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DIFFERENTIAL EFFECT OF 1-(5-ISOQUINOLINESULFONYL)-2-METHYLPIPERAZINE (H-7) ON αB-*crystallin* AND *hsp70* GENE EXPRESSION IN MURINE CELL LINES

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Abstract—We studied the effect of isoquinolinesulfonamide derivatives (H-7, H-8, and HA1004) on the expression of two heat shock genes (αB-*crystallin* and *hsp70*) in NIH 3T3 and Swiss 3T3 cells after heat shock at 45° for 10 min. Western blots and northern blots showed that H-7 effectively suppressed the accumulation of HSP70 and αB-crystallin mRNA as well as the synthesis of their proteins. The degree of suppression was dependent upon the concentration of the drug. Moreover, the expression of the *hsp* genes was differentially suppressed by H-7. The expression of the αB-*crystallin* gene was more effectively inhibited than that of the *hsp70* gene by H-7. Nuclear run-on assay demonstrates that this difference was due to the differential effect of H-7 on the elongation of transcription of different *hsp* genes.

Key words: H-7; αB-crystallin; hsp70; elongation of transcription

Cells respond to variety of stresses by turning on certain genes, such as heat shock genes [1]. Consequently, a number of specific proteins that are generally referred to as HSPs‡ accumulate in the cells [2–4]. The HSPs can be subdivided into a number of groups such as the high molecular weight HSPs (HSP70, HSP90, and HSP110) and the low molecular weight HSPs (HSP27, HSP25, and αB-crystallin). Although these proteins have similar physiological and biochemical functions, e.g. acting as molecular chaperones [5–8], they are regulated independently [9, 10]. Recently, several researchers reported that the isoquinolinesulfonamide derivatives H-7 or H-8 selectively inhibit heat shock gene expression in mammalian cells [11–13]. It is interesting to study whether H-7 or H-8 differentially inhibits different members of HSP groups. For this study, we chose HSP70 from the high molecular weight HSPs and αB-crystallin from the low molecular weight HSPs, because these proteins, when compared with other HSPs, are prominently induced by heat shock in murine cell lines [14, 15]. We observed that the synthesis of αB-crystallin was inhibited more effectively than that of HSP70 by treatment with H-7 and H-8. Moreover, our data from gel retardation assay, nuclear run-on assay, and northern blots suggest that the drug differentially affects the downstream process of transcriptional regulation rather than the binding activity of transcription factor to the promoter of the genes.

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‡ Abbreviations: HSPs, heat shock proteins; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; HA1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TCA, trichloroacetic acid; HSF, heat shock transcription factor; HSE, heat shock element; CTD, carboxyl-terminal domain; Pol IIA, RNA polymerase IIA; Pol IIO, RNA polymerase IIO; HSP70, 70 kDa heat shock protein; HSP90, 90 kDa heat shock protein; HSP110, 110 kDa heat shock protein; HSP27, 27 kDa heat shock protein; HSP25, 25 kDa heat shock protein; and 1× SSC, 150 mM NaCl, 15 mM $Na_3C_6H_5O_7$.

MATERIALS AND METHODS

Two types of exponentially growing murine cells (Swiss 3T3, and NIH 3T3) were cultured in McCoy's 5a medium (Cellgro). The medium was supplemented with 26 mM sodium bicarbonate and 10% iron-supplemented calf serum (HyClone). T-75 flasks or 35-mm petri dishes containing cells were kept in a 37° humidified incubator with a mixture of 95% air and 5% CO_2.

For hyperthermic treatment, T-75 flasks or 35-mm petri dishes containing cells were heated by total immersion in a circulating water bath (Heto) maintained within ±0.05° of the desired temperature.

Actinomycin D and isoquinolinesulfonamide derivatives (H-7, H-8, and HA1004) were obtained from the Sigma Chemical Co. Drug treatment was accomplished by replacing the culture medium with drug-containing medium or adding a drug from concentrated stock solution into the culture medium. The drug treatment was terminated by aspiration and rinsing with Hanks' balanced salt solution.

