

# Rapid Effect of Heat Shock on Two Heterogeneous Nuclear Ribonucleoprotein-associated Antigens in HeLa Cells

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**Abstract.** During severe heat shock, which is known to interrupt both splicing of RNA transcripts and nucleocytoplasmic transport, it is to be expected that the substructure of heterogeneous nuclear ribonucleoproteins (hnRNP) is altered in some way. Recently, we have shown that such a stress actually induces rapid alterations at the level of individual proteins (Lutz, Y., M. Jacob, and J.-P. Fuchs. 1988. *Exp. Cell Res.* 175:109–124). Here we report further investigations on two related 72.5–74-kD hnRNP proteins whose behavior is also rapidly modified by a heat shock at 45°C, whereas no effect is observed at 42°C. Using a monoclonal antibody, we show that in situ the antigens are available only when the cells are heat shocked at 45°C. Subcellular fractionation shows that in normal

cells the antigens are associated with the bulk of hnRNP (50–200S). During heat shock, whereas the overall characteristics of the bulk of preexisting hnRNP are unchanged, these antigens rapidly switch to a subpopulation of hnRNP with larger average size (50 to >300S) and increased stability. Substructural analysis of the associated hnRNP in normal and stressed cells shows that in both cases the antigens are associated with the nuclear matrix subcomplex of hnRNP, which in situ is part of the internal nuclear matrix. Such hnRNP antigens, which are rapidly redistributed during a heat shock at the upper temperature range of the stress response, might well be involved in splicing and/or transport control.

A temperature elevation a few degrees Celsius above normal growth temperature induces in all cell types or organisms a series of functional and morphological alterations, termed cellular heat shock or stress response (9, 24, 41), which are gradually reversed over a period of several hours once the physiological temperature is restored.

At the posttranscriptional level, it has been shown, both in insect (42) and mammalian cells (5, 21), that a heat shock at the upper temperature range of the stress response transiently inhibits pre-mRNA splicing and nucleocytoplasmic transport. Since these two functions are linked to heterogeneous nuclear ribonucleoproteins (hnRNP)<sup>1</sup> (2, 8, 15, 16, 27, 38), we postulated, as suggested earlier (29), that heat shock turns off these functions by modifying the substructure of hnRNP. Therefore, the hnRNP, which contain numerous proteins ranging from 30 to 200 kD (17, 20), were analyzed before and after heat shock. However, studies based on the determination of the buoyant density in CsCl gradients and of the velocity in sucrose gradients did not reveal any differences in the overall characteristics of hnRNP (22, 26). We then started to search for alterations at the level of individual

proteins by using monoclonal antibodies we have raised against hnRNP purified from HeLa cells. This approach recently allowed us to confirm our hypothesis. Indeed, we detected two related 35–37-kD antigens that are redistributed during a heat shock at 45°C, whereas no effect was observed at 42°C (26). Interestingly, this redistribution, which can only be triggered at a temperature range known to interrupt splicing and transport (5, 21, 42), occurs very rapidly after the onset of heat shock.

To further investigate the rapid effect of heat shock on hnRNP substructure, we searched for other hnRNP proteins whose behavior might also be modified in such stress conditions. Therefore, we screened our monoclonal antibody library for antibodies that in immunocytofluorescence tests would reveal differences in the antigen distribution and/or signal intensity between normal cells or mildly stressed cells (42°C) and severely stressed cells (45°C). In this paper, we report the characterization and the behavior of two such hnRNP-associated antigens of molecular masses 72.5 and 74 kD. In contrast to the 35–37-kD hnRNP antigens, which in normal cells are associated with the 30–50S subcomplexes of hnRNP (26), the 72.5–74-kD antigens are bound to the nuclear matrix subcomplex of hnRNP. Rapidly, after the onset of heat shock at 45°C, they are found on a particular subpopulation of very resistant hnRNP.

1. *Abbreviations used in this paper:* hnRNA, heterogeneous nuclear ribonucleic acid; hnRNP, heterogeneous nuclear ribonucleoproteins; PG, perichromatin granules; RNP, ribonucleoproteins.

## Materials and Methods

### Cell Culture, Labeling, and Heat Shock

HeLa S3 cells were grown in suspension ( $1-5 \times 10^5$  cells/ml) at 37°C in Joklik's minimal essential medium supplemented with 7.5% newborn calf serum and 2.5% inactivated FCS. For protein labeling, cells were resuspended at  $5 \times 10^5$  cells/ml in fresh medium containing 5% of the standard methionine concentration and incubated in the presence of [<sup>35</sup>S]methionine (10  $\mu$ Ci/ml) for 22 h.

For subcellular fractionation, HeLa S3 cells were resuspended at  $3 \times 10^6$  cells/ml in fresh medium, labeled with [<sup>3</sup>H]uridine (2  $\mu$ Ci/ml) for 1 h, and processed as described below. When investigating the effect of heat shock,  $10^8$  cells were labeled in the same way but, after 50 min of incubation, the cells were transferred to a 175-cm<sup>2</sup> tissue culture flask that was immersed in a circulating water bath for 10 min at 45°C before fractionation.

### Isolation, Fractionation, and Dissociation of hnRNP

hnRNP from HeLa S3 cells were prepared and fractionated as described previously, all steps being performed in the presence of RNasin,  $\beta$ -2 glycerophosphate, and protease inhibitors (26). Briefly, nuclei were purified from  $10^8$  cells, resuspended in reticulocyte standard buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>), and sonicated. After adding 50 mM KCl, the sonicate was briefly centrifuged at high speed. The supernatant or nuclear extract was then layered onto a 10–25% (wt/vol) linear sucrose gradient (2  $\times$  15.5 ml) with a 3-ml 40% sucrose cushion in TEA buffer (10 mM triethanolamine-HCl, pH 7.4, 100 mM KCl, 1 mM MgCl<sub>2</sub>) and centrifuged at 82,500 g for 2.5 h in a rotor (SW-27; Beckman Instruments, Inc., Fullerton, CA).

Ribonuclease treatment of hnRNP was carried out by incubating a nuclear extract for 20 min at 25°C with 0.1  $\mu$ g/ml RNase A, the products being fractionated on a sucrose gradient as described above. For studying hnRNP dissociation by salt, a nuclear extract was brought to 0.4 M in KCl, incubated at 0°C for 20 min, and centrifuged on a sucrose gradient containing the same concentration of KCl.

### CsCl Density Gradients

100  $\mu$ l of [<sup>3</sup>H]uridine-labeled nuclear extract was brought to 450  $\mu$ l with TEA buffer and fixed for 4 h at 4°C after adding 50  $\mu$ l of 30% neutralized formaldehyde. The nuclear extract was then applied to a 1.3–1.7-g/cm<sup>3</sup> preformed CsCl gradient in TEA buffer and centrifuged at 36,000 rpm for 16 h at 20°C in a rotor (SW-65; Beckman Instruments, Inc.).

### Whole Cell Extracts

HeLa S3 cells were cultured in suspension as described above. An aliquot of the cell suspension, which will serve as a control, was centrifuged, and the cells were washed twice with Ca<sup>++</sup>- and Mg<sup>++</sup>-free PBS, pH 7.4, and further sonicated in the presence of Laemmli's sample buffer (23). The remaining cell suspension was immersed in a circulating water bath for 10 min at 45°C, and aliquots were treated as the control after various periods of recovery in the incubator at 37°C.

Kc167 *Drosophila* cells in Echalier medium supplemented with 2% inactivated FCS were grown at 25°C in 25-cm<sup>2</sup> tissue culture flasks. For the control flask, the cells were scraped, washed, and sonicated as above. The other flasks were immersed in the water bath for 10 min at 37°C, and the cells were treated as the controls after various periods of recovery at 25°C.

### Monoclonal Antibody

Hybridoma cell line IS-6D12.3 (or 6D12) was obtained as previously described (26) after immunizing BALB/c mice with hnRNP purified on sucrose gradients and fusing the spleen cells with myeloma cell line SP2/0-Ag14. Hybridoma cell line 6D12, which produces an IgM antibody, was cloned on soft agar.

Hybridoma 6D12 was cultured in RPMI-1640 supplemented with 10% inactivated FCS or expanded as ascites tumors in syngenic mice. Both culture supernatant and ascites fluid were used as monoclonal antibody sources.

### Protein Blots

For Western blots, protein samples were resolved on a SDS–9% polyacrylamide gel (23) and electrophoretically transferred to nitrocellulose (0.45  $\mu$ m; Schleicher & Schuell, Inc., Keene, NH) (37). The fractionated proteins were probed with hybridoma supernatant as described before. The second antibody was goat anti-mouse Ig-F(ab)<sub>2</sub> (Cappel Laboratories, Malvern, PA) labeled with <sup>125</sup>I.

For immunodetections along sucrose and CsCl gradients, an aliquot of each fraction was diluted with PBS and adsorbed on nitrocellulose using a slot blot apparatus (Manifold II; Schleicher & Schuell, Inc.). Immunodetection was as described for Western blots.

Autoradiography was at –80°C using X-Omat films (Eastman Kodak Co., Rochester, NY) and intensifying screens.

### Immunoprecipitation

Monoclonal antibodies were coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer, using ascites fluids as antibody sources. 2 vol of unlabeled or [<sup>35</sup>S]methionine-labeled nuclear extract containing 0.5% Tween-20 were preincubated twice for 1 h at 4°C with 1 vol of an unrelated murine monoclonal antibody coupled to Sepharose. The antigen was then immunoprecipitated from the nuclear extract for 1.5 h at 4°C with 1 vol of 6D12 antibody coupled to Sepharose. After extensive washing with 0.5% Tween-20 in PBS, the pellet was treated as indicated below.

