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Heat Shock Gene Expression and Cytoskeletal Alterations in Mouse Neuroblastoma Cells

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The cytoskeleton of neuroblastoma cells, clone Neuro 2A, is altered by two stress conditions: heat shock and arsenite treatment. Microtubules are reorganized, intermediate filaments are aggregated around the nucleus, and the number of stress fibers is reduced. Since both stress modalities induce similar cytoskeletal alterations, no thermic denaturation of one or more cytoskeletal components can be involved in this process. Heat shock proteins are induced both by heat and by arsenite. However, cells treated with arsenite synthesize hsp28 which is not detected in heat-treated cells. Synthesis of all hsps is prevented by addition of actinomycin D or cycloheximide. Under these conditions no alterations are observed in the organization of microtubules and intermediate filaments during heat or arsenite treatment. However, these drugs are not able to prevent the rapid loss of stress fibers. A re-formation of the cytoskeleton during the recovery period proceeds within 3 h and is also found to occur in the presence of a protein synthesis inhibitor. These data suggest that reorganization of microtubules and intermediate filaments during a stress treatment requires the synthesis of a new protein(s), probably hsp(s).

Most living cells respond to exposure to temperatures a few degrees above their normal physiological temperature by activation of a small set of genes and preferential synthesis of proteins encoded by these genes: heat shock proteins (hsps) (for reviews see Refs. [1–10]). This response was first described to occur in *Drosophila* [11], and since then it has been observed in several other organisms [12]. Besides a heat treatment, hsp synthesis can also be induced by a variety of agents like sodium arsenite [13–16], ethanol [15], and amino acid analogs [17, 18] (for a summary of inducers see Ref. [8]). In the last few years the understanding of the mechanism of heat shock gene activation has increased considerably [2, 9, 10], but the function of the heat shock response is still not well understood.

We have been studying the heat shock response in cultured mammalian cells. We could show both by immunofluorescence microscopy and by immunoelectron microscopy that heat treatments resulting in hsp synthesis concomitantly induce drastic alterations in the cytoskeleton [19]. These cytoskeletal changes were observed 15–20 min after the temperature elevation and were found to be cell specific.

The mechanism which leads to the cytoskeletal alteration during the heat treatment is not known. Recent studies have shown that calmodulin activation is

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required early in this process [20]. In this paper we have extended this study and have searched for other factors involved in the cytoskeletal reorganization of N2A neuroblastoma cells, which are activated during the heat treatment. A direct involvement of heat on this process could be excluded since arsenite was able to induce similar cytoskeletal changes. The stress-induced changes in microtubules and intermediate filaments were prevented by actinomycin D or cycloheximide, while the rapid loss of stress fibers during both heat and arsenite treatment was not affected by cycloheximide. These results suggest that both RNA and protein syntheses are required for the stress-induced cytoskeletal changes in N2A neuroblastoma cells to occur. However, the subsequent re-formation of the cytoskeleton during a recovery period is independent of newly synthesized proteins.

MATERIALS AND METHODS

Materials. Sodium arsenite, actinomycin D, cycloheximide, and dihydrocytochalasin B were from Sigma (St. Louis, MO); NBD-phallicidin was obtained from Molecular Probes, Inc. (Junction City, OR); nocodazole was obtained from Janssen Pharmaceutica (Beerse, Belgium); and mouse monoclonal anti-β-tubulin and rabbit polyclonal anti-vimentin were characterized as described previously [19, 21].

Cell culture, heat, and arsenite treatment. Mouse neuroblastoma cells, clone Neuro 2A, were grown in 35-mm dishes (Costar, Cambridge, MA) in 2 ml Dulbecco's modified Eagle's medium (DMEM) buffered with 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.4 and supplemented with 10% fetal calf serum (Seromed, Berlin). Heat treatment of the cell was performed by placing the dishes in a water bath, thermoregulated at ± 0.02°C with a circulating Thermomix 1420 (Braun Ag., Melsungen, FRG) as described [19]. Sodium arsenite treatment was done by adding 20 μl from an arsenite stock solution to the culture medium resulting in a final concentration of 75 μ*M*.