For one-dimensional SDS–PAGE and western blot, cells were solubilized with lysis buffer [2.4 M glycerol, 0.14 M Tris (pH 6.8), 0.21 M SDS, 0.3 mM bromophenol blue]. Samples were boiled for 5 min. Protein content was measured with BCA* Protein Assay Reagent (Pierce). The samples were diluted with 1× lysis buffer containing 1.28 M mercaptoethanol. An equal amount of protein (30 μg) was analyzed on a 5% SDS–PAGE for the stacking gel and 10–18% linear gradient SDS–PAGE

for the separating gel. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting with the αB-crystallin polyclonal antibody (J. Horwitz, University of California at Los Angeles) or the HSP70 monoclonal antibody (Amersham). Both antibodies were diluted 1:500. Alkaline phosphatase-conjugated goat-anti-rabbit IgG (diluted 1:500, Zymed) was used to detect αB-crystallin antibody. Alkaline phosphatase-conjugated rabbit-anti-mouse IgG (diluted 1:800; Zymed) was used to detect HSP70 antibody. The antigen–antibody complexes were visualized by a colorimetric reaction.

HSP70 or αB-crystallin mRNA levels were determined by northern blot analysis. Total cellular RNA was extracted by the LiCl-urea method of Tushinski *et al.* [16]. For RNA analysis, 30 μg of total RNA was electrophoresed in a 1% agarose-formaldehyde gel [17]. The RNA was blotted from the gels onto nitrocellulose membranes and baked at 80° for 2 hr in a vacuum oven. Membranes were prehybridized at 42° in 50% formamide, 1× Denhardt's solution, 25 mM KPO_4 (pH 7.4), 5× SSC, and 50 μg/mL denatured and fragmented salmon sperm DNA. Hybridizations were at 42° in prehybridization solution containing 10% dextran sulfate and radiolabeled human *hsp70* cDNA probes (R. Morimoto, Northwestern University), mouse *αB-crystalline* specific 29-mer double-stranded synthetic oligonucleotide (5′-AGCCTGCTGTCGCCGCAGCCCCTAAGAAG-3′), or *GAPDH* cDNA (American Type Culture Collection) at a concentration of 1.5×10^6, 4×10^6, or 1×10^6 cpm/mL, respectively. The *hsp70* and *GAPDH* cDNA probes were labeled with ^{32}P by using the Prime-It®II random primer kit (Stratagene Inc.). The *αB-crystalline* oligonucleotide was labeled with ^{32}P by Klenow fill-in reaction. The fill-in reaction was set up in a microcentrifuge tube containing 200 ng of oligonucleotide, 20 μL of 5× LB solution [0.2 M Tris (pH 8.0), 25 mM $MgCl_2$, 0.3% β-mercaptoethanol, 90 μM dTTP, 90 μM dGTP, 1 M 4-morpholinepropanesulfonic acid (MOPS, pH 6.6)], 5 μL of [^{32}P]dATP (sp. act. 3000 Ci/mmol, ICN), 5 μL of [^{32}P]dCTP (sp. act. 3000 Ci/mmol, ICN), and 15 U Klenow (Promega). Post-hybridization, blots were washed in 2× SSC for 15 min at room temperature, washed once in 0.5× SSC and 0.1% SDS for 25 min at 50°, and washed twice in 0.2× SSC and 0.1% SDS for 1 hr at 50°. Blots were placed into a stainless steel cassette with Kodak X-Omatic intensifying screen and autoradiographed on Fuji RX X-ray film.