### Isoelectric Focusing

A nuclear extract was precipitated, and the pellet was washed twice with cold acetone, dried, and dissolved in 8 M deionized urea. Isoelectric focusing was performed on a polyacrylamide gel containing 8 M urea and ampholines with a pH range of 3–10 (LKB Instruments, Inc., Gaithersburg, MD). The proteins were then transferred to nitrocellulose and probed with hybridoma supernatant, using <sup>125</sup>I-labeled goat anti-mouse Ig-F(ab)<sub>2</sub> as a second antibody.

Alternatively, antigens were immunoprecipitated from a [<sup>35</sup>S]methionine-labeled nuclear extract as described above. After elution with 3.5 M sodium isothiocyanate, pH 8, the antigens were further precipitated with 10% TCA in the presence of 0.4 mg/ml sodium desoxycholate. The precipitate was spun down at high speed and the pellet treated as described above. After resolving the antigens by isoelectric focusing, the gel was treated for fluorography (6) and autoradiographed as for immunoblots.

### Radiolabeling of Proteins and Peptidic Maps

Peptidic maps were essentially carried out as previously described (11). Immunoprecipitated polypeptides were resolved by SDS-PAGE, and the stained bands were cut out, dried, and further submitted to iodination using the chloramine-T method in the presence of carrier-free Na<sup>125</sup>I (Amersham Corp., Arlington Heights, IL). After extensive washing in 7.5% acetic acid, 5% methanol, the bands were cut into small fragments and dried. Each sample was incubated for 24 h at 37°C in 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.8, containing 15 U/ml of trypsin (Sigma Chemical Co., St. Louis, MO) or thermolysin (Merck & Co., Rahway, NJ). The supernatant was then lyophilized, and the residue was dissolved in water, lyophilized again, and redissolved in electrophoresis solution.

Peptide samples were resolved in two dimensions on cellulose-coated TLC plates (10  $\times$  10 cm; Merck & Co.). The first dimension was electrophoresis in acetic acid/formic acid/water (3:1:16 vol/vol/vol); the second dimension was ascending chromatography in butanol/pyridine/acetic acid/water (32.5:25:5:20 vol/vol/vol/vol). After air drying, the plates were autoradiographed as for immunoblots.

### Indirect Immunofluorescence

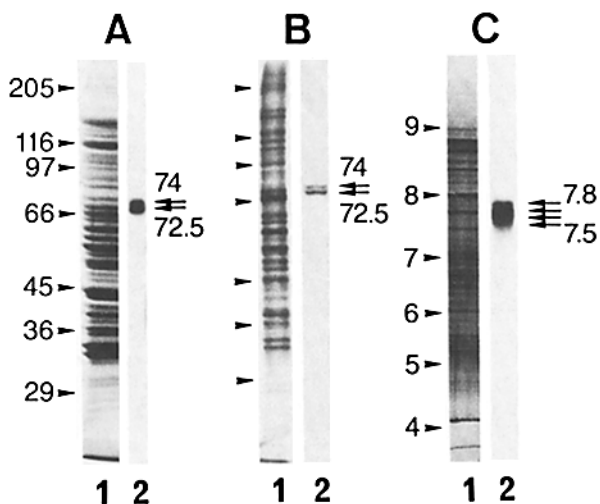
Schaffner HeLa cells (1) ( $10^5$  cells/ml) in Dulbecco's medium supplemented with 7.5% newborn calf serum and 2.5% inactivated FCS were grown in Leighton tubes (Costar, Cambridge, MA) at 37°C for 3 d, washed with PBS, and fixed for 4 min with 2% paraformaldehyde. When heat shock effects were investigated, Leighton tubes were immersed in a water bath for 10 min at 45°C before fixation, which was carried out after various periods of recovery at 37°C. The cells were then permeabilized at –20°C with methanol and acetone for 4 and 2 min, respectively. After washing the cells with 0.1% Triton X-100, 0.05% NaN<sub>3</sub> in PBS, they were incubated for 30 min with 20% normal inactivated goat serum, 0.1% Triton X-100 in PBS

and again rinsed with 0.1% Triton X-100, 0.05% NaN<sub>3</sub> in PBS. The cells were incubated overnight with hybridoma supernatant, washed with several changes of 0.1% Triton X-100, 0.05% NaN<sub>3</sub> in PBS over 2 h and reacted for 45 min with TRITC-conjugated goat anti-mouse Ig, 20% normal inactivated goat serum, 0.1% Triton X-100 in PBS. After extensive washings with 0.1% Triton X-100, 0.05% NaN<sub>3</sub> in PBS followed by PBS, DNA was counterstained with Hoechst 33258 dye. The preparations were again washed with PBS, mounted on glass slides in glycerol/PBS (4:1 vol/vol) containing 5% propylgallate as a photobleaching retardant (19), and examined with a photomicroscope (Carl Zeiss, Inc., Thornwood, NY) fitted for epifluorescence and phase contrast. Photographs were taken on Technical Pan 2415 films (Eastman Kodak Co.). Film exposure and photographic printing are always carried out in the same conditions.

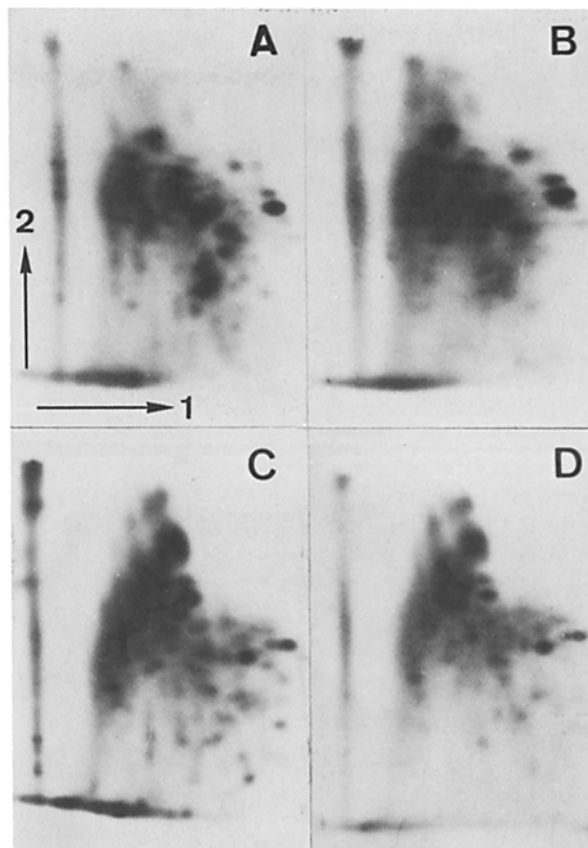
### Nuclear Matrices

For immunocytofluorescence experiments, Schaffner HeLa cells were grown in Leighton tubes and then either submitted or not to heat treatment as described above. Nuclear matrices were prepared as whole mounts as described before (26). Briefly, the plastic strips were dipped in PBS and the cells permeabilized for 3 min at 0–4°C with 0.5% NP-40 in TSKM buffer (25 mM Tris-HCl, pH 7, 100 mM NaCl, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM PMSF). After rinsing with TSKM buffer, the cells were incubated for 15 min at 22°C in the same buffer containing 0.5 mg/ml RNase-free DNase I with or without 0.1 mg/ml RNase A. Digested material was extracted with several changes of high ionic strength buffer (30 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 0.4 M [NH<sub>4</sub>]<sub>2</sub> SO<sub>4</sub>, 1 mM PMSF) over 15 min at 0–4°C. After washing with TSKM buffer, the skeletons were fixed and processed for immunofluorescence as for whole cells. The complete removal of the digested chromatin was checked by the absence of Hoechst 33258 fluorescence. Film exposure and photographic printing were carried out as above.

For Western blot analysis, nuclei purified from normal or heat-shocked HeLa S3 cells were washed with TSKM buffer and pelleted at 800 g for 5 min. 5 × 10<sup>7</sup> nuclei were resuspended in 0.5 ml of the same digestion



**Figure 1.** Molecular mass and isoelectric point determinations of the 6D12 antigens. All procedures were as described under Materials and Methods. (A) Western blot: a nuclear extract was resolved on a 9% SDS-polyacrylamide gel and either stained with Coomassie blue (lane 1) or analyzed by immunoblotting (lane 2). (B) Immunoprecipitation: the 6D12 antigens were immunoprecipitated from a [<sup>35</sup>S]methionine-labeled nuclear extract and then electrophoresed on a 9% SDS-polyacrylamide gel and fluorographed (lane 2) along with the whole nuclear extract (lane 1). (C) Isoelectric focusing: a nuclear extract was resolved in the presence of ampholines pH 3–10 and either stained with Coomassie blue (lane 1) or analyzed by immunoblotting (lane 2). In A–C no signal was detected when antibody 6D12 was replaced by culture supernatant from the parent myeloma line SP2/O. Values indicate molecular masses in kilodaltons.



**Figure 2.** Peptidic maps of the 72.5- and 74-kD antigens recognized by antibody 6D12. The procedure was as described under Materials and Methods. The isolated antigens were digested with either trypsin (A and B) or thermolysin (C and D), and the peptides were further separated by electrophoresis (→1) followed by chromatography (→2). 72.5-kD antigen maps are shown in A and C and 74-kD antigen maps are in B and D.

medium as above and incubated for 15 min at 22°C. After sedimentation at 600 g for 15 min through a 2-ml 30% sucrose layer in TSKM buffer, the depleted nuclei were resuspended in 2.5 ml high ionic strength buffer, incubated at 0–4°C for 15 min, and again sedimented through a sucrose layer as above. The nuclear matrices were then processed for Western blots as described above.

