

# Activation of Heat Shock Transcription Factor 3 by c-Myb in the Absence of Cellular Stress

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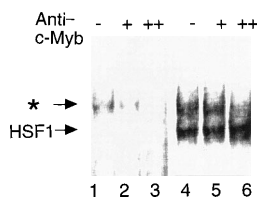
In vertebrates, the presence of multiple heat shock transcription factors (HSFs) indicates that these factors may be regulated by distinct stress signals. HSF3 was specifically activated in unstressed proliferating cells by direct binding to the *c-myc* proto-oncogene product (c-Myb). These factors formed a complex through their DNA binding domains that stimulated the nuclear entry and formation of the transcriptionally active trimer of HSF3. Because c-Myb participates in cellular proliferation, this regulatory pathway may provide a link between cellular proliferation and the stress response.

Transcriptional induction of heat shock genes in response to stress is mediated by HSF that binds to the heat shock element (HSE) (1). Members of the HSF gene family contain a conserved DNA binding domain, an extended hydrophobic repeat (HR-A/B) involved in trimerization, and a transactivation domain (2). HSF is thought to exist in cells as an inactive monomer that acquires high DNA binding affinity by trimerization after exposure to stress (2–4). The COOH-terminal hydrophobic repeat (HR-C) suppresses trimer formation by interaction with the NH<sub>2</sub>-terminal hydrophobic repeats (5). Vertebrates express multiple HSFs (6) whose functions are largely unknown. HSF1 and HSF3 are activated by exposure to different heat shock temperatures (4, 7, 8), and HSF2 appears to be regulated by distinct environmental and developmental cues (9). We previously found that c-Myb can stimulate transcription of the *hsp70* gene through HSE, although c-Myb itself does not bind directly to this sequence (10). Because heat shock protein gene expression is itself growth-regulated (11) and heat shock proteins and molecular chaperones are essential for the activities of proteins involved in proliferation and apoptosis (12), we expected that further analyses of the HSE-dependent activation by c-Myb would shed light on the uncharacterized link between the stress response and proliferation control. Here, we demonstrate that HSF3 can be activated in unstressed

proliferating cells by direct binding to the *c-myc* proto-oncogene product (c-Myb).

We first examined whether c-Myb is involved in the protein complex that binds to HSE. In gel mobility shift assays with the HSE probe and whole cell extracts (WCEs) from heat-shocked Molt-4 cells, a slower migrating complex was detected in addition to the expected HSF1:HSE complex (Fig. 1). This slower migrating complex was eliminated by the addition of c-Myb-specific antibody, suggesting that c-Myb interacts with a HSF.

c-Myb activates transcription through the HSE in CV-1 cells but not in NIH 3T3 cells (10). Cotransfection of HSF1 or HSF2 into NIH 3T3 cells did not stimulate the activity of the HSE-containing promoter in the presence or absence of c-Myb; however, expression of HSF3 restored c-Myb responsiveness (Fig. 2). This effect was sensitive to the amount of exogenous HSF3; large amounts of HSF3 resulted in increased basal expression and attenuation of the positive effect of c-Myb. Consistent with our previous results (10), the c-Myb mutant lacking the activation domain enhanced HSF3-mediated transcription. The small degree of enhancement of HSF3 activity by c-Myb in



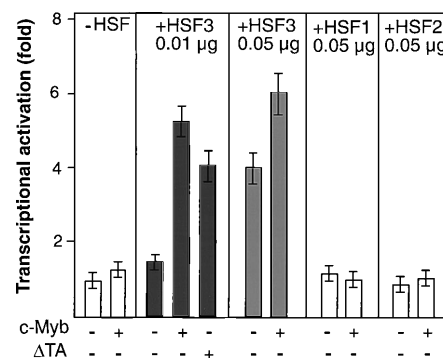
**Fig. 1.** Presence of c-Myb in the HSE:protein complex. A gel retardation assay with a HSE probe and WCEs from unstressed (lanes 1 to 3) and heat-shocked (lanes 4 to 6) Molt-4 cells was performed (20). WCEs were incubated with 0.1 (+) or 1  $\mu$ l (++) of c-Myb-specific monoclonal antibody MAb1.1 (anti-c-Myb) (21) or normal immunoglobulin G (-). The complex that was eliminated by c-Myb-specific antibody is indicated by an asterisk.

NIH 3T3 cells could be due to a negative regulator that binds to c-Myb and HSF3.