Conditions for the gel mobility-shift assay, a description of the ^{32}P-labeled HSE oligonucleotide, and preparation of whole-cell extracts were as published previously [18]. A double-stranded HSE oligonucleotide of the mouse *hsp70* gene promoter (5′-GACGCGAAACTGCTGGAAGATTCCTGG-3′, [19]) or the mouse *αB-crystallin* gene promoter (5′-TGACCTCACCATTCCAGAAGCTTCAGAAGACTG-3′, [15]) was used. The HSE oligonucleotide was labeled with ^{32}P Klenow fill-in reaction as described above. Binding reactions with 20 μg of whole-cell extracts were performed for 15 min at 25° in a final volume of 25 μL of binding buffer [10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol] containing about 0.5 ng radiolabeled probe, 10 μg yeast tRNA (Boehringer Mannheim), 1 μg *Escherichia coli* DNA (Sigma Chemical Co.), 10 μg poly(d[I–C]) (Pharmacia LKB), and 50 μg BSA (Sigma Chemical Co.). Samples were electrophoresed on a nondenaturing 4.5% polyacrylamide gel for 2.5 hr at 140 V. After electrophoresis, gels were fixed with 7.5% acetic acid for 15 min and rinsed with water for 3 min and then dried. Autoradiography was carried out at −70° with intensifying screens as described above.

The nuclear run-on assay procedure of Banerji *et al.* [20] was adapted for these experiments. For measurement of elongation of transcription, cells in a T-150 tissue culture flask were washed twice with cold phosphate-buffered saline, scraped, and pelleted by centrifugation at 4° for 5 min at 300*g*. The pellets were resuspended with lysis buffer [10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$] containing 0.5% Nonidet P-40 and set on ice for 5 min. The nuclei were then pelleted by centrifugation for 5 min at 500*g*. The nuclei were resuspended in glycerol storage buffer [50 mM Tris–HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0), 40% glycerol] and stored at −70°. Nascent RNA transcripts were allowed to elongate in reaction buffer [25 mM HEPES (pH 7.5), 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol,

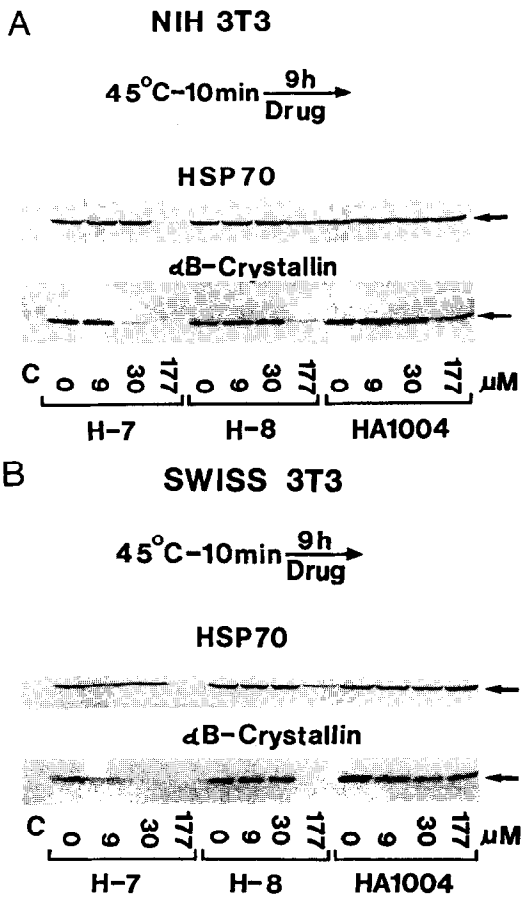

Fig. 1. Western blots with an anti-HSP70 antibody or an anti-αB-crystallin antibody. NIH 3T3 (panel A) or Swiss 3T3 (panel B) cells were heated at 45° for 10 min and immediately treated with various concentrations (0–177 μM) of H-7, H-8 or HA1004 for 9 hr at 37°. The drug concentration is indicated at the bottom of each lane. Lysates containing approximately equal amounts of protein (30 μg) were separated by SDS–PAGE, transferred onto a nitrocellulose membrane and processed for immunoblotting with HSP70 monoclonal antibody (HSP70) or αB-crystallin polyclonal antibody (αB-crystallin). C = lysates from untreated unheated control cells.