Radioisotope labeling and gel electrophoresis. After the appropriate treatments, the culture medium was removed and replaced by DMEM lacking methionine. Subconfluent cell cultures (3×10^4 cell/cm^2) were labeled for 3 h with [^{35}S]methionine (sp act 250 μCi/mol, Amersham, UK) (5–15 μCi/ml). The medium was removed and the cells were washed extensively with cold phosphate-buffered saline (PBS) at pH 7.4. The cells were processed for one-dimensional gel electrophoresis according to Laemmli [22] as described previously [19]. The gels were finally fixed in 40% methanol and 7.5% acetic acid, prepared for fluorography, dried, and exposed to Kodak SB panoramic dental X-ray film at −70°C.

Immunofluorescence. For indirect immunofluorescence studies cells were grown until a confluency of 10%, and labeling of cytoskeletal components was done as described previously [19]. For double-labeling experiments Rhodamine-conjugated goat anti-rabbit (GAR-TRITC) and fluorescein-conjugated goat antimouse (GAM-FITC) (both from Nordic, Tilburg, The Netherlands) were applied. To reduce fading of fluorescein, the mounting solution (10% Mowiol 4–88 (Hoechst, Frankfurt) 10% glycerol and 0.1 *M* Tris–HCl, pH 8.3) was supplemented with 30 m*M* *n*-propylgallate (Sigma) according to Giloh and Sedat [23]. Quantification of cells with an intact cytoskeleton was done as described elsewhere [20].

RESULTS

Effects of Heat and Arsenite Treatment on Protein Synthesis

The changeover in the pattern of protein synthesis in mammalian cells undergoing the stress response is illustrated in Fig. 1. [^{35}S]Methionine incorporation in proteins of control cells is shown in lane *1* of both panels. After heat shock (30 min at 43°C) four additional proteins were synthesized with apparent molecular masses of 100, 84, 70, and 68 kDa (Fig. 1*A*). Neuroblastoma cells treated for 1 h

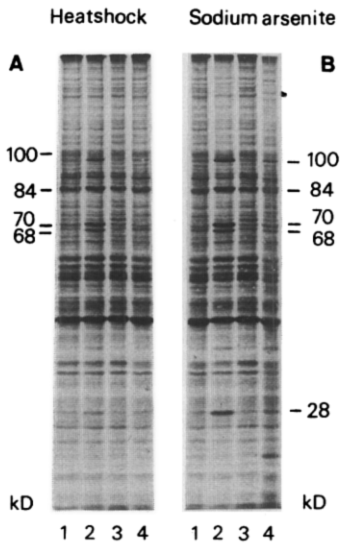

Fig. 1. Protein composition of normal and stressed neuroblastoma cells. Neuroblastoma cells were incubated at 37°C (lane *1*, *A* and *B*) at 43°C for 30 min (*A*) or with 75 μ*M* sodium arsenite for 60 min (*B*). The cells were stressed without drug addition (lane *2*, *A* and *B*), in the presence of 1 μg/ml actinomycin D (lane *3*, *A* and *B*), or in the presence of 3 μg/ml cycloheximide (lane *4*, *A* and *B*). The cells were subsequently labeled for 3 h with [^{35}S]methionine at 37°C and the proteins were separated on a 10% SDS–polyacrylamide gel.

with 75 μ*M* arsenite showed an increased synthesis of five proteins. In addition to the large hsps, the synthesis of a small hsp with an apparent molecular mass of 28 kDa was increased (Fig. 1*B*).

Effect of Heat and Arsenite Treatment on the Cytoskeleton

Treatment conditions used to induce hsp synthesis were also applied for analysis of stress-induced changes in the cytoskeleton of neuroblastoma cells. This cell type remained flat during both stress treatments. Therefore the cytoskeleton of these cells could very well be studied with immunofluorescence microscopy. Microfilaments were stained with NBD-phallicidin and are shown in Fig. 2. As a result of both heat and arsenite treatments, the number of stress fibers was found to be reduced. Microtubules and intermediate filaments were analyzed using double labeling. As shown in Fig. 3, similar alterations in these cytoskeletal systems were observed after both stress treatments. Intermediate filaments were aggregated around the nucleus and, concomitantly, microtubules were reorganized. Most prominent was the disappearance of the microtubule organizing center (MTOC), normally present in the vicinity of the nucleus which was observed after both stress treatments (Fig. 3*A*). Minor differences however were apparant in cell shape changes which were also visible in stress fiber and microtubule patterns. A more complete loss of stress fibers was observed during heat shock. Therefore the loss of cell extensions was more elaborate during the heat treat-