### Immunoelectron Microscopy

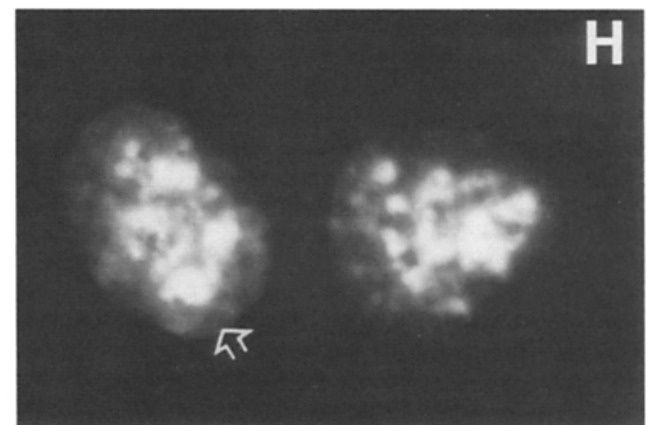
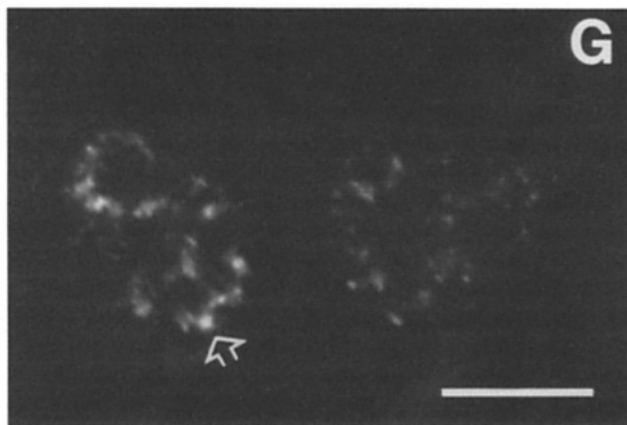
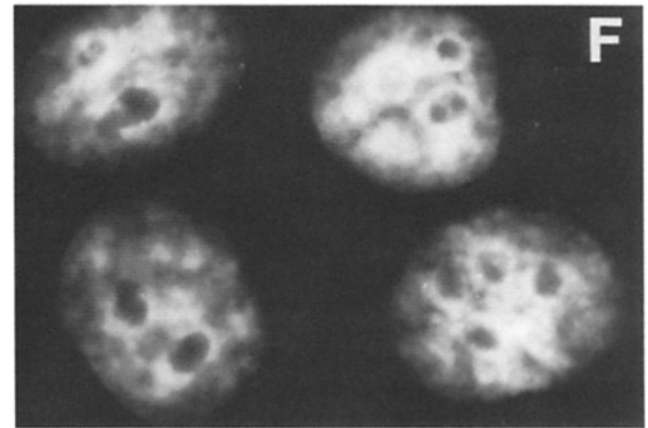
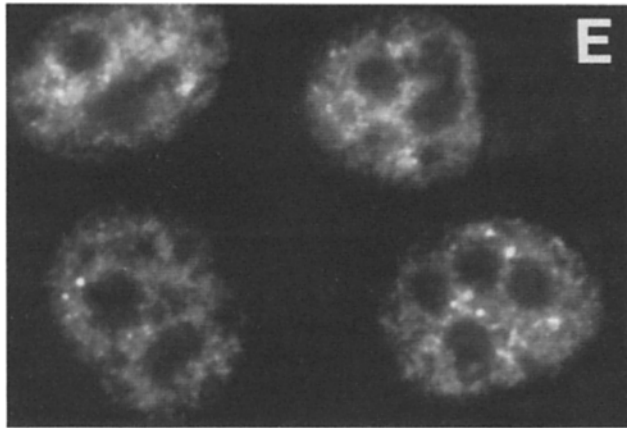
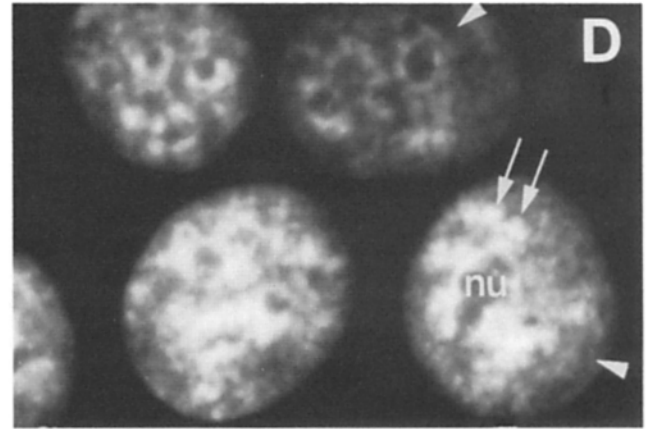
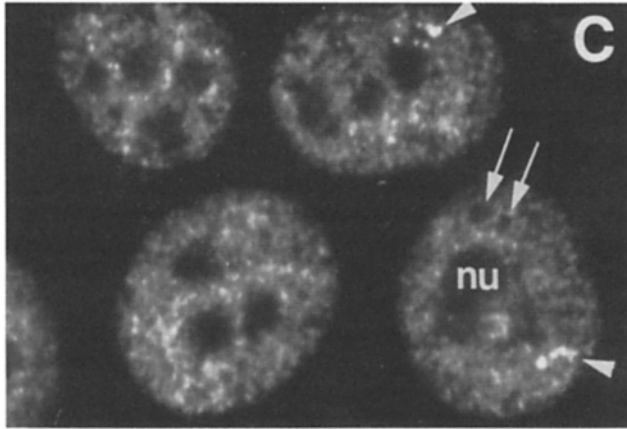
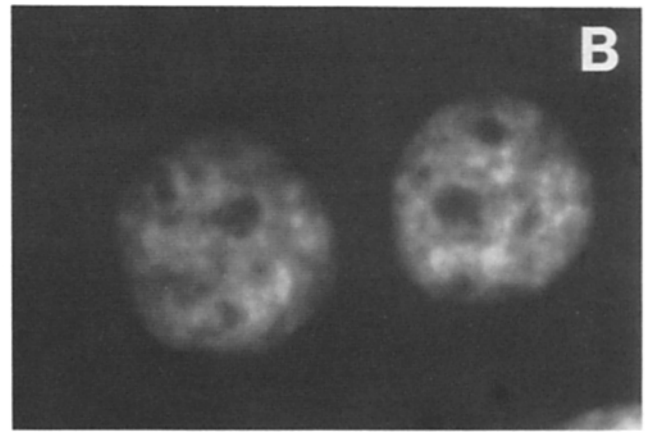
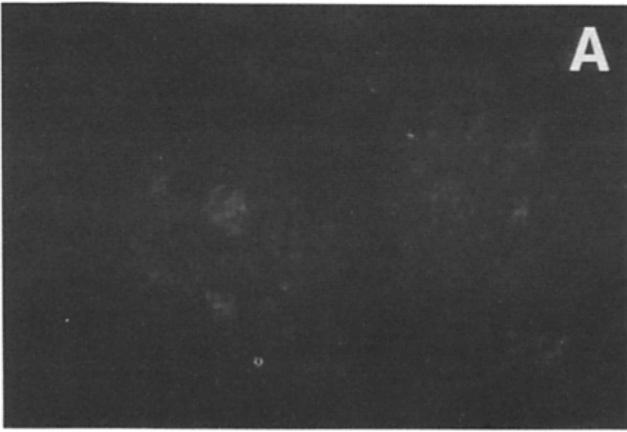
HeLa S3 cells were heat shocked for 10 min at 45°C and either fixed immediately or returned at 37°C for 4 h. Fixation was with 4% paraformaldehyde in 0.1 M Sørensen phosphate buffer for 1 h at 4°C. After dehydration with methanol, the cells were embedded in Lowicryl K4M (7). Thin sections mounted on formvar-carbon-coated nickel grids were incubated with 1% BSA in PBS and then with hybridoma supernatant for several hours.

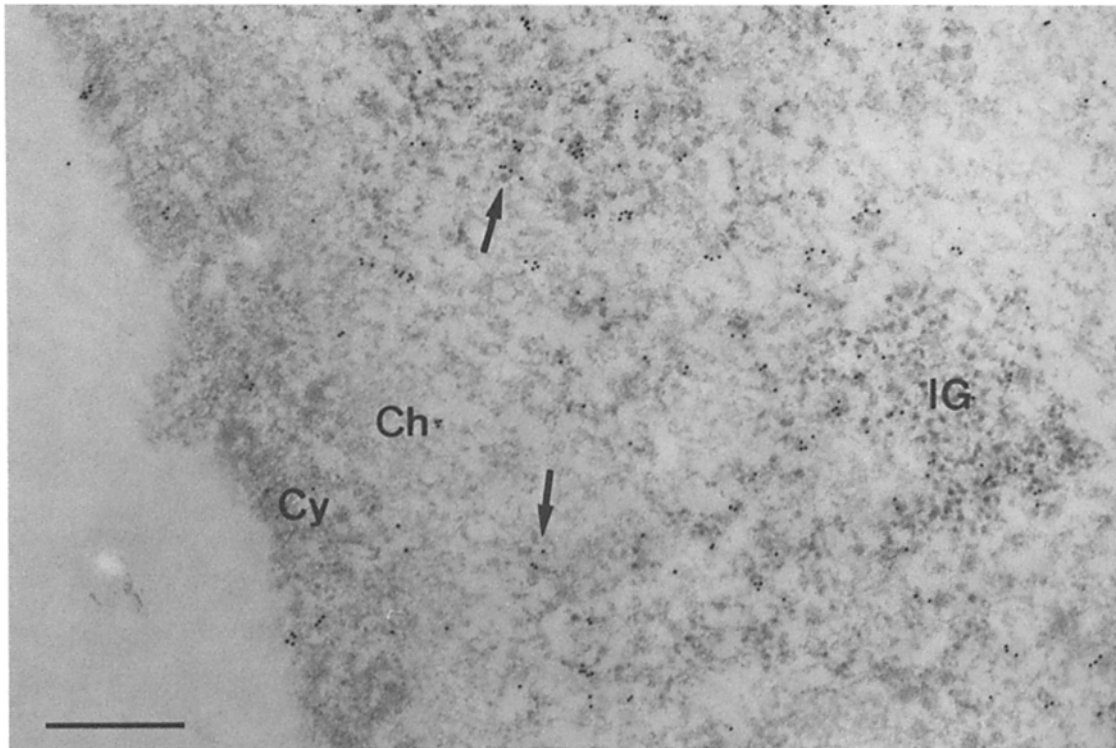
After rinsing the sections in PBS for 15 min with three changes, they were incubated with goat anti-mouse IgG conjugated to 10-nm-diameter gold particles (Janssen Pharmaceutica, Beerse, Belgium) diluted 10–20 times with Tris-HCl buffer, pH 8.2. The sections were then rinsed in the same buffer for 10 min with two changes and finally rinsed in water. After air drying the thin sections, ribonucleoproteins (RNP) were preferentially stained by the regressive EDTA method of Bernhard (3).