5% glycerol, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.1 mCi [^{32}P]UTP (sp. act. 700 Ci/mmol, ICN)] containing approximately 200 μg (50 μL of nuclei) of DNA for 20 min at 26°. Reactions were stopped by the addition of 5 U RNase-free DNase I (Promega) and further incubated for 15 min at 37°. The nuclei were lysed with 3 vol. of SDS/urea buffer [2% SDS, 7 M urea, 0.35 M NaCl, 1 mM EDTA, 10 mM Tris–HCl (pH 8.0), 0.5 mg proteinase K/mL] and incubated for 1 hr at 45°. Then cold TCA (20%) and tRNA (100 μg) were added into the lysate, and the samples were incubated for 30 min at 4° and then centrifuged at 5000*g* for 15 min. The pellet was washed twice with cold ethanol to remove any trace of TCA and then resuspended in hybridization buffer [50% formamide, 5× Denhardt solution, 6× SSC, 0.2% SDS, 100 μg/mL tRNA]. For RNA–DNA dot hybridization, plasmids containing the *hsp70* cDNA gene (pH 2.3; [21]), or the *αB-crystallin* genomic DNA gene (pRD8; [22]) were linearized by restriction enzyme (*Pst*I or *Hind*III) digest, denatured by 0.1 N NaOH, and neutral-