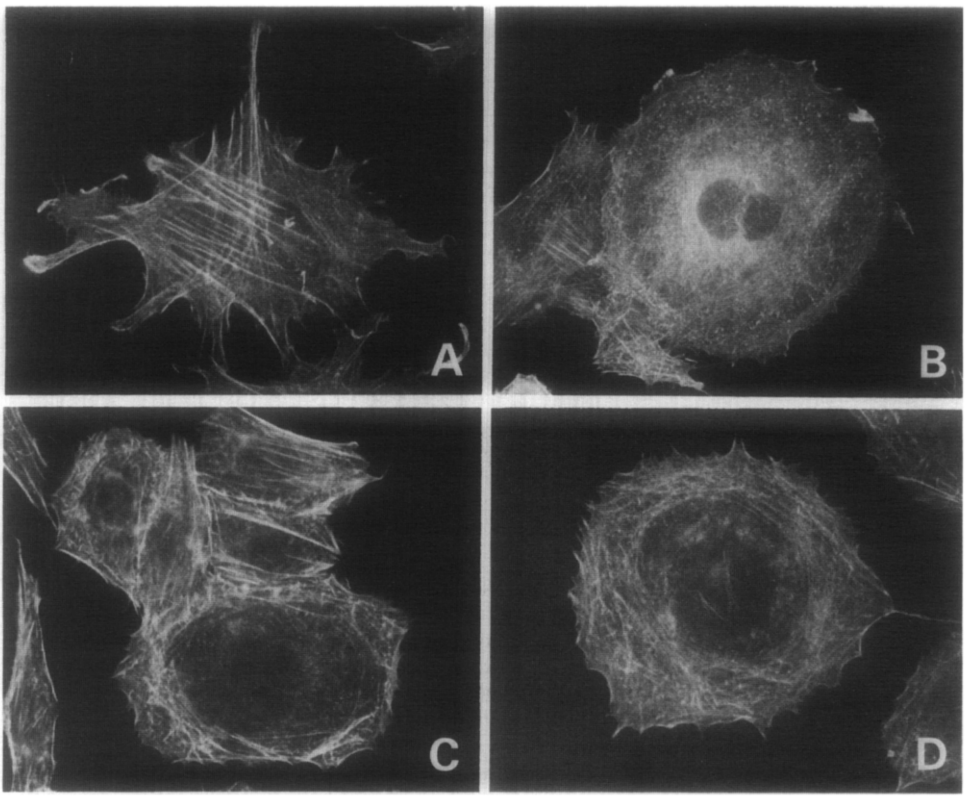

Fig. 2. Stress fiber patterns of neuroblastoma cells. The cells were fixed with 3% paraformaldehyde in PBS, pH 7.4, and permeabilized with 0.1% Triton X-100 in PBS, pH 7.4, for 5 min. The stress fibers were stained with 0.3 μg/ml NBD-phallicidin. Shown are cells grown at 37°C (*A*), cells incubated 30 min at 43°C (*B*), cells incubated 60 min with 75 μ*M* arsenite (*C*), and cells incubated 60 min with 75 μ*M* arsenite in the presence of 3 μg/ml cycloheximide (*D*).

ment and, as a consequence, microtubules became organized more concentrically throughout the cytoplasm.

Effect of Transcription and Translation Inhibitors on the Stress-Induced Alterations in the Cytoskeleton

Cytoskeletal changes as described here were observed 15–20 min after the temperature shift [19]. Since the induction of stress protein synthesis occurs very rapidly, a possible role for hsps in this process was investigated with inhibitors for either transcription or translation. Addition of actinomycin D (1 μg/ml; ACD) 15 min prior to the stress treatment inhibited the induction of hsp synthesis (Fig. 1, lane *3*, both panels). Addition of cycloheximide (3 μg/ml; CHX) 15 min prior to the stress treatment resulted not only in a 90% inhibition of total protein synthesis (data not shown) but also in the inhibition of the induction of hsp synthesis (Fig. 1, lane *4*, both panels). Other control experiments were performed to exclude a direct effect of these inhibitors on the cytoskeleton. As formerly shown by Sharpe *et al.* [24] treatment of mammalian cells with cycloheximide (10 μg/ml)