In vitro binding experiments revealed that c-Myb selectively bound to the glutathione-S-transferase (GST)-HSF3 fusion protein and not to GST-HSF1 or GST-HSF2 (Fig. 3A). The domain of c-Myb that was previously shown to interact with DNA was also necessary for interaction with HSF3; two of the three imperfect tandem repeats of 51 to 53 amino acids in the DNA binding domain (13), R2 and R3, were required for binding (Fig. 3B). The V103L mutation [valine (V) at position 103 is mutated to leucine (L)], which abrogates the conformational flexibility in R2 (14), did not affect binding to HSF3, whereas the leucine zipper mutation in the negative regulatory domain of c-Myb (15) decreased binding, suggesting that the leucine zipper region enhances the interaction with HSF3. The domain previously shown to interact with DNA, located in the NH<sub>2</sub>-terminal region of HSF3, was necessary for interaction with c-Myb (Fig. 3C).

To examine binding in vivo, we generated chimeric proteins in which the DNA binding domains of HSF3 and c-Myb were fused in frame to the DNA binding domain of Gal4 and the transcriptional activation domain of VP16, respectively, and transcriptional activation in NIH 3T3 cells was examined (Fig. 3D). The Myb-VP16 fusion protein stimulated Gal4-HSF3 activity about 6.8-fold, whereas VP16 alone had no effect. This effect appeared to be relatively specific because stimulation of Myb-VP16 by Gal4-HSF1 or Gal4-HSF2 was only 2.2- or 1.8-fold, respectively.

In the presence of c-Myb, HSF3 localized to the nucleus as intense areas of punc-



**Fig. 2.** HSE-dependent transactivation by c-Myb mediated by HSF3. Chloramphenicol acetyltransferase (CAT) assays were performed after the transfection of NIH 3T3 cells with a mixture of DNA containing the HSE-containing CAT reporter plasmid, the plasmid to express either wild-type c-Myb or a c-Myb mutant lacking the activation domain ( $\Delta$ TA), and the indicated amount of HSF1, HSF2, or HSF3 expression plasmid (22). Data represent means  $\pm$  SEM.

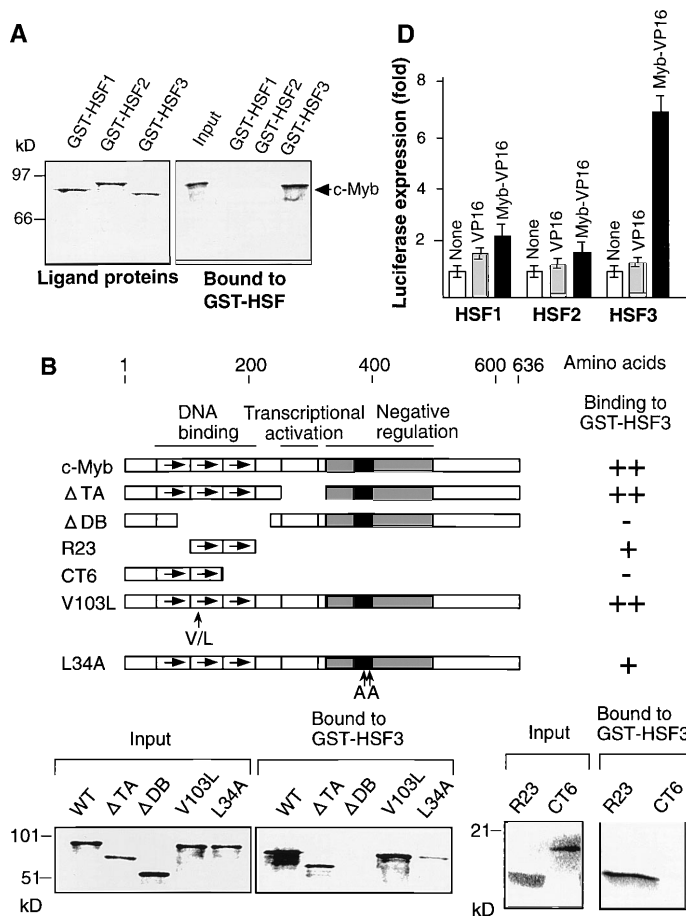
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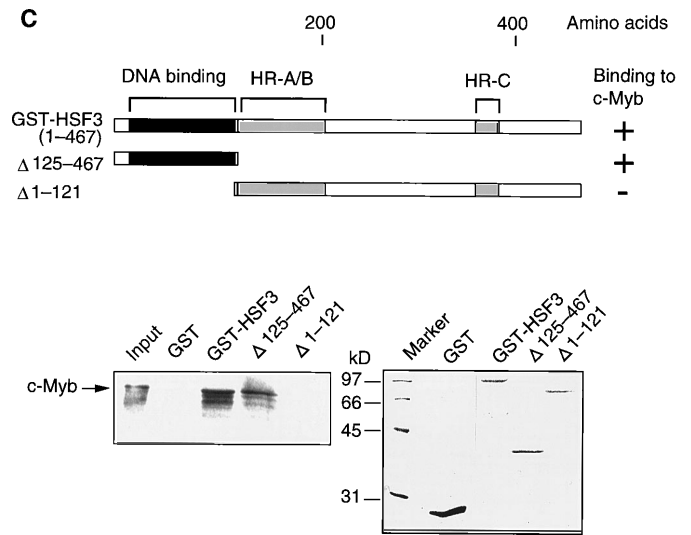
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**Fig. 3.** Interaction between HSF3 and c-Myb. (A) Comparison of binding between HSFs 1, 2, or 3 and c-Myb. (Left) GST-HSF proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. (Right) [<sup>35</sup>S]c-Myb that bound to GST-HSF was analyzed (23). (B) Deletion analysis of c-Myb. The derivatives of c-Myb are shown (A, Ala), and results of in vitro binding assays are below. (+ +), (+), and (-) indicate the binding of 33 to 40%, 9 to 16%, and less than 1% of the input protein, respectively. (C) Deletion analysis of HSF3. (Lower left) The amount of [<sup>35</sup>S]c-Myb that bound to GST-HSF3 was analyzed. (Lower right) GST-HSF3 proteins were analyzed by SDS-PAGE and Coomassie staining. (D) In vivo two-hybrid interaction. NIH 3T3 cells were cotransfected with the luciferase reporter plasmid containing the Gal4-binding sites, the expression plasmid for the Gal4-HSF fusion protein, and the expression plasmid for the c-Myb-VP16 fusion protein or VP16 alone (24). Degree of activation is indicated (means ± SEM).