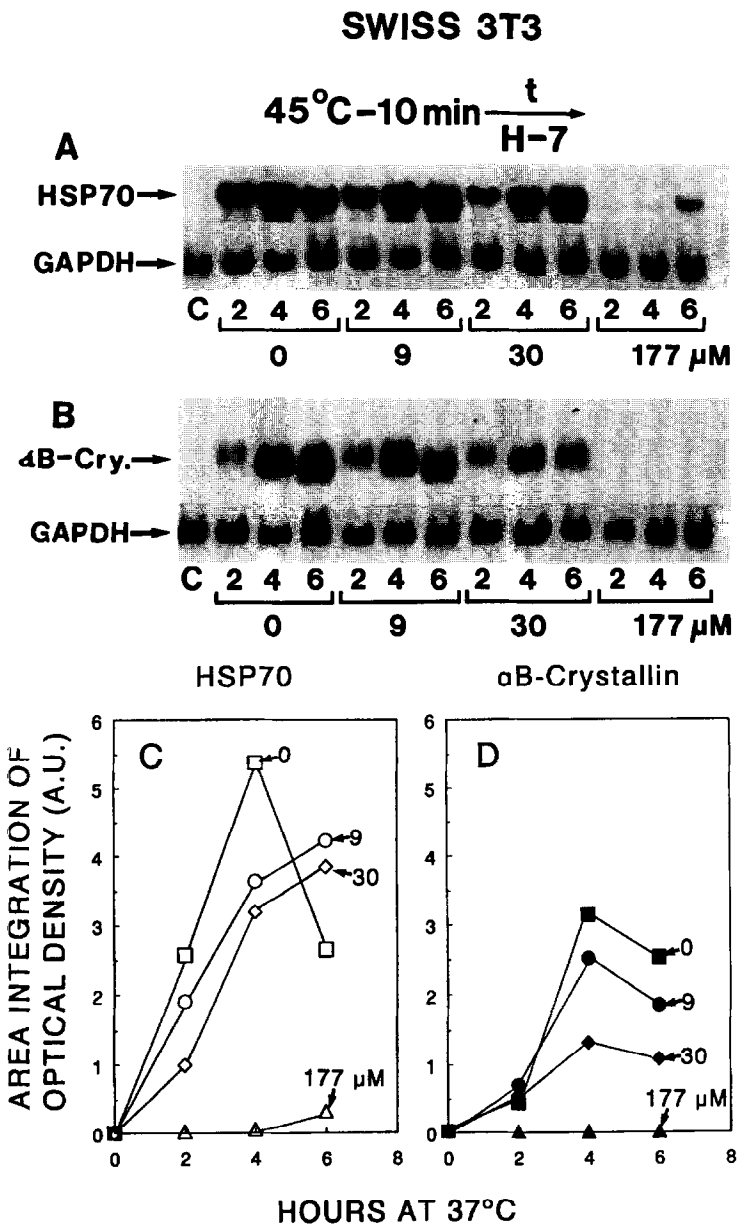

Fig. 2. Effect of H-7 on accumulation of HSP70 mRNA or αB-crystallin mRNA. Swiss 3T3 cells were heated at 45° for 10 min and incubated at 37° for various times (2–6 hr) indicated at the bottom of each lane. Various concentrations of H-7 (0–177 μM) were added immediately after heat shock and left on during incubation at 37°. RNA was isolated, separated, and then hybridized with HSP70 cDNA or αB-crystallin cDNA fragments. Hybridization of mRNA from cytoplasm to *hsp70* gene (panel A) or *αB-crystallin* gene (panel B) was autoradiographed, and analyzed with a densitometer (panels C and D). Differences in RNA loading were examined by rehybridizing the nitrocellulose membranes with the housekeeping gene, *GAPDH* cDNA. Panels A and B: the locations of the HSP70 mRNA (HSP70), αB-crystallin mRNA (αB-Cry.), or GAPDH mRNA (GAPDH) are identified by arrows. C = RNA from unheated cells. Panels C and D: each number next to a symbol indicates the drug concentration (μM). A.U. = arbitrary unit.

ized with 6× SSC. Plasmid DNA was spotted onto nitrocellulose (prewet in distilled water) using a Bio-Dot microfiltration apparatus (Bio-Rad) and washed with 6× SSC. The nitrocellulose was air-dried, and baked under vacuum at 80° for 2 hr. For hybridization, blots were preincubated with hybridization solution for 1 hr at 42°. The ^{32}P-labeled RNA samples were heated for 10 min at 60° before addition to the hybridization solution. Hybridization was conducted for 72 hr at 42°. After hybridization, blots were washed once in 6× SSC/0.2% SDS at room temperature and twice in 2× SSC/0.2% SDS for 30 min at 65°. Blots were placed into a stainless steel cassette with an intensifying screen and autoradiographed. Quantitation of autoradiographs was carried out by scanning densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA) using area integration.

RESULTS

The western blots in Fig. 1 show the effect of isoquinolinesulfonamide derivatives (H-7, H-8, or HA1004) on the levels of HSP70 and αB-crystallin in NIH 3T3 or Swiss 3T3 cells. The levels of HSP70 and αB-crystallin were not detectable in either unheated cell line (lanes C). Heat shock at 45° for 10 min increased the levels of both proteins (lanes 0). Treatment with H-7 as well as H-8 after heat shock suppressed the levels of both proteins. The level of suppression was dependent upon the drug concentrations. In contrast, HA1004 did not affect the levels of HSP70 and αB-crystallin. H-7 suppressed the levels of both proteins more effectively than H-8 did (lanes 177 at H-7 vs lanes 177 at H-8). Drug treatment also inhibited overall protein synthesis. Total protein synthesis was inhibited 10 or 50% by 30 or 177 μM H-7, respectively. Interestingly, we observed that H-7 or H-8 suppressed the level of αB-crystallin more effectively than that of HSP70 in both cell lines. This differential inhibition of different members of HSP groups by treatment with the drug was also observed in the process of transcription. Northern blots in Fig. 2 demonstrated that the levels of HSP70 mRNA and αB-crystallin mRNA increased rapidly and reached maximal values within 4 hr after heat shock at 45° for 10 min in Swiss 3T3 cells. H-7 delayed and suppressed the accumulation of HSP70 mRNA when the drug was added after heat shock (Fig. 2C), whereas the drug simply suppressed the accumulation of αB-crystallin mRNA (Fig. 2D). The level of suppression was dependent upon the drug concentrations. We also observed that H-7 inhibited the accumulation of αB-crystallin mRNA more effectively than that of HSP70 mRNA. Similar results were observed in NIH 3T3 cells (data not shown). We further investigated the differential effect of H-7 on the accumulation of different members of HSP mRNA by studying (a) stability of HSP mRNAs, (b) elongation of transcription of *hsp* genes, or (c) regulation of transcription at the promoter region of the *hsp* genes.