## Results

### Characterization of the Antigens

A nuclear extract from normal (nonstressed) HeLa S3 cells





**Figure 4.** Immunoelectron microscopy. Localization of the 6D12 antigens in HeLa cells heat shocked for 10 min at 45°C and immediately fixed. The thin section was reacted with antibody 6D12 and then with a second antibody coupled to gold particles. The gold particles decorate peri- and interchromatin fibrils (arrows). The bleached condensed chromatin (Ch), the cluster of interchromatin granules (IG), and the cytoplasm (Cy) are not labeled. RNP were preferentially stained by the regressive EDTA method. Bar, 0.5  $\mu$ m.

was resolved by SDS-gel electrophoresis, transferred to nitrocellulose, and probed with monoclonal antibody 6D12 (Fig. 1 A). Within the complex pattern revealed by Coomassie blue staining, which mainly represents hnRNP, small nuclear RNP, and nucleosol proteins, antibody 6D12 detected two bands at 72.5 and 74 kD. The same two bands could be immunoprecipitated from a [<sup>35</sup>S]methionine-labeled nuclear extract (Fig. 1 B). From now on, these antigens will be termed 6D12 antigens.

When a nuclear extract was fractionated by isoelectric focusing, transferred to nitrocellulose, and reacted with antibody 6D12, four bands between pI 7.5 and 7.8 were detected (Fig. 1 C). Isoelectric focusing carried out after the antigens were immunoprecipitated from a [<sup>35</sup>S]methionine-labeled nuclear extract revealed the same four bands (not shown). These results suggest that one of the 72.5–74-kD polypeptides or both of them bear posttranslational modifications. Isoelectric focusing, performed after the antigens were immunoprecipitated from a <sup>32</sup>P-labeled nuclear extract, indicated that these charge isomers are not due to phosphoryla-

tion (not shown), though many hnRNP proteins were previously shown to be phosphorylated (14, 17).

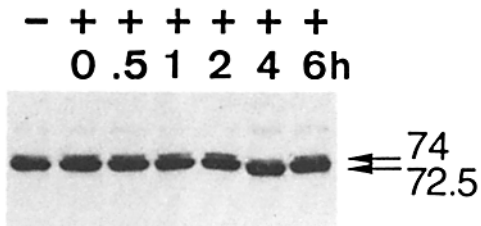
To rapidly find out whether or not the two 6D12 antigens are structurally related, peptidic mapping was performed. Fig. 2 shows that after trypsin as well as after thermolysin digestion the two proteins display similar patterns, which indicates a fair degree of homology.

Analysis of cell lines from other species revealed that the epitope recognized by antibody 6D12 is at least conserved down to the insects. However, it is noteworthy that in *Drosophila Kc167* cells antibody 6D12 detects a single band at 140 kD, as will be shown below (Fig. 6).

#### ***Immunocytochemical Localization of the Antigens in Normal and Heat-shocked Cells***

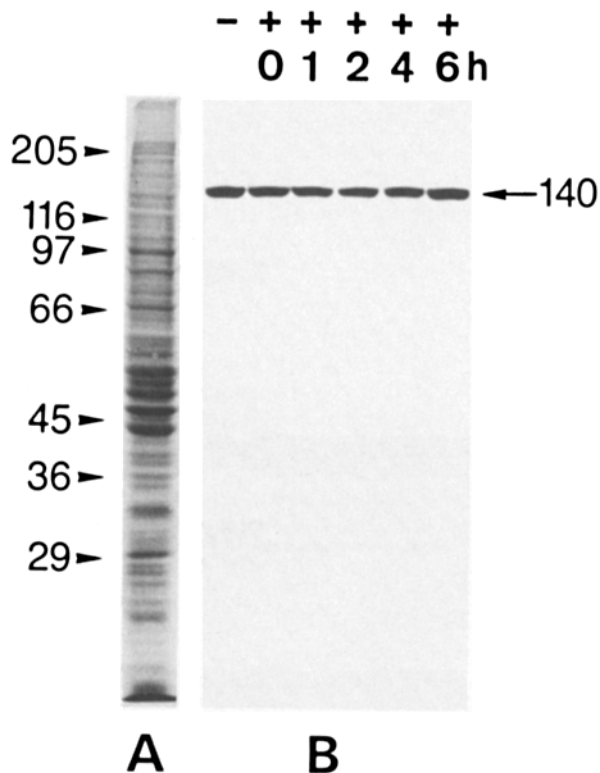
Although the 6D12 antigens were well detected when a nuclear extract was analyzed by Western blot (Fig. 1 A), antibody 6D12 did not reveal any signal by indirect immunocytofluorescence on HeLa Schaffner cells cultured in standard

**Figure 3.** Immunocytofluorescence localization of the 6D12 antigens in normal and heat-shocked HeLa cells. The procedures were as described under Materials and Methods. Cells were either nonstressed (A and B) or submitted to a 10-min heat shock at 45°C followed by 0 (C and D), 1 (E and F), and 5 h (G and H) of recovery at 37°C. Shown in A, C, E, and G are the rhodamine immunofluorescence micrographs and in B, D, F, and H are the corresponding Hoechst 33258 counterstain micrographs. The condensed chromatin (arrows) and the nucleoli (nu) are free of rhodamine fluorescence, the fluorescent granules and the bright granule clusters (arrowheads) being merely located in the interchromatin space. The open arrow points to an area where the rhodamine fluorescence persists during the later phase of recovery. Controls where antibody 6D12 was replaced by culture supernatant from the parent myeloma line SP2/O did not reveal any signals. Bar, 10  $\mu$ m.

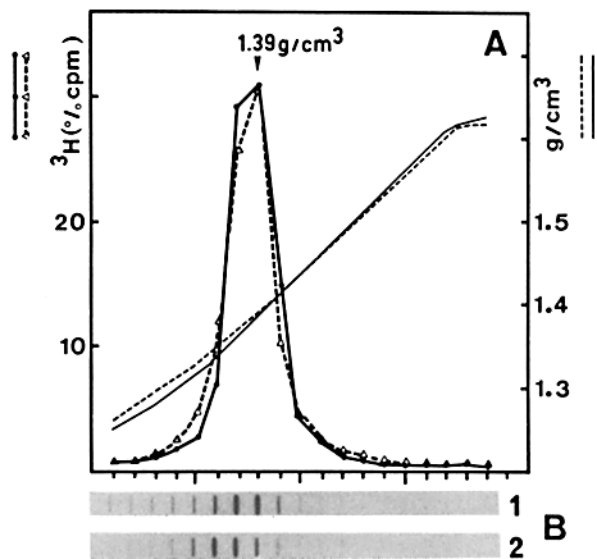


**Figure 5.** Quantitative comparison of the 6D12 antigens present in normal and heat-shocked HeLa cells. The procedures were as described in Materials and Methods. Cellular extracts from either normal cells (-) or cells given a 10-min heat shock at 45°C (+) and further allowed to recover at 37°C for increasing times were analyzed by Western blotting. Cellular extracts from equal amounts of cells were analyzed.

conditions (Fig. 3, *A* and *B*). When HeLa cells were processed for immunocytofluorescence immediately after being heat shocked for 10 min at 42°C, no difference was observed (not shown). In contrast, when the cells were heat shocked for 10 or even only 5 min at 45°C, a bright nuclear signal was displayed (Fig. 3, *C* and *D*). The nuclear signal was distributed as small granules, and some of them, organized in small clusters, were much brighter. When comparing the rhodamine distribution and the Hoechst 33258 counterstain, it appeared that the antibody-binding sites were absent from the nucleoli, the perinucleolar chromatin, as well as from the smaller chromatin clumps. Despite the limits of this method,



**Figure 6.** Western blot analysis and quantitative comparison of the 6D12 antigens present in normal and heat-shocked *Drosophila Kc167* cells. The procedures were as described in Materials and Methods. (*A*) Cellular extract from normal cells stained with Coomassie blue. (*B*) Western blot analysis of cellular extracts from either normal cells (-) or cells given a 10-min heat shock at 37°C (+) and then allowed to recover at 25°C for increasing times. Cellular extracts from equal amounts of cells were analyzed.



**Figure 7.** Distribution of the 6D12 antigens in nuclear extracts from normal and heat-shocked HeLa cells after fractionation on CsCl density gradients. All procedures were as described in Materials and Methods. (*A*) Distribution of the [<sup>3</sup>H]uridine acid-insoluble radioactivity in normal cells (●-●) and cells heat shocked for 10 min at 45°C (Δ-Δ), the density curves being represented by a solid and a dashed line, respectively. (*B*) Distribution of the 6D12 antigens in normal cells (lane 1) and in heat-shocked cells (lane 2), as determined by a slot blot procedure. Controls where antibody 6D12 was replaced by culture supernatant from the parent myeloma line SP2/O did not reveal any signal.