**Fig. 4.** Activation of HSF3 by c-Myb in unstressed cells. (A) Effect of c-Myb on the cellular localization of HSF3. Immunostaining of HSF3 was performed with 293T cells transfected with the plasmids to express the indicated proteins (25). (B) Protein immunoblot of HSF3. 293T cells were transfected with plasmids to express the designated proteins, and HSF3 expression was examined by protein immunoblot (25). (C) Induction of HSF3 DNA binding activity by c-Myb. Gel retardation assays were performed with a HSE probe and WCEs from 293T cells transfected with plasmids that expressed the designated proteins (25). WCEs were incubated with no antibody (lanes 1 to 4), the HSF3-specific antibody (8) (lane 5), the c-Myb-specific monoclonal antibody MAb5.1 (lane 6), or normal mouse immunoglobulin G as a control (lane 7). Arrows indicate specific protein-DNA complexes. The c-Myb-specific complex is denoted by an asterisk. Multiple bands of HSF3-HSE complex are visible as previously reported (6, 8).

stress. c-Myb protein is highly expressed during the G<sub>1</sub> to S transition of the cell cycle and is required for the G<sub>1</sub> to S transition and maintenance of the proliferative state (17). The expression of the gene *hsp70*

tate staining, whereas in the absence of c-Myb, HSF3 was uniformly distributed throughout the nucleus (Fig. 4A). A similar pattern of punctate staining of activated HSF was observed for *Drosophila* HSF in Schneider cells and polytene nuclei (16) and for HSF1 in stressed HeLa cells (4). Nuclear granules correlate with HSF trimerization and activation (4, 16), suggesting that c-Myb affects the subnuclear localization and may affect trimerization and activation of HSF3. The increased detection of nuclear localized HSF3 staining in the presence of c-Myb was not accompanied by an increased total amount of HSF3 protein (Fig. 4B). This could be due to failure to detect the inactive form of HSF3 in the cytosol with the antibody to HSF3, which was raised to the activation domain in the COOH-terminus. Alternatively, c-Myb may enhance the nuclear translocation of HSF3.

Although WCE from cells transfected with either plasmid exhibited almost no HSE-binding activity, the WCE from cells expressing both HSF3 and c-Myb gave rise to multiple slowly migrating bands (Fig. 4C). Further shifting of these bands after incubation with specific antibodies indicated that the lower multiple bands contained HSF3 and the upper one band contained

both HSF3 and c-Myb. c-Myb and HSF3 interact directly through their DNA binding domains, resulting in the activation of HSF3, which is normally inactive in the absence of cell

is also induced at the G<sub>1</sub>-S transition in non-heat-shocked cells (11). Our results raise the possibility that the c-Myb-induced activation of HSF3 contributes to the cell cycle-dependent expression of stress-responsive genes.