The effect of 30 μM H-7 on the stability of HSP70 mRNA and αB-crystallin mRNA is shown in Fig. 3. Treatment with 2 μg/mL actinomycin D inhibits mRNA synthesis by 98% [23]. We observed the differences in the decay rates of HSP70 mRNA and αB-crystallin mRNA in the presence of 2 μg/mL actinomycin D; HSP70 mRNA decayed faster. H-7 somewhat facilitated the decay of both mRNAs. However, the effect of H-7 on the decay of both mRNAs was not significantly different. In contrast, data from the nuclear run-on assay clearly demonstrated that H-7-induced inhibition of the elongation of αB-crystallin mRNA was much greater than that of HSP70 mRNA (Fig. 4). H-7 (30 μM) inhibited the elongation of HSP70 mRNA or that of αB-crystallin mRNA by 56 or 78%, respectively. Figure 4 also shows that this differential inhibition was virtually identical at 89 μM. However, there was no difference in the inhibition of the two mRNAs at 177 μM. The plot of relative ratios of the elongation of the two mRNAs shows that the percent of inhibition for the HSP70 mRNA was roughly equal both above and below 30 μM

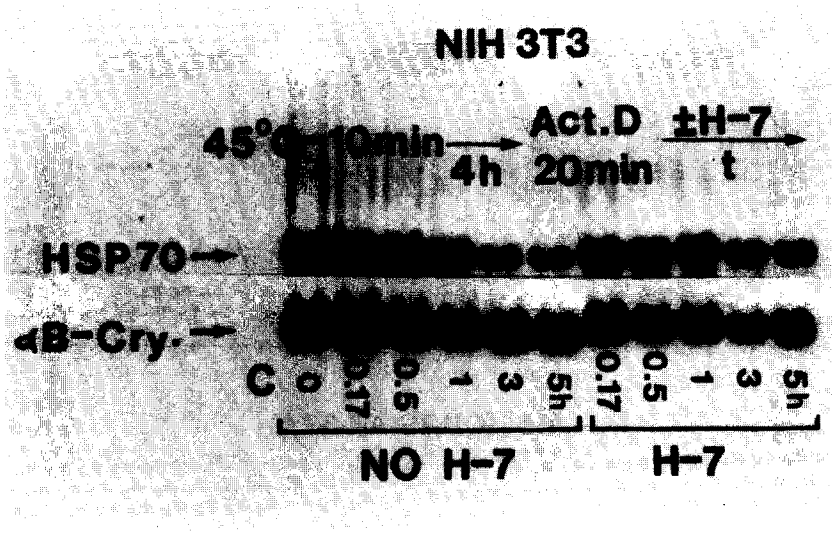

Fig. 3. Effect of 30 μM H-7 on the decay of HSP70 mRNA or αB-crystallin mRNA. NIH 3T3 cells were heated at 45° for 10 min, incubated at 37° for 4 hr, and treated with 2 μg/mL actinomycin D for 20 min. Cells were then treated without (NO H-7) or with 30 μM H-7 (H-7) in the presence of actinomycin D for various times (0–5 hr) indicated at the bottom of each lane. RNA was isolated, separated, and then hybridized with HSP70 cDNA or αB-crystallin cDNA fragments. The locations of the HSP70 mRNA (HSP70), or αB-crystallin mRNA (αB-Cry.) are identified by arrows. C = RNA from unheated cells.

H-7, whereas the majority of the elongation inhibition for αB-crystallin mRNA occurred at H-7 concentrations equal to or below 30 μM (Fig. 4B).

