig. 4).

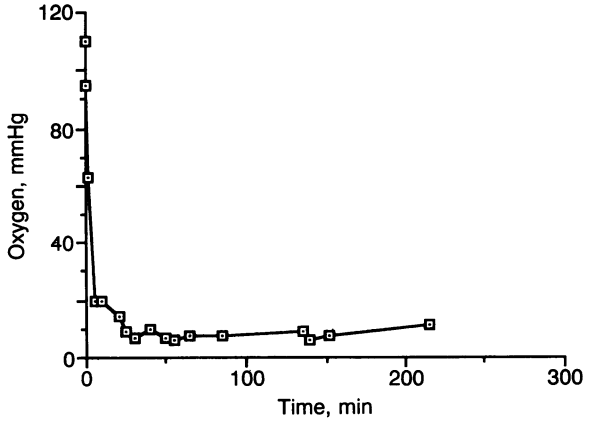

FIG. 1. Time course of changes in oxygen tension in medium overlying cell cultures after the transition from air to nitrogen atmosphere. Oxygen tension was measured polarographically with a glass microelectrode.

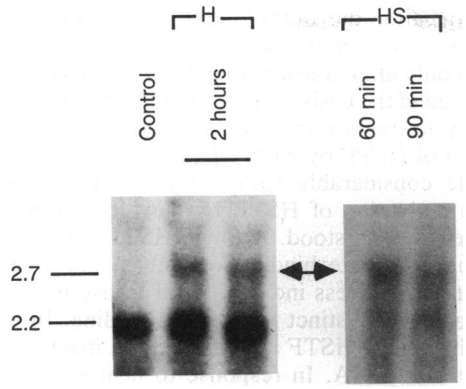

FIG. 2. Effects of hypoxia and heat stress on expression of endogenous HSP70 genes in mouse C2C12 myogenic cells. The figure shows total RNA from control, hypoxic (lanes H), and heat-stressed (lanes HS) cells hybridized to a human HSP70 cDNA probe. The duration of hypoxia was 2 hr and heat-stressed cells were held at 42.5°C for 60 and 90 min. The position of the stress-inducible 2.7-kilobase transcript is indicated (arrow). The lower band is the constitutive form of the message (HSC70). The blots prepared from control and heat-stressed cells were exposed for 24 hr and 6 hr, respectively. Molecular sizes are in kilobases.

C2 myogenic cells stably transformed with the CAT gene under the control of the human HSP70 gene promoter with its native HSE (Δ5N/-105) expressed only low levels of CAT activity under nonstressed conditions, but 4–6 hr of hypoxia resulted in a substantial induction of reporter gene expression (Fig. 5). Expression of CAT from constructs driven by the promoter with a native HSE also was evoked in these cells by shorter durations of heat stress. However, a 4-base mutation in the HSE (LSPN) abolished the responsiveness of the promoter to both heat stress and hypoxia.

Similar results were observed in transient-expression assays performed in primary cultures of skeletal myotubes derived from chicken embryo myoblasts. CAT activity was 6-fold higher after hypoxia in cells transfected with Δ5N/-105

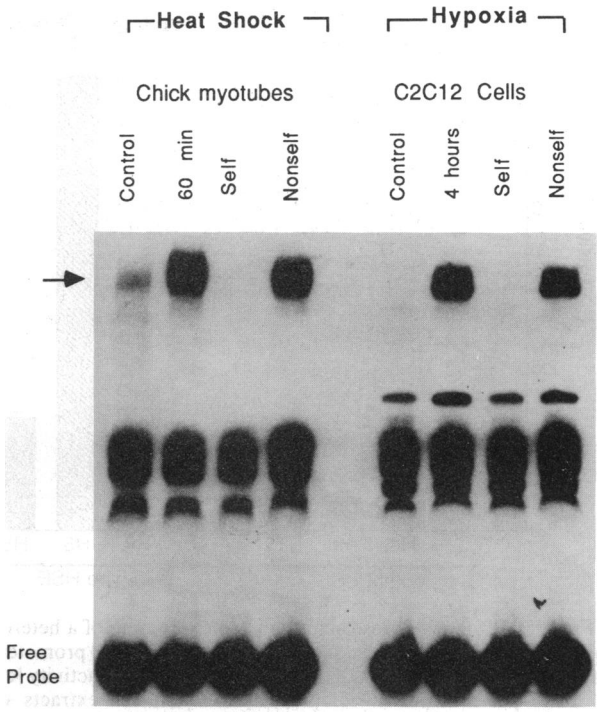

FIG. 4. Sequence specificity of HSE-binding activities in heat-stressed and hypoxic cells. Gel-retention assays were performed with extracts from chicken embryo myotubes or mouse C2C12 myogenic cells under conditions of heat shock or hypoxia, as indicated. Stress-inducible HSE-binding activity was abolished by competition with a 200-fold molar excess of unlabeled HSE oligonucleotide (lanes Self), but not by an identical concentration of oligonucleotide containing a mutant HSE (lanes Nonself).