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- Preparation of WCEs from Molt-4 human T cells and gel retardation assays were done as described (18). A double-stranded oligonucleotide derived from the distal HSE of the human *hsp70* promoter (10) was used as a probe. HSE-containing complexes were analyzed on 3.2% polyacrylamide gels. Antibodies were preincubated with WCEs for 50 min on ice before initiation of the binding reaction.
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- The plasmids to express chicken HSF1, HSF2, and HSF3 were constructed by inserting the corresponding cDNAs downstream of the chicken cytoplasmic  $\beta$ -actin promoter. A mixture containing 4  $\mu$ g of the HSE-containing CAT reporter plasmid pA10CATdHSE2, various amounts of HSF expression plasmid, 4  $\mu$ g of the c-Myb expression plasmid, and 2  $\mu$ g of pRSV- $\beta$ -gal was transfected into NIH 3T3 cells. The total amount of plasmid DNA was adjusted to 18  $\mu$ g by the addition of pact-1 DNA. CAT assays were performed as described (10).
- The preparation of GST-HSF fusion proteins, in vitro translation of c-Myb, and binding assays were as described (19) except for the binding buffer used [20 mM Hepes (pH 8.5), 1 mM EDTA, 5 mM dithiothreitol, and 0.1% NP-40], which contained 70 or 150 mM NaCl (for Fig. 3A).
- We constructed the plasmid to express the Gal4-HSF fusion proteins containing the Gal4 DNA binding domain (amino acids 1 to 147) joined to either the DNA binding domain of HSF1 (amino acids 1 to 132), HSF2 (amino acids 1 to 130), or HSF3 (amino acids 1 to 125), by the polymerase chain reaction-based method with the use of the cytomegalovirus promoter-containing expression vector. The plasmid to express the Myb-VP16 fusion protein containing the DNA binding domain of c-Myb (amino acids 1 to 193) joined to the VP16 activation domain was made similarly with the use of the pcDNA3 vector (Invitrogen). In the reporter plasmid, the thymidine kinase promoter containing three copies of the Gal4-binding site was linked to the luciferase gene. A mixture containing 4  $\mu$ g of the luciferase reporter plasmid, 2  $\mu$ g of the Gal4-HSF expression plasmid, 8  $\mu$ g of either the VP16-Myb or VP16 expression plasmid, and 1  $\mu$ g of pact- $\beta$ -gal was transfected into NIH 3T3 cells, and luciferase assays were performed. Total plasmid DNA was adjusted to 17  $\mu$ g by the addition of pact-1 DNA.
- The 293T cells were transfected with either 0.3  $\mu$ g of the chicken HSF3 expression plasmid or 8  $\mu$ g of the mouse c-Myb expression plasmid, or a mixture of both plasmids. The transfected DNA also contained 0.5  $\mu$ g of pact- $\beta$ -gal DNA (10), and the total amount of plasmid DNA was adjusted to 10  $\mu$ g by the addition of pact-1 DNA (10). Immunostaining and protein immunoblotting of HSF3 were performed as described (8).
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## Influence of Food Web Structure on Carbon Exchange Between Lakes and the Atmosphere

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Top predators and nutrient loading in lakes were manipulated to assess the influence of food web structure on carbon flux between lakes and the atmosphere. Nutrient enrichment increased primary production, causing lakes to become net sinks for atmospheric carbon (C<sub>atm</sub>). Changes in top predators caused shifts in grazers. At identical nutrient loading, C<sub>atm</sub> invasion was greater to a lake with low grazing than to one with high grazing. Carbon stable-isotope distributions corroborated the drawdown of lake carbon dioxide and traced C<sub>atm</sub> transfer from algae to top predators. Thus, top predators altered ecosystem carbon fixation and linkages to the atmosphere.

In many lakes, carbon (C) inputs from terrestrial systems are sufficiently high that lakes are supersaturated with CO<sub>2</sub>, and there is net diffusion of CO<sub>2</sub> out of surface water, making lakes conduits of C from the terrestrial environment to the atmosphere (1–3). In productive lakes, primary production by algae and C storage in biota and sediments are high, so that aqueous CO<sub>2</sub> is depleted and C<sub>atm</sub> diffuses into surface waters (2, 4).

Primary production by algae in lakes is determined by interactions among a variety of factors that include nutrient loading (5) and food web structure (6). In general, primary production is high in lakes with large nutrient loads. Food web structure is often determined by the feeding characteristics of

fishes. Lakes with planktivorous fishes are generally characterized by small bodied zooplankton grazers (7) that are less effective at suppressing algal abundance and growth than are communities dominated by large bodied grazers that often coexist with piscivorous fishes (6). Thus, food web structure as determined by the dominant feeding modes of predatory fishes has the potential to regulate aquatic primary production and C fluxes between lakes and the atmosphere.

We independently manipulated fish communities and nutrient loading rates in four lakes to test the interactive effects of nutrient loading and food web structure on lake productivity and C exchange with the atmosphere (Table 1). Two lakes were dominated by zooplanktivorous fishes (minnows), and two by piscivores (bass) (8). From 1993 to 1995, one lake from each food web configuration was enriched with nitrogen and phosphorus, and the two other (reference) lakes were monitored without fertilization (Table 1).

At ambient nutrient loads, mean summer primary production rates by pelagic algae ranged from 170 to 414 mg C·

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