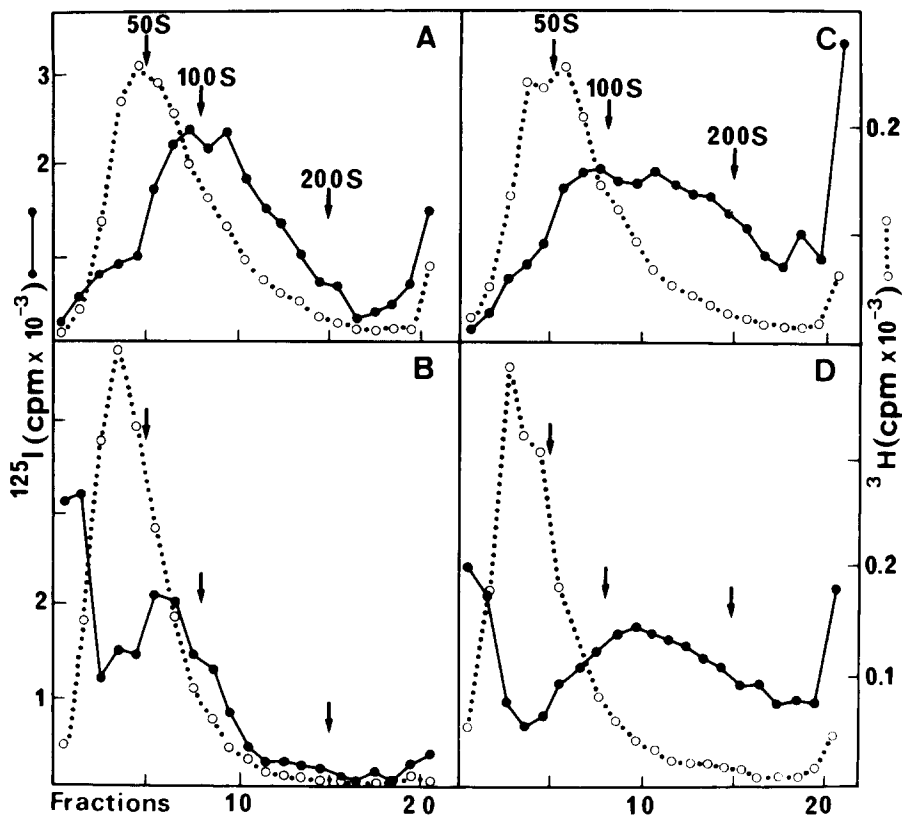
it can be inferred from these data that the rhodamine-stained granules are merely located in the interchromatin space, as would be expected for hnRNP (31).

Additional studies by immunoelectron microscopy confirmed that only cells treated at 45°C for 10 min displayed a nuclear signal (Fig. 4), though between normal and heat-shocked cells there was no apparent difference in nuclear morphology (normal cells not shown). The gold particles are exclusively detected on the preferentially contrasted peri- and interchromatin fibrils, which are located at the border of the bleached condensed chromatin and throughout the entire nucleoplasm. Earlier investigations showed that these fibrils correspond to *in situ* hnRNP (12, 13, 31, 32).

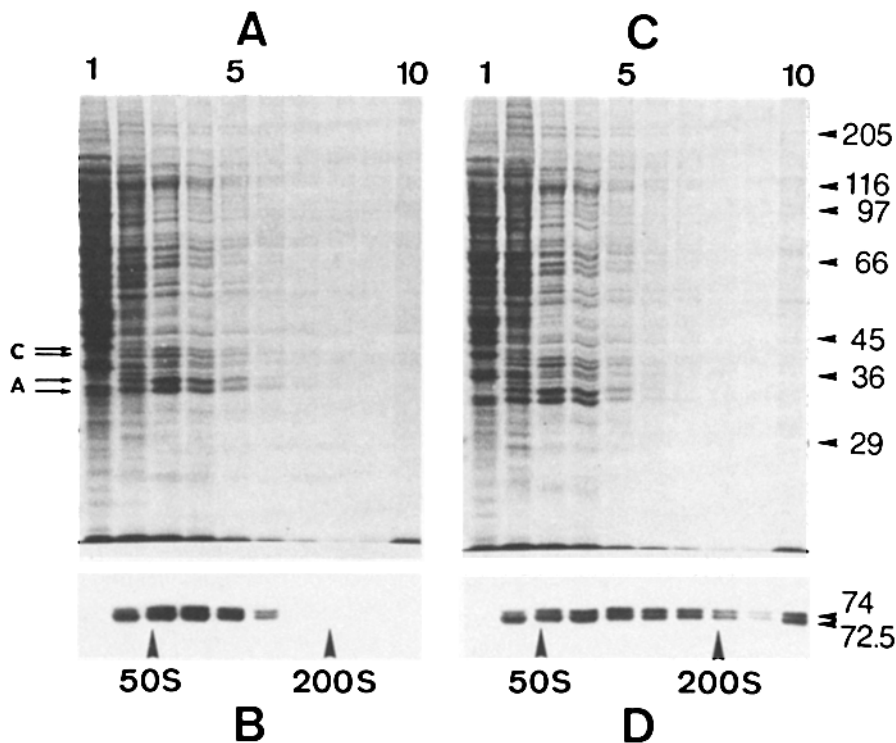
Taken together, these data show that the behavior of the 6D12 antigens is clearly modified when cells are heat shocked at 45°C for times as short as 5 or 10 min, whereas no effect is observed at 42°C. In addition, the location of the antigens in stressed cells strongly suggests that they are associated with hnRNP.

#### **Relative Amounts of Antigens Present in Normal and Heat-shocked HeLa and *Drosophila* Cells**

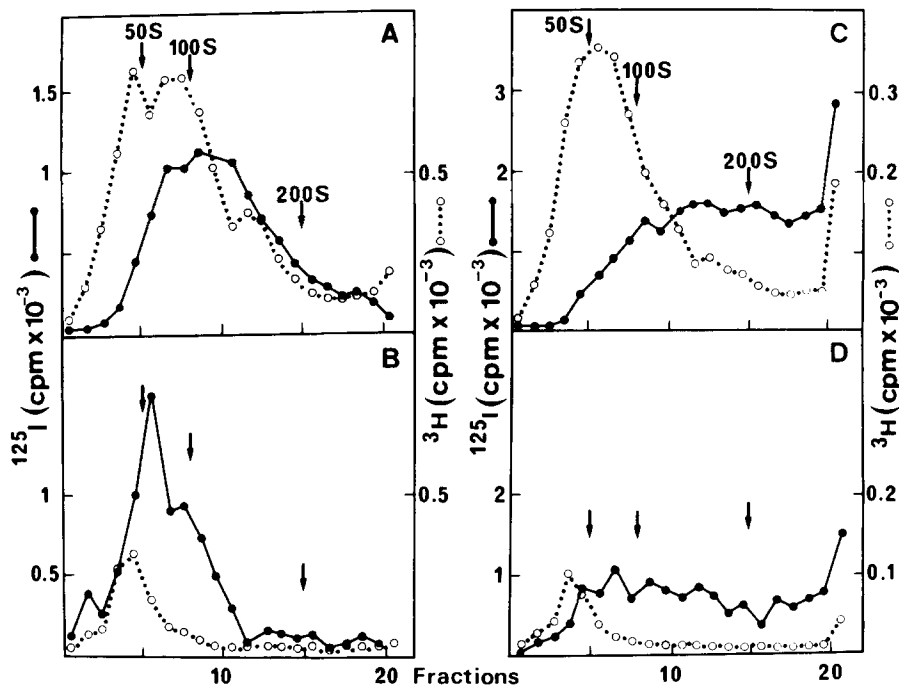
As it was shown above, the antigens recognized by antibody 6D12 are present in normal cells (Fig. 1, *A* and *B*), although by immunocytofluorescence they were only detected in stressed cells (Fig. 3). At this point, the question arose whether these antigens are heat inducible and therefore might accumulate with time, as does for instance the heat shock protein hsp72 (9, 24, 39). To investigate such a possibility, cellular extracts, prepared from equal aliquots of normal and of heat-shocked HeLa S3 cells that recovered for 0,



**Figure 8.** Effect of KCl treatment on the distribution of the 6D12 antigens in nuclear extracts from normal and heat-shocked HeLa cells fractionated on sucrose gradients. The procedures were as described in Materials and Methods. Cells grown at 37°C were pulse labeled for 1 h with [<sup>3</sup>H]uridine. Before preparing the nuclear extracts, half of the cells were heat shocked at 45°C during the last 10 min of labeling. Nuclear extracts from equal amounts of cells were used in the assays A–D. Fractionations were carried out on 10–25% sucrose gradients, and the [<sup>3</sup>H]uridine acid-insoluble activities were determined. The 6D12 antigen distributions were analyzed using a <sup>125</sup>I-labeled second antibody. (A) Nuclear extract from normal cells. (B) Nuclear extract from normal cells treated with 400 mM KCl. (C and D) Nuclear extracts from heat-shocked cells processed as in A and B, respectively. The two fractions from the top correspond to the volume of the sample layered on the gradient. The fraction from the bottom also contains the material that might have pelleted.