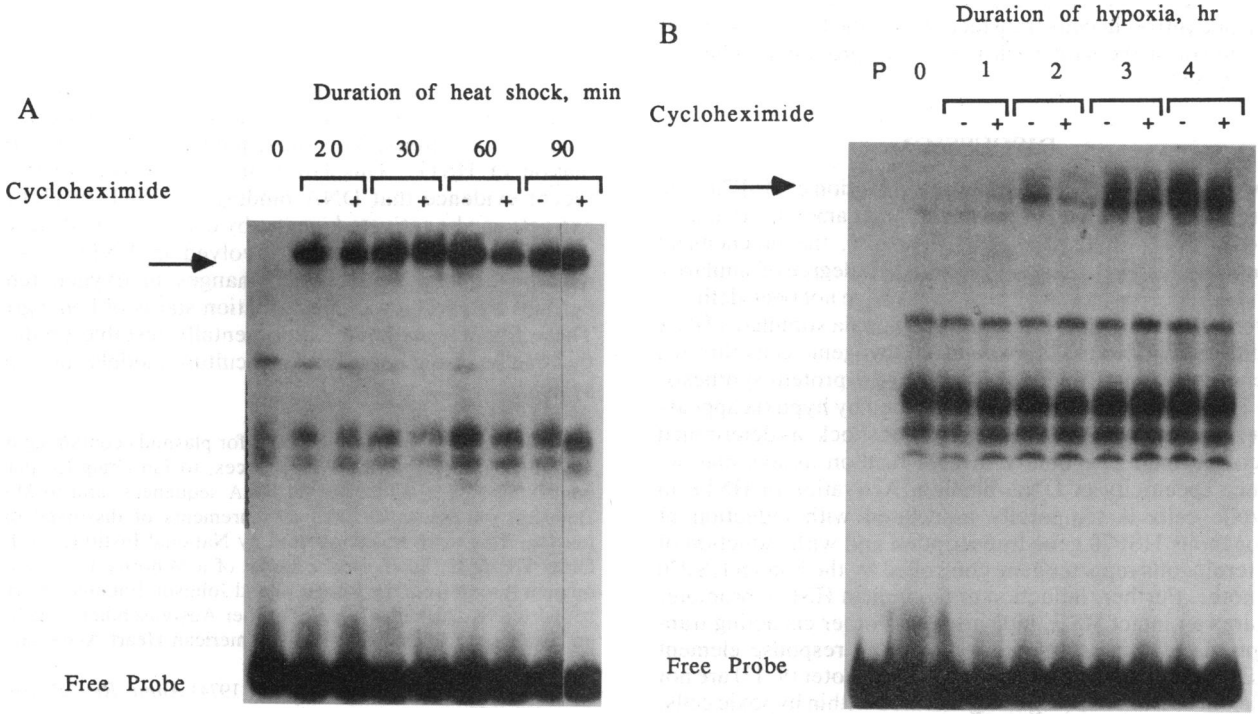

FIG. 3. Time course of effects of heat stress (*A*) and hypoxia (*B*) on DNA-binding activity in protein extracts from cultured C2C12 myogenic cells. Gel-retention assays were performed by using a synthetic double-stranded oligonucleotide containing a consensus HSE as the probe. The position of migration of the unbound (free) probe in the gel is indicated in lane P. The stress-inducible complexes formed after exposure of the cells to elevated temperature are indicated (arrow), and bands of similar mobility are observed after hypoxia. The duration of each stress condition is indicated, and cells were cultured in the absence (−) or presence (+) of cycloheximide.