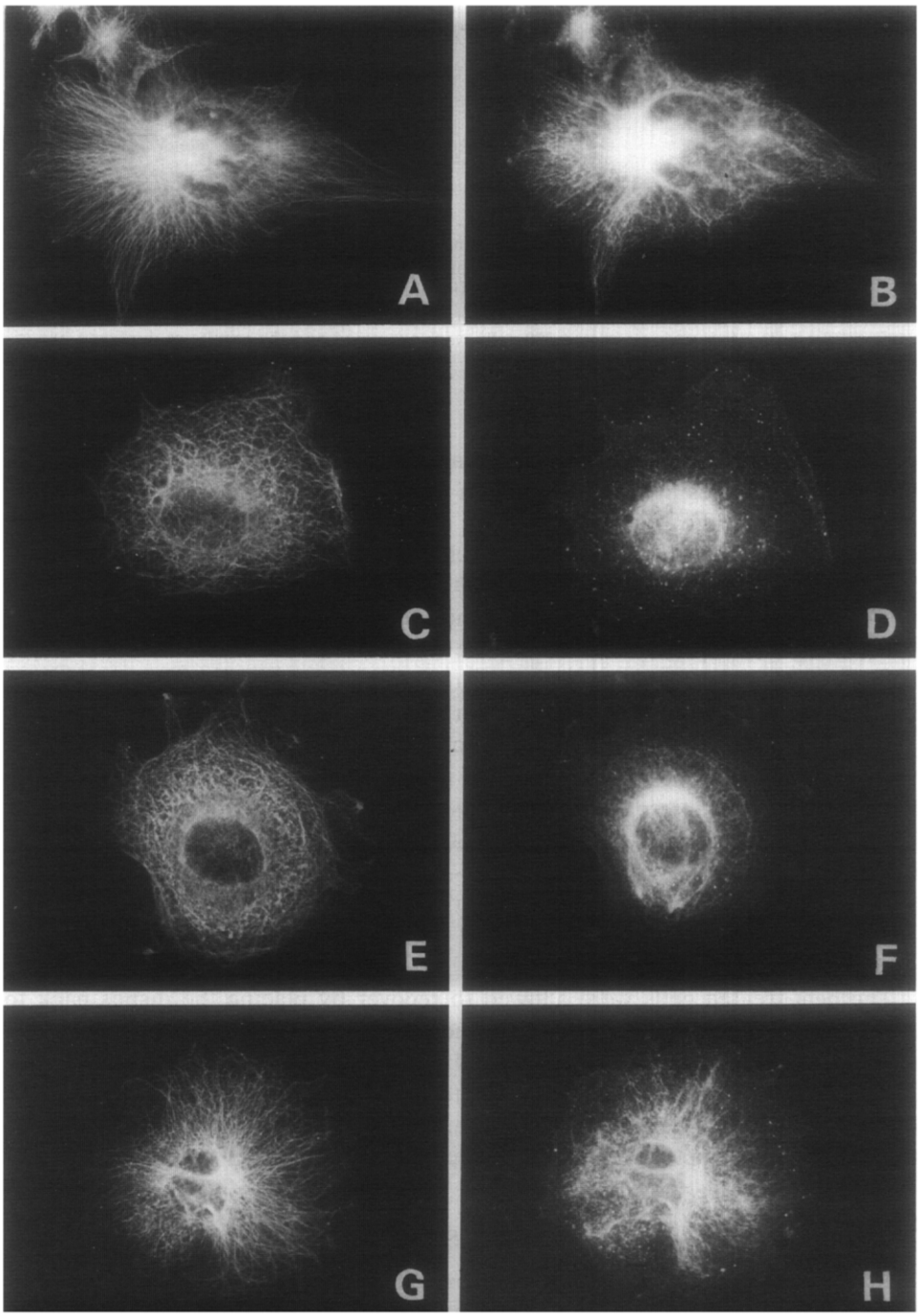

Fig. 3. Double-label indirect immunofluorescence analysis of the distribution of microtubules and intermediate filaments (vimentin) in neuroblastoma cells. Cells were grown in culture dishes, fixed for 5 min in −20°C methanol, and incubated with a mouse monoclonal antibody against β-tubulin (*A, C, E, G*) and a rabbit polyclonal antibody against vimentin (*B, D, F, H*). Shown are cells incubated at 37°C (*A, B*), cells incubated 30 min at 43°C (*C, D*), cells incubated 60 min with 75 μ*M* sodium arsenite (*E, F*), and cells incubated 30 min at 43°C in the presence of 3 μg/ml cycloheximide (*G, H*).