Gel mobility-shift analysis of whole cell extracts from heated cells showed the formation of HSF-*hsp70* or *αB-crystallin*-HSE complex (H in Fig. 5). Little or no HSF binding activity was detected in extracts from cells grown at 37° (C in Fig. 5). The level of heat-induced HSF–HSE complex decreased during incubation at 37°. The dissociation of HSF–HSE complex occurred within 5 hr after heat shock in both *hsp* gene oligonucleotides.

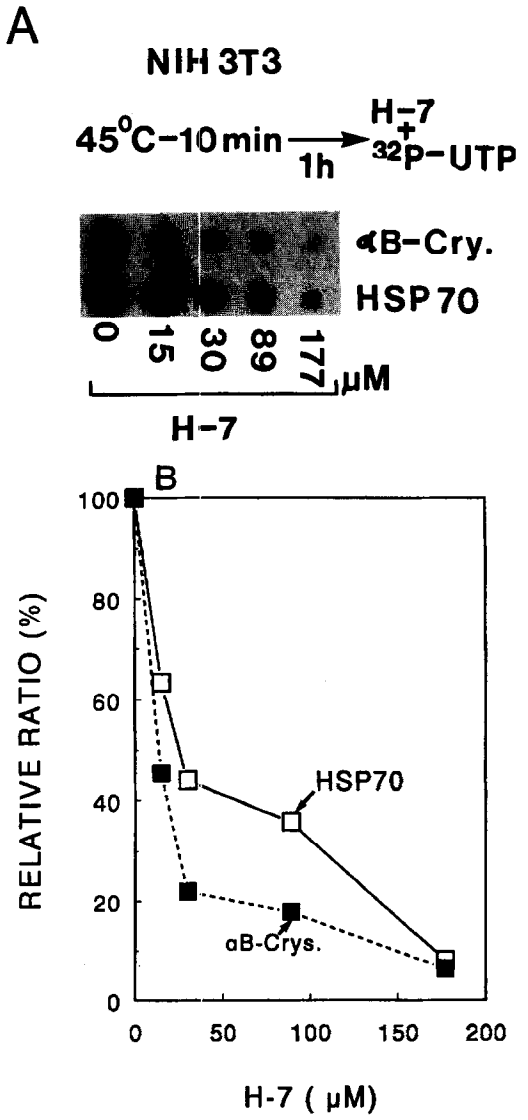

Fig 4. Effect of H-7 treatment on the elongation of HSP70 and αB-crystallin mRNA. NIH 3T3 cells were heated at 45° for 10 min, and incubated at 37° for 1 hr. After incubation, nuclei were isolated and labeled with [^{32}P]UTP for 20 min in the presence of various concentrations of H-7 (0–177 μM) indicated at the bottom of each dot. Hybridization of [^{32}P]UTP-labeled *in vitro* transcribed RNA from the nuclei to *hsp70* gene or *αB-crystallin* gene was autoradiographed (panel A), and analyzed with a densitometer (panel B). The ratio (%) of the intensity of the H-7-treated sample spot to the intensity of the untreated sample spot is plotted as a function of various concentrations of H-7 for HSP70 mRNA (□) or αB-crystallin mRNA (■).