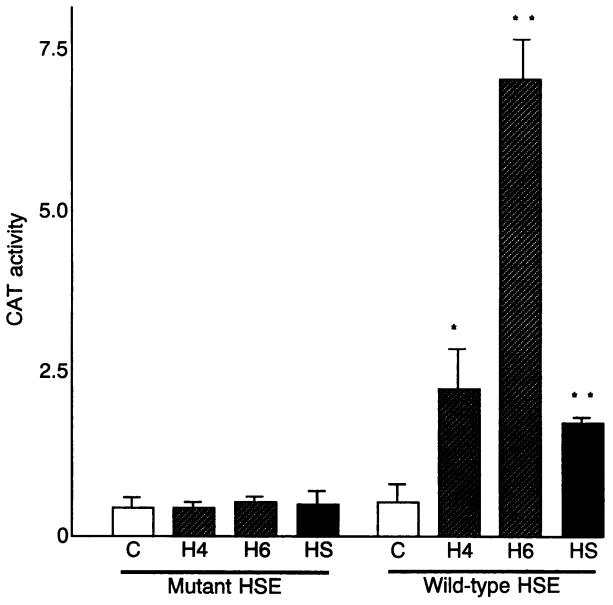

FIG. 5. Effects of stress conditions on expression of a heterologous gene (CAT) under the control of the human HSP70 promoter in stably transformed mouse C2C12 myogenic cells. CAT activity [cpm ($\times 10^{-2}$) per min per mg of protein] in whole-cell extracts was measured in cells grown under control (bars C) conditions or subjected to 4 or 6 hr of hypoxia (bars H4 or H6, respectively) or 1 hr of heat stress (bars HS), followed by 4 hr of recovery. CAT activity was inducible by either stress while linked to a native HSE (Δ5N/-105) but was uninducible when driven by a promoter bearing a 4-base mutation within the HSE (LSPN). Differences in group means were compared by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$ vs. control).

as compared to background levels observed under nonstressed conditions or during hypoxia in cells transfected with the construct (LSPN) bearing the mutant HSE (data not shown). However, CAT activity was unaffected by hypoxia (96% of control) in cells transfected with the CAT gene under the control of the simian virus 40 early promoter/enhancer (pSV2CAT).

DISCUSSION

Previous studies have demonstrated induction of HSP70 gene expression by ischemia in tissues of intact animals (4) and by hypoxia in cultured cells (29). However, the biochemical events that lead to this induction and their degree of similarity with pathways triggered by heat stress have not been defined.

Our current findings indicate that hypoxia stimulates DNA binding activity of HSTF in cultured myogenic cells through mechanisms that are independent of new protein synthesis. The HSE-binding activity that is induced by hypoxia appears to be identical to HSTF induced by heat shock, as determined by comparison of mobility in gel-retention assays and sequence specificity of DNA binding. Activation of HSTF in hypoxic cells is temporally associated with induction of endogenous HSP70 gene transcription and with induction of a heterologous reporter gene controlled by the human HSP70 promoter. Further, induction of the human HSP70 promoter requires an intact HSE, indicating that other cis-acting transcriptional control elements [e.g., serum response element (SRE)] contained within this complex promoter (9, 13) are not sufficient to transduce signals generated within hypoxic cells.

These findings provide strong evidence that hypoxia and heat shock induce expression of the HSP70 gene by similar, if not identical, mechanisms. The most notable difference between heat shock and hypoxia in this study concerned the time course of activation of HSTF binding and HSP70 gene expression after the onset of the stress condition. These responses were rapid during heat stress (<20 min) but occurred only after considerable delay (2 hr) during hypoxia. To understand the basis for this temporal difference, it will be necessary to explore the biochemical events that result in activation of HSTF by each type of stress.

Despite considerable study, the proximate stimuli that result in activation of HSTF by elevated temperature are incompletely understood. In fungi, HSTF is bound constitutively to its cognate binding site, even under nonstressed conditions, and stress inducibility of transcription is attributable to events distinct from DNA binding. In unstressed mammalian cells, HSTF is present in an inactive form that does not bind DNA. In response to heat stress, the DNA binding activity of a preexistent pool of HSTF is unmasked. Transcriptional activation of heat-responsive promoters requires DNA binding of HSTF, but recent data indicate that DNA binding and transcriptional activation may be separable and independently regulated functions of mammalian, as well as fungal, HSTF (30). Activation of HSTF by heat shock has been attributed both to direct temperature-dependent conformational changes in HSTF itself or in other proteins complexed to HSTF and to heat-inducible pathways of posttranslation modification, including, but probably not limited to, protein kinases and phosphatases for which HSTF is a substrate (31). Different states of phosphorylation of HSTF may account for the multiple bands observed in gel-retention assays of extracts from hypoxic cells (Fig. 3*A*). However, there was no consistent relationship between the type of stress (hypoxia vs. heat) or the duration of stress exposure and the presence of multiple forms of stress-inducible DNA-binding activity.

Hypoxic activation of HSTF could result from several potential mechanisms that are not mutually exclusive. Changes in cellular energy charge or redox potential due to diminished oxidative metabolism may destabilize the structure of certain proteins (32), possibly HSTF itself, and trigger the same pathway(s) induced by heat shock. Such conformational changes could occur directly through allosteric effects of nucleoside phosphates. In addition, ATP depletion may alter the activity of membrane ion channels, resulting in changes in the cytoplasmic concentrations of divalent or monovalent cations, with subsequent effects on the conformation of HSTF. This latter mechanism is supported by recent evidence that DNA binding of HSTF in HeLa cell extracts can be activated *in vitro* by calcium or hydrogen ions (33). Alternatively, proteins involved in HSTF activation may be directly sensitive to changes in oxygen tension, perhaps by variation in the oxidation status of heme groups. These hypotheses have experimentally testable predictions that can be tested using both cell culture models and cell-free systems.

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