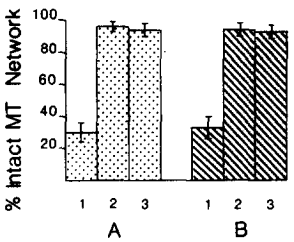

Fig. 4. Quantification of the effect of actinomycin D and cycloheximide on microtubule reorganization. Neuroblastoma cells were heat-shocked (30 min at 43°C) (*A*) or treated with sodium arsenite (60 min with 75 μ*M*) (*B*), without drug addition (*1*), in the presence of 1 μg/ml actinomycin D (*2*) or in the presence of 3 μg/ml cycloheximide (*3*). The cells were fixed for 5 min in −20°C methanol and stained with anti-β-tubulin. For each treatment 200 cells were examined and the results of duplicate experiments are given.

could induce an aggregation of intermediate filaments around the nucleus. We did not observe changes in the cytoskeleton during the first 4 h of treatment with 3 μg/ml cycloheximide (data not shown). Furthermore, we did not observe any alteration in the sensitivity of the cytoskeleton for nocodazole or dihydrocytochalasin B after a treatment with either ACD or CHX. This implies that neither ACD nor CHX affects the cytoskeletal dynamics at the concentration used.

Prevention of hsp synthesis during the stress treatment did not alter the rapid loss of stress fibers (Fig. 2*D*). However, a reorganization of microtubules and intermediate filaments did not occur in the presence of either ACD or CHX during both stress treatments (Figs. 3*G* and 3*H*). For a quantitative comparison, 200 cells per experiment were analyzed to see whether their microtubules were intact and whether intermediate filaments had aggregated. Both stress treatments induced changes in the cytoskeleton in 60–70% of the cell population. However, in the presence of the inhibitors only 5–10% of the cell population showed an altered organization of the cytoskeleton (Fig. 4). These results indicate that both RNA and protein synthesis are required for stress-induced changes in the cytoskeleton to occur.

The heat-treated cells were allowed to recover by placing the dishes back to 37°C. Within 3 h, the cytoskeleton in most cells was found to be normal again (Fig. 5). Addition of cycloheximide did not prevent this process (Fig. 5). Although many recovery processes seem to be mediated by proteins synthesized during the recovery period (see for instance Refs. [25, 26]), the re-formation of the cytoskeleton appeared to occur independently of protein synthesis.

DISCUSSION

In the present study we report our investigations on the mechanism of the heat-induced changes in the cytoskeleton. The results show that treatment of neuroblastoma cells with either heat or arsenite induced similar changes in protein synthesis and in the cytoskeletal organization. Arsenite, however, induced the synthesis of a hsp of 28 kDa in addition to the hsps also induced by a heat shock.

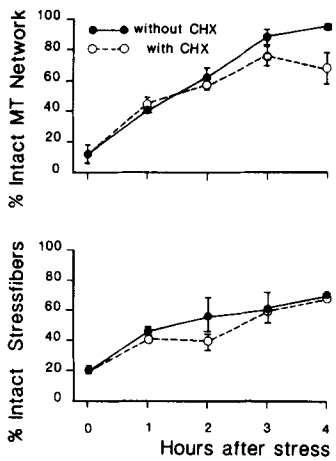

Fig. 5. Quantification of the effect of cycloheximide on the reappearance of stress fibers and normal microtubule patterns after a heat treatment. Neuroblastoma cells were heat-shocked for 30 min at 43°C and replaced at 37°C, either in the presence of 3 μg/ml cycloheximide (○) or without drug addition (●). The cells were fixed for 30 min with 3% paraformaldehyde in PBS, pH 7.4, permeabilized for 5 min with 0.1% Triton X-100 in PBS, pH 7.4, and stained with NBD-phallicidin or fixed for 5 min with −20°C methanol and incubated with anti-β-tubulin. At each time point 200 cells were examined and the results of duplicate experiments are given.