**Figure 9.** Western blot analysis across sucrose gradients of the 6D12 antigen distribution in nuclear extracts from normal and heat-shocked HeLa cells. The procedures were as described in Materials and Methods. Cells grown at 37°C were pulse labeled for 1 h with [<sup>3</sup>H]uridine and half of them were heat shocked at 45°C for the last 10 min of labeling. Nuclear extracts from equal amounts of normal and heat-shocked cells were fractionated on sucrose gradients as in Fig. 8, and the [<sup>3</sup>H]uridine acid-insoluble activities determined. Gradient fractions were then pooled two by two (lanes 1–10), and equal aliquots were processed for Western blot analysis. Nuclear extract fractions from normal cells stained by Coomassie blue (A) or analyzed by immunoblotting (B). (C and D) Nuclear extract fractions from heat-shocked cells processed as in A and B, respectively. Distribution of the 6D12 antigens, expressed as a percentage (B/D), is as follows: (lanes 1–10, respectively) 1.0/0.4; 18.9/12.9; 25.7/14.1; 26.9/15.6; 19.6/15.6; 8.0/11.6; 0/8.5; 0/5.1; 0/3.0; and 0/13.1. Lane 1 corresponds to the volume of sample layered on top of the gradient and lane 10 contains the material that might have pelleted. Arrows point to C and A major hnRNP proteins (4).



**Figure 10.** Effect of RNase A treatment on the distribution of the 6D12 antigens in nuclear extracts from normal and heat-shocked HeLa cells fractionated on sucrose gradients. All procedures were as described in Materials and Methods. (A and B) Cells were grown at 37°C and pulse labeled for 1 h with [<sup>3</sup>H]uridine. Equal volumes of nuclear extracts were incubated for 20 min at 25°C with 0 (A) or 0.1 μg/ml RNase A (B) and further fractionated on 10–25% sucrose gradients. The [<sup>3</sup>H]uridine acid-insoluble activities were determined, and the 6D12 antigen distribution was analyzed using a <sup>125</sup>I-labeled second antibody. (C and D) Cells grown as before were heat shocked at 45°C for the last 10 min of the labeling, and equal volumes of nuclear extract were processed as in A and B, respectively. 40% less 6D12 antigens were detected in D when compared with C. Distribution of the antigens in the different regions of the gradients expressed as a percentage (C/D) is as follows: (<50S) 2.8/11.4; (50–100S) 11.4/18.1; (100–200S) 42.9/37.6; (>200S) 42.9/32.9. Fractions 1, 2, and 21 are defined as in Fig. 8.

0.5, 1, 2, 4, or 6 h at 37°C, were analyzed by Western blot. Fig. 5 shows that the 6D12 antigens are always present in the same amount. Thus, the 6D12 antigens are not heat inducible. A polyclonal antibody directed against hsp72 and hsc73 (a gift from Dr. P. May, Institut de Recherches Scientifiques sur le Cancer, Villejuif, France) further revealed that these stress proteins are different from the 6D12 antigens. Indeed, by Western blot analysis, slight but significant differences in the migrations were observed (not shown).

Since the epitope is conserved in *Drosophila*, Western blot analysis was also performed on cellular extracts prepared from equal amounts of normal *Kc167* cells and of cells heat shocked for 10 min at 37°C and allowed to recover for 0, 1, 2, 4, or 6 h at 25°C. Fig. 6 shows that antibody 6D12 revealed a strong single band at 140 kD, which is not heat inducible. Moreover, as expected, the anti-hsp72/hsc73 antibody revealed a band at 70 kD, but did not cross react with the 140-kD antigen (not shown).

#### Antigen-associated Structures in Normal HeLa Cells

Since antibody 6D12 was obtained after immunizing mice with purified hnRNP, one would expect the 6D12 antigens to be associated with these structures in normal cells. In the following series of experiments, we analyzed the behavior of the antigens and further compared the characteristics of the associated structures with those of hnRNP (20).

To determine the density of the antigen-associated structures, HeLa S3 cells were pulse labeled for 1 h with [<sup>3</sup>H]uridine, and the nuclear extract was fixed with formaldehyde and analyzed on a CsCl gradient. Fig. 7 A (solid line) shows that the [<sup>3</sup>H]uridine-labeled heterogeneous nuclear ribonucleic acid (hnRNA) is distributed as a sharp peak at 1.39 g/cm<sup>3</sup>, which fits the values previously determined for hnRNP

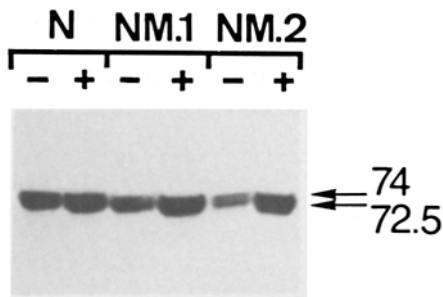
(34). Immunodetection along the gradient shows that the antigen distribution exactly follows that of hnRNP.

For studying the distribution of the antigens as a function of hnRNP size, cells were labeled as above, and the nuclear extract was fractionated on a sucrose gradient. Fig. 8 A shows that hnRNP sediment as a broad peak, between 30 and >200S, with a maximum of ~50S. Immunodetection along the gradient reveals a heterogeneous distribution, showing that the 6D12 antigens seem to be primarily associated with the hnRNP between 50 and 200S and to a much lesser extent with hnRNP <50S. Western blot analysis along such a sucrose gradient shows that there is no difference in the distribution of the two polypeptides (Fig. 9, A and B).

We then tried to localize the 6D12 antigens more precisely within the hnRNP structure, which is in fact comprised of a nuclear matrix fibril and 30–50S monomeric particles also called core particles, the hnRNA being alternately associated with these two types of subcomplexes (20). To study the antigen distribution with respect to these subcomplexes, we took advantage of a difference in sensitivity to KCl and RNase A, which allows further discrimination of the two types of subcomplexes or of their components on sucrose gradients (14, 20).

When hnRNP are treated with 400 mM KCl, the monomeric proteins are entirely solubilized and therefore remain on the top of sucrose gradients, whereas the nuclear matrix fibrils, which are still associated with the hnRNA, continue to enter the gradient (14, 18). Fig. 8 B shows that, due to solubilization of the monomeric proteins, the hnRNP profile is shifted to smaller sedimentation values. In these conditions, the bulk of 6D12 antigens (75%) comigrates with the hnRNA peak, which is typical of the nuclear matrix subcomplex of hnRNP. Only ~25% of the antigens are solubilized in these conditions.





**Figure 11.** Western blot analysis and quantitative comparison of the 6D12 antigens in nuclear matrices from normal and heat-shocked HeLa cells. All procedures were as described in Materials and Methods. Nuclei from normal cells (-) and from cells heat shocked for 10 min at 45°C (+) were isolated. For nuclear matrix preparation, the nuclei were treated with either DNase I (*NM.1*) or DNase I plus RNase A (*NM.2*) followed by high ionic strength extraction. Aliquots containing nuclei (*N*) and nuclear matrices (*NM.1* and *NM.2*) from equal amounts of cells were processed for Western blot analysis.

When hnRNP are treated with RNase A, the nuclear matrix fibril remains heterogeneous in size, even if most hnRNA is hydrolyzed (35), whereas the 30–50S monoparticles are broken down, with part of the products being entirely solubilized and the other part being reassembled in RNP that migrate as a sharp peak at 40–45S (25, 36). Fig. 10 B shows an experiment where the RNase treatment was carried out so that the major part of hnRNA was digested. In these conditions, the antigens were still distributed in a heterogeneous manner, though the profile was shifted towards smaller sedimentation values due to hnRNA and monoparticle protein loss. Again, this behavior is that expected for proteins associated with the nuclear matrix subcomplex of hnRNP.

Thus, various experimental approaches show that in normal HeLa cells the 6D12 antigens are part of hnRNP and also that they mainly behave as proteins from the nuclear matrix subcomplex of hnRNP.

### Rapid Effect of Heat Shock on Antigen Distribution

The *in situ* experiments described above (Fig. 3 C and Fig. 4) suggested that after heat shock the 6D12 antigens are still associated with hnRNP. To further investigate the localization of the 6D12 antigens in heat-shocked cells, we carried out the same series of experiments as for normal cells, using nuclear extracts from cells pulse labeled as before but treated at 45°C during the last 10 min of labeling. In these conditions, the labeled hnRNA mainly represents preexisting hnRNA.

According to earlier data (22, 26), all our experiments show that the general properties of the preexisting hnRNP are not affected by this heat treatment. Indeed, when compared with normal cells, the hnRNA distribution remains unchanged on CsCl gradients (Fig. 7 A) as well as on sucrose gradients (Fig. 8, C and A). Even after salt treatment (Fig. 8, D and B) or RNase treatment (Fig. 10, D and B) no difference is observed. In addition, overall hnRNP protein distribution along sucrose gradients does not show any detectable difference either (Fig. 9, C and A).

As to the 6D12 antigens, CsCl density gradient analysis of a nuclear extract from heat-shocked cells shows that they are still present within the hnRNA peak but that their distribution is now shifted towards smaller hnRNP densities (Fig. 7

B, lane 2). It should be noticed that this shift, which might perhaps appear as relatively small, is nevertheless significant since it has been observed in a reproducible manner in all our experiments. The redistribution of the 6D12 antigens could in fact also be observed on sucrose gradients, where the antigen profile is clearly shifted towards higher average sedimentation values (50 to >300S) (Fig. 8 C). The magnitude of the shift always showed some variations from one experiment to another (see also Fig. 10 C). Analysis by Western blot along such sucrose gradients confirmed this shift (Fig. 9 D) and also showed that after heat shock the two polypeptides are mainly distributed in the same way.