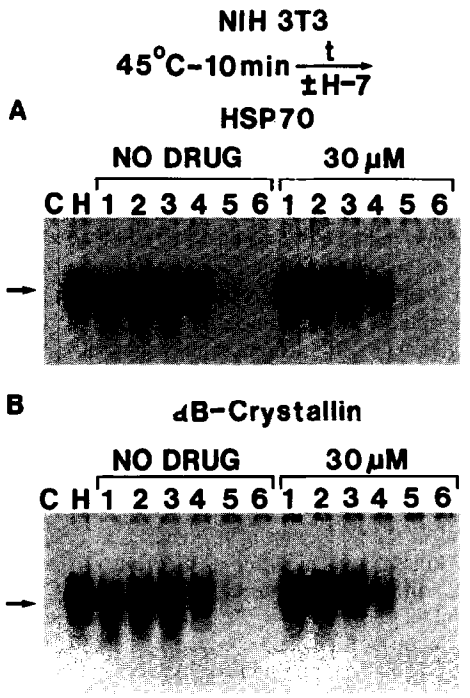

Fig. 5. Effect of 30 μM on dissociation of the heat shock transcription factor-heat shock element (HSF–HSE) complex. NIH 3T3 cells were heated at 45° for 10 min, and incubated at 37° for various times (1–6 hr) indicated at the top of each lane with or without 30 μM H-7. Aliquots of whole cell extracts containing 20 μg protein were used to assay for binding to [^{32}P]HSP70 heat shock element (A) or [^{32}P]αB-crystallin heat shock element (B) oligonucleotide. C = untreated unheated control cells. H = cells heated at 45° for 10 min. Arrows indicate the position of the HSF–HSE complex.

The dissociation of this complex was not affected by treatment with 30 μM H-7 in either *hsp* gene oligonucleotide.

DISCUSSION

The data presented in this paper demonstrated that H-7-induced differential inhibition of *αB-crystallin* and *hsp70* gene expression occurs at the transcriptional level. Moreover, the drug differentially affected the elongation of transcription of the *hsp* genes. In contrast, H-7 did not affect the dissociation of HSF–HSE complex. These results suggest that downstream regulation of transcription (elongation of transcription) rather than upstream regulation of transcription (dissociation of HSF–HSE complex on the promoter) was mainly affected by treatment with H-7. However, a fundamental question that remains unanswered is how H-7 differentially affects *αB-crystallin* and *hsp70* gene expression. At the present time, we can only speculate on the mechanisms for differential effects of H-7 on the regulation of *hsp* gene transcription. In eukaryotic cells, three different nuclear RNA polymerases (I, II, and III) have been identified, each of which transcribes a different class of genes. Polymerase II, responsible for the synthesis of mRNA, is a multisubunit enzyme [24]. It is composed of two large subunits of molecular weight in excess of 100,000 and a collection of 8–10 smaller subunits. The largest subunit of RNA

polymerase II contains at its COOH terminus multiple repeats of the consensus motif Tyr-Ser-Pro-Thr-Ser-Pro-Ser, designated CTD [24]. Two forms of RNA polymerase II, which are referred to as Pol IIA and Pol IIO, co-exist within the cells. They differ by the extent of phosphorylation in the CTD. Pol IIA and Pol IIO contain hypo- and hyperphosphorylated CTD, respectively. Recent evidence suggests that the Pol IIA interacts with the promoter, transcription factor TFIID, or TATA factor to form a stable preinitiation complex, whereas the Pol IIO form would be generated upon entry into initiation of transcription and subsequently involved in transcript elongation [25, 26]. Recent studies show that basal transcription occurs in the absence of the highly phosphorylated Pol IIO form [27]. In contrast, heat shock genes are transcribed by a mixture of Pol IIA and IIO [28, 29]. During heat shock, the CTD of RNA polymerase II converts from the dephosphorylated IIA form to the phosphorylated IIO form. This conversion process may contribute to the regulation of *hsp* gene [28]. Two distinct CTD kinases, which differ in their nucleotide requirements, differentially phosphorylate CTD [30]. H-7 and H-8 inhibit the activity of CTD kinases, promote dephosphorylation of CTD, and consequently inhibit the activity of CTD kinases, promote dephosphorylation of CTD, and consequently inhibit stress-induced *hsp* gene expression [13, 31]. We speculate that inhibition of CTD by H-7 results from inhibition of protein kinase C. Moreover, our data show that H-7 differentially inhibited the expression of two different *hsp* (*αB-crystallin* and *hsp70*) genes. Since multiple kinases are involved in CTD phosphorylation, a diverse pathway for the phosphorylation of CTD, which has a specific role for the expression of an *hsp* gene, would be a possible explanation. We present this model only as a framework for future experiments. Obviously, further studies at the molecular level are necessary to understand the mechanism of the H-7 effect on elongation of *hsp* gene transcription.

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