In *Drosophila* and plants the small hsps are synthesized abundantly after heat shock [7, 8]. Mammalian cell types, however, seem to be incapable of synthesizing such hsps, although in some cell lines the synthesis of a small hsp after heat shock is reported [19, 27]. It is suggested that this apparent absence of small hsps in most mammalian cell types is due mainly to the low content or even absence of methionine residues in these proteins [14, 28]. By incorporation of [^{3}H]leucine instead of [^{35}S]methionine, synthesis of several small hsps in mammalian cells was demonstrated [14, 28]. However, as shown in this paper, arsenite treatment of neuroblastoma cells resulted in the induction of a methionine-containing small hsp which was not detected in these cells after heat shock. Furthermore, Kim and co-workers [14] reported the synthesis of two arsenite-induced small hsps which could be detected by [^{35}S]methionine incorporation in rat fibroblasts, the synthesis of which was not induced by heat shock. From these results it is evident that the expression of methionine-containing small hsps is variable with respect to cell type as well as to the inducer of the stress response. Whether different gene activation mechanisms or different processing events are responsible for the differences in the expression patterns of small hsps in mammalian cells remains unclear.

An important result of the present study is the observed similarity in cytoskeletal reorganization induced by the two different stress treatments. A direct influence of heat on this reorganization can therefore be excluded. The experiments with actinomycin D and cycloheximide showed that newly synthesized proteins are required for the changes in microtubules and intermediate filaments to occur. On the other hand, such proteins were not required for the rapid loss of stress

fibers. A disappearance of stress fibers after heat shock was also reported for H35 hepatoma cells [19] and Chinese hamster overy (CHO) cells [29]. In contrast to our observations with neuroblastoma cells, novel synthesized proteins were required for the reappearance of stress fibers in CHO cells [29].

The stress-induced alterations in microtubules as reported here are in agreement with the results of several other studies. Rieder and Bajer [30] have shown that nonkinetochore microtubules were destroyed by a heat treatment, while other microtubules had formed closely packed structures. Disassembly of microtubules after a heat shock was described by Coss *et al.* [31] for Chinese hamster ovary cells and by Lin *et al.* [32] for 3T3 fibroblasts. Possible conflicting results have been obtained by Thomas *et al.* [18] and Welch and Suhan [33]. They did not observe changes in the microtubular organization, but the intermediate filaments were also found to be aggregated around the nucleus after heat shock. However, these authors have not investigated the rapid cytoskeletal changes occurring within the first hour of the stress treatment. On the other hand, cell-specific changes in the cytoskeleton, as described previously [19], may also account for this difference in cytoskeletal changes upon stress.

As demonstrated with the transcription and translation inhibitors, newly synthesized proteins were required for the stress-induced changes in microtubules and intermediate filaments of neuroblastoma cells to occur. It is tempting to speculate about the identity of the novel synthesized protein(s) which could be involved. We have shown in a previous paper that the cytoskeletal changes occur 15–20 min after the temperature elevation [19]. As described for *Drosophila* tissue culture cells, the first transcripts for hsp70 are completed within 4 min after the temperature shift [34] and after 8–10 min hsp70 is already visible on polyacrylamide gels [35]. Furthermore, it is shown for mammalian cells that the synthesis of normal proteins is inhibited within 5 min after the temperature shift [36]. Therefore, the involvement of hsps in the cytoskeletal reorganization is likely, since most of the proteins synthesized during the heat shock are hsps and the synthesis of the hsps occurs very rapidly.

In the second place it is worthwhile to consider which hsp(s) is (are) eventually involved in the cytoskeletal reorganization. Several hsps have been described as being associated with the cytoskeleton [12, 37]. Recently, it was shown that hsp70 is identical to a microtubule-associated protein (MAP) with a molecular mass of 68 kDa [38–40]. Copurification of hsp70 with neurofilaments from rat brain and microtubules from HeLa cells was reported by Wang *et al.* [39] and, furthermore, the coding sequence of a 68-kDa MAP was shown to have close homology with the hsp70 gene from *Drosophila* [38] and with hsp73 from HeLa cells [40]. Since the hsp70 can be considered as a microtubule-associated protein, the involvement of hsp70 in the stress-induced alterations in the cytoskeleton of N2A neuroblastoma cells as described here is very likely but remains to be shown.