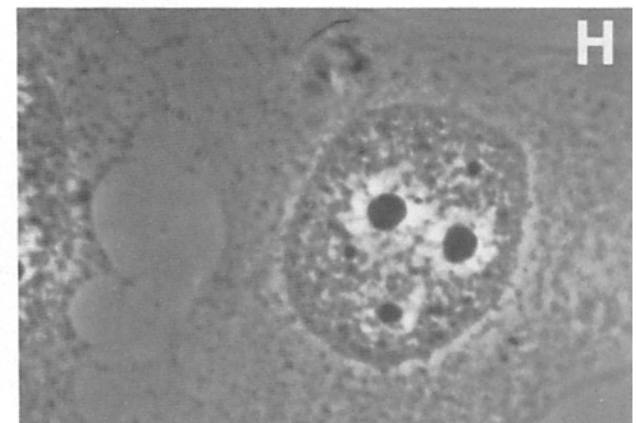
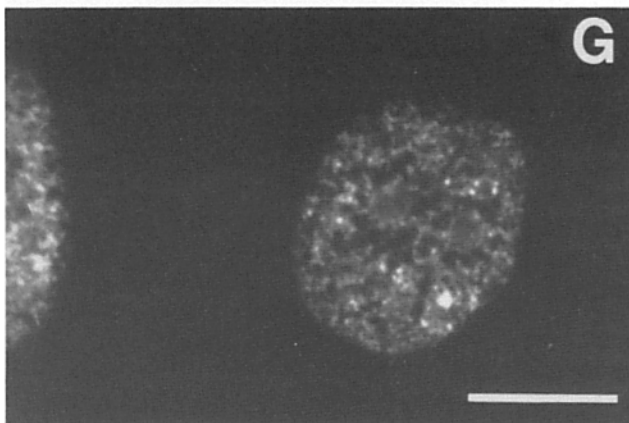
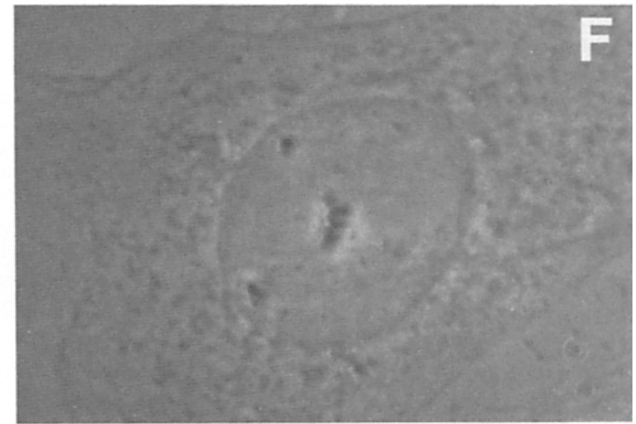
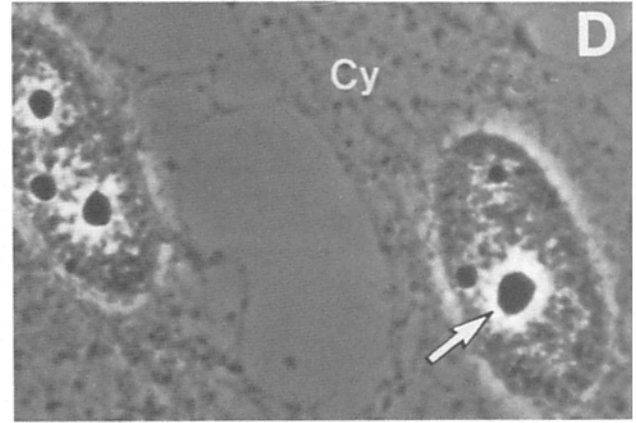
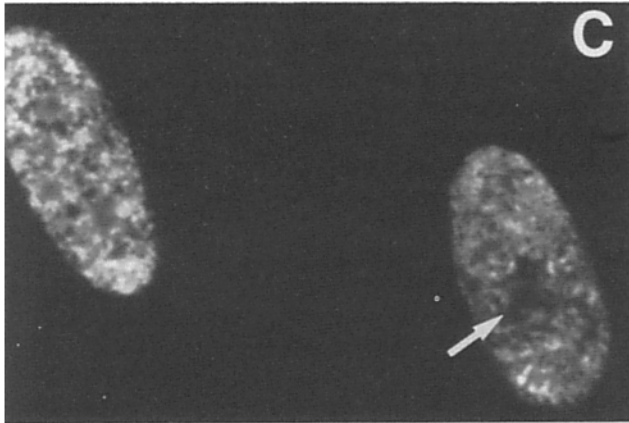
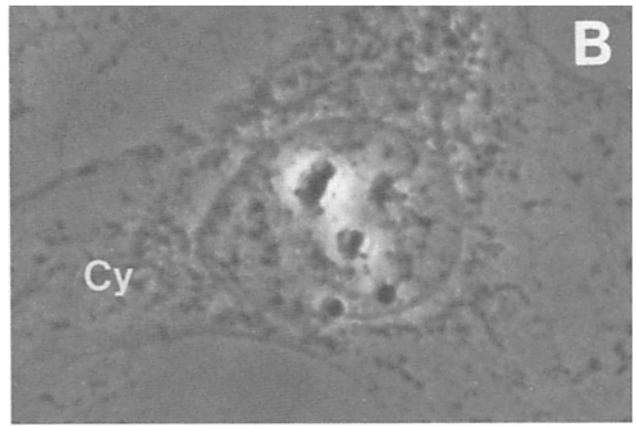
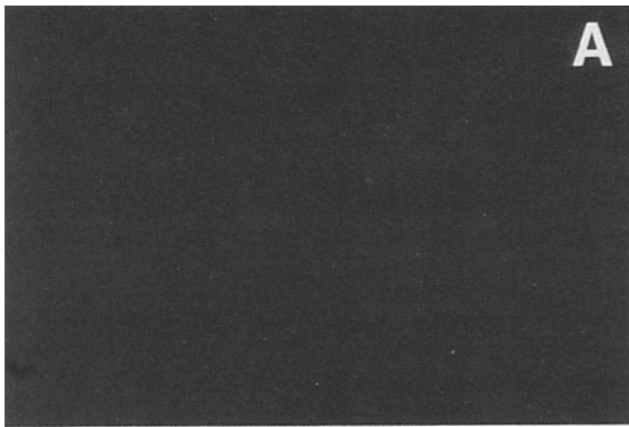
After salt treatment (Fig. 8 D), and in contrast to normal cells, the bulk of 6D12 antigens (80%) did not follow the shift of the hnRNA profile any more but mainly kept on sedimenting in the heavier region of the gradient. Further analysis of these antigen-associated structures shows that they are also relatively resistant to RNase A (Fig. 10 D). Here too, the 6D12 antigens remained basically distributed as in the control (Fig. 10 C), in spite of some decrease of the signal that is probably due to some alteration of the associated structures induced by the degradation of RNA. Thus, these experiments show that after heat shock the 6D12 antigens are strongly bound to the associated structures, which are themselves very resistant.

Taken together, all these data show that after a 10-min heat shock at 45°C, the 6D12 antigens are no longer associated with the bulk of preexisting [<sup>3</sup>H]uridine-labeled hnRNP, whose general properties nevertheless remain unchanged. Instead, they are found on a subpopulation of hnRNP that have a slightly lower average density, a larger average size, and an increased resistance to salt and RNase. This increased stability shows that these hnRNP essentially contain the nuclear matrix subcomplex and only little 30–50S subcomplexes, if any (20, 35).

### Immunodetection of the Antigens on Nuclear Matrices Isolated from Normal and Heat-shocked HeLa Cells

Our experiments on isolated hnRNP showed that the 6D12 antigens are always part of the nuclear matrix subcomplex of hnRNP whether they are associated with the bulk of hnRNP in normal cells or with the resistant hnRNP in stressed cells. Since *in situ* these subcomplexes are known to be part of the internal nuclear matrix (15), we also investigated the behavior of the 6D12 antigens within nuclear matrices prepared from normal and stressed cells. These matrices were either isolated from purified nuclei or prepared as whole mounts and further analyzed by Western blot and immunocytofluorescence, respectively. These two types of preparations, basically carried out in the same way, include a treatment with DNase I or DNase I plus RNase A followed by an extraction with high ionic strength buffer which, of course, also solubilizes the 30–50S monoparticle proteins of hnRNP (15).

Western blot analysis of control nuclei from normal and stressed cells showed, as expected, that the same amounts of 6D12 antigens are present (Fig. 11). When nuclei from normal cells were further processed, it turned out that an important fraction of the antigens definitely remains associated with the nuclear matrix. Even after a drastic additional RNase treatment, a smaller, yet significant, amount of antigens remains associated with the nuclear matrix. Thus, these



data clearly confirm that in normal cells the 6D12 antigens are mainly associated with the nuclear matrix. They further show that the two polypeptides are equally resistant to the salt and RNase treatments. However, as already shown in whole cells, the antigens still remain unavailable for antibody 6D12 in nuclear matrix whole mounts whether or not they are treated with RNase (Fig. 12, *A* and *E*).

Immediately after a 10-min heat shock at 45°C, the 6D12 antigens are still associated with the nuclear matrix (Fig. 11) but, in contrast to normal cells, most or all antigens remain on this structure. An additional RNase treatment does not modify the signal (Fig. 11), which confirms the dramatic increase in stability of this association after heat shock. Immunocytofluorescence analysis of the whole mount counterparts, treated or not with RNase (Fig. 12, *C* and *G*), shows that in both cases the signal appears as small granules associated with the nuclear matrix, the nucleoli and the remnant cytoskeleton being excluded. Moreover, the distribution as well as the intensity of this signal is basically the same as in whole stressed cells (Fig. 3 *C*), which again demonstrates that after heat shock the 6D12 antigens are strongly associated with the nuclear matrix.

Thus, these experiments show that in normal as well as in stressed cells, the 6D12 antigens are definitely associated with the internal nuclear matrix, though in a much stronger manner after heat shock. These results are clearly in line with those obtained by substructural analysis on isolated hnRNP.

#### *Immunocytological Localization of the Antigens during Recovery*

When cells heat shocked for 10 min at 45°C were analyzed by immunocytofluorescence after various periods of recovery at 37°C, the original signal (Fig. 3 *C*) remained unchanged for the first 2–3 h (Fig. 3 *E*) and then decreased gradually (Fig. 3 *G*). Finally, after 6–7 h of recovery, the signal was again the same as in normal cells (Fig. 3 *A*), thus showing that this heat shock effect is entirely reversible. If at this time the cells were challenged by a second heat shock carried out in the same manner, the whole cell population again displayed the same signal as after the first heat shock. An experiment carried out in the same manner as in Fig. 3 revealed that, in contrast to the 6D12 antigens, hsp72 and hsc73 gradually accumulate in the nucleoli during recovery (not shown), as earlier described (39).

A closer examination of the antigen distribution at the later period of recovery (Fig. 3 *G*) shows that the number of fluorescent granules decreased drastically, whereas some bright loci persisted for longer times. Indeed, immunoelectron microscopy studies show that the signal was predominantly located within few loci that gradually appear during recovery and reach a maximum 4–5 h after the onset of heat shock. We have recently established that these loci are active sites of hnRNA synthesis and that they are comprised, as

shown in Fig. 13 *A*, of an area of condensed chromatin, an adjacent area of contrasted fibrils where hnRNA appears first, and clusters of perichromatin granules (PG) (10, 31) to which hnRNA is rapidly shifted and where it is then transiently stored (Puvion, E., A. Viron, F. Harper, P. Mähl, and J. P. Fuchs, manuscript in preparation). Interestingly, Fig. 13 *A* reveals that, within the locus, antibody 6D12 only decorates the hnRNA-containing structures, namely the contrasted fibrils and the perichromatin granules. An enlargement of a cluster of PG (Fig. 13 *B*) clearly shows that part of the PG are individually labeled, the unlabeled ones being probably buried within the section and therefore unavailable for the antibody.

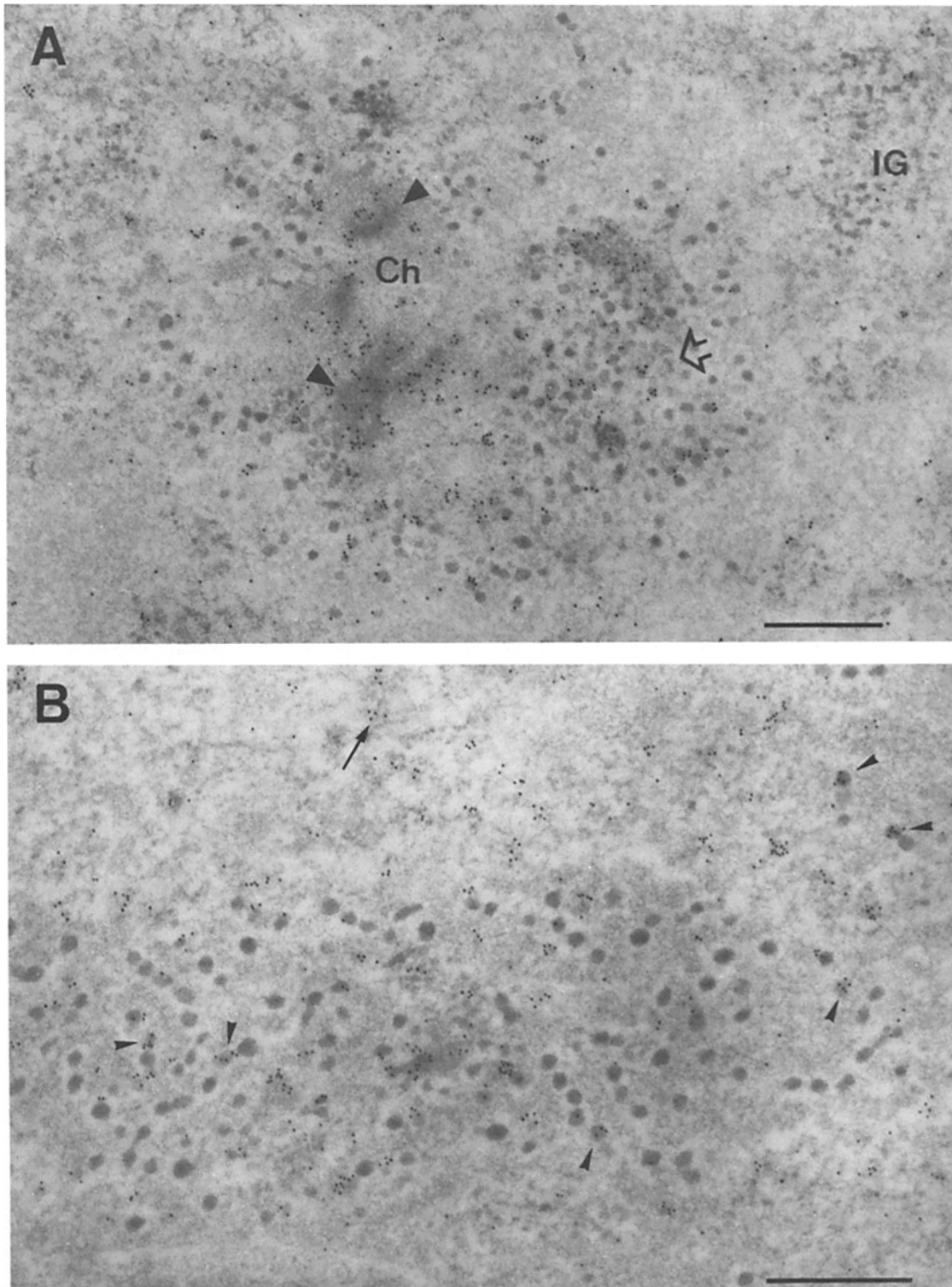
Thus, the early effect of heat shock, which in situ is characterized by a strong signal located on the peri- and interchromatin fibrils all over the interchromatin space, is not simply reversed during recovery. Indeed, during the later phase of recovery the 6D12 antigens are predominantly detected within loci of accumulation of PG, which were not seen by preferential RNP staining early after heat shock.

#### *Discussion*

Previous studies we carried out on HeLa cells first showed that a heat shock performed at a temperature range known to interrupt splicing and nucleocytoplasmic transport (5, 21, 42) in fact rapidly alters the substructure of hnRNP at the level of individual proteins (26). The investigations presented here show that rapid alterations do not only concern the related 35–37-kD antigens we recently described (26) but also extend to other hnRNP antigens with different substructural localization. Interestingly, monoclonal antibody 6D12, which was selected after screening our library for alterations only induced at 45°C and detectable in situ, also recognizes a doublet of polypeptides but with molecular masses of 72.5 and 74 kD. Structural analysis by peptidic mapping shows that the two 72.5–74-kD polypeptides not only share a common epitope but have in fact a good degree of structural homology.

Since the behavior of these two polypeptides is modified by heat shock, an early question in this work was to know whether or not these antigens belong to the hsp70 family. In fact, some of the characteristics of the 6D12 antigens argue against this possibility. First, the 6D12 antigens are not heat inducible, in the sense that they do not accumulate with time. Second, the 6D12 charge isomers are clearly basic (pI 7.5–7.8), whereas all hsp70 charge isomers are acidic (40). Third, the 6D12 antigen distribution revealed by immunocytofluorescence in normal and stressed cells is entirely different from that observed under the same conditions with a polyclonal anti-hsp72/hsc73 antibody and is also different from the individual distributions of hsp72 and hsc73 in both tolerant and nontolerant cells (40). And, finally, in *Drosophila* *KC167* cells antibody 6D12 detects a single noninducible band

*Figure 12.* Immunocytofluorescence localization of the 6D12 antigens in whole mount nuclear matrices from normal and heat-shocked HeLa cells. The procedures were as described in Materials and Methods. Normal cells were treated with DNase I (*A* and *B*) or with DNase I plus RNase A (*E* and *F*) followed by high ionic strength extraction. (*C* and *D* and *G* and *H*) Cells heat shocked for 10 min at 45°C were processed as in *A* and *B* and *E* and *F*, respectively. Shown in *A*, *C*, *E*, and *G* are the fluorescent micrographs and in *B*, *D*, *F*, and *H* are the corresponding phase-contrast micrographs. Arrows show residual nucleolus. Cy, remnant cytoskeleton. Bar, 10  $\mu$ m.



**Figure 13.** Immunoelectron microscopy. Localization of the 6D12 antigens in HeLa cells heat shocked for 10 min at 45°C and allowed to recover for 4 h at 37°C. RNP were preferentially stained and antibody-binding sites were revealed with gold particles. (A) Locus of accumulation of PG. The condensed chromatin (*Ch*) of the locus is not labeled, the gold particles being concentrated on the contrasted fibrils (*arrowheads*) and on the PG (*open arrow*). The cluster of interchromatin granules (*IG*) is not labeled. (B) Enlargement of a cluster of PG. Many PG are significantly labeled (*arrowheads*). Some gold particles are still present on peri- and interchromatin fibrils (*arrow*). Bar, 0.5  $\mu\text{m}$ .

at 140 kD, whereas the anti-hsp72/hsp73 antibody detects a band at 70 kD as expected. Taken together, these data unambiguously show that the 6D12 antigens do not belong to the hsp70 family.

Monoclonal antibody 6D12, which is part of the library we raised against hnRNP from normal cells, recognized antigens that were found to be associated with these structures, as expected. Indeed, studies based on sedimentation properties in both CsCl and sucrose gradients showed that in normal cells the 6D12 antigens are associated with the bulk of hnRNP. At this point, it was interesting to check whether similar proteins, in terms of molecular mass and isoelectric point, are present among the proteins from immunopurified HeLa cell hnRNP previously described by Pinol-Roma et al. (30). Although the 6D12 antigens are not major hnRNP proteins, there might be some similarities with some of the multiple protein species this group has detected in the 68-kD region. However, possible relationships remain