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REFERENCES

1. Atkinson, B. G., and Walden, S. B. (Eds.) (1985) Changes in Eukaryotic Gene Expression in Response to Environmental Stress, Academic Press, Orlando, FL.
2. Bienz, M. (1985) *Trends Biochem. Sci.* **10**, 157.
3. Lanks, K. W. (1986) *Exp. Cell Res.* **165**, 1.
4. Craig, E. A. (1985) *CRC Crit. Rev. Biochem.* **18**, 239.
5. Subjeck, J. R., and Shyy, T. T. (1986) *Amer. Phys. Soc.* **363**, CL.
6. Schlesinger, M. J. (1986) *J. Cell Biol.* **103**, 321.
7. Kimpel, J. A., and Key, J. L. (1985) *Trends Biochem. Sci.* **10**, 353.
8. Novel, L. (Ed.) (1985) Heat Shock Response of Eukaryotic Cells, Springer Verlag, Berlin, FRG.
9. Pelham, H. R. B. (1985) *Trends Gen.* **1**, 31.
10. Voellmy, R. (1985) *Bio. Essays* **1**, 213.
11. Ritossa, F. (1962) *Experientia* **18**, 571.
12. Schlesinger, M. J., Ashburner, M., and Tissières, A. (Eds.), (1982) Heat Shock: From Bacteria to Man, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
13. Johnston, D., Opperman, H., Jackson, J., and Levinson, W. (1980). *J. Biol. Chem.* **255**, 6975.
14. Kim, Y. J., Shuman, J., Sette, M., and Przybyla, A. (1983) *J. Cell Biol.* **96**, 393.
15. Li, G. C. (1983) *J. Cell. Physiol.* **115**, 116.
16. Mirault, M. E., Southgate, R., and Delwart, E. (1982) *EMBO J.* **1**, 1279.
17. Kelley, P. M., and Schlesinger, M. J. (1978) *Cell* **15**, 1286.
18. Thomas, G. P., Welch, W. J., Mathews, M. B., and Feramisco, J. R. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **46**, 985.
19. Van Bergen en Henegouwen, P. M. P., Jordi, W. J. R. M., Van Dongen, G., Ramaekers, F C. F., Amesz, H., and Linnemans, W. A. M. (1985) *Int. J. Hyperthermia* **1**, 69.
20. Wiegant, F. A. C., Tuyl, M., and Linnemans, W. A. M. (1985) *Int. J. Hyperthermia* **1**, 157.
21. Ramaekers, F. C. S., Puts, J. J. G., Kant, A., Moesker, O., Jap, P. H. K., and Vooijs, G. P. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **46**, 331.
22. Laemmli, U. K. (1970) *Nature (London)* **277**, 680.
23. Giloh, H., and Sedat, J. W. (1982) *Science* **217**, 1252.
24. Sharpe, A. H., Bo Chen, L., Murphy, J. R., and Fields, B. N. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7267.
25. Lewis, M. J., and Pelham, H. R. B. (1985) *EMBO J.* **4**, 3137.
26. Pelham, H. R. B. (1984) *EMBO J.* **3**, 3095.
27. Landry, J., and Chrétien, P. (1982) *Canad. J. Biochem. Cell Biol.* **61**, 428.
28. Welch, W. J. (1985) *J. Biol. Chem.* **260**, 3058.
29. Glass, J. R., DeWitt, R. G., and Cress, A. E. (1985) *Cancer Res.* **45**, 258.
30. Rieder, C., and Bajer, A. S. (1977) *J. Cell Biol.* **74**, 717.
31. Coss, R. A., Dewey, W. C., and Bamberg, J. R. (1982) *Cancer Res.* **42**, 1059.
32. Lin, P. S., Turi, A., Kwock, L., and Lu, R. C. (1982) *Natl. Cancer Inst. Monogr.* **61**, 463.
33. Welch, W. J., and Suhan, J. P. (1985) *J. Cell Biol.* **101**, 1198.
34. Lindquist, S. (1980) *Dev. Biol.* **77**, 463.
35. Mirault, M. E., Goldschmidt-Clermont, M., Moran, L., Arrigo, A. P., and Tissières, A. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 819.
36. McGormick, W., and Penman, S. (1969) *J. Mol. Biol.* **39**, 315.
37. Wang, C., Gomer, R. H., and Lazarides, E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3531.
38. Lim, L., Hall, C., Leung, T., and Whatley, S. (1984) *Biochem. J.* **224**, 677.
39. Wang, C., Asai, D. J., and Lazarides, E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1541.
40. Welch, W. J., Feramisco, I. R., and Blose, S. H. (1985) *Ann. N.Y. Acad. Sci.* **455**, 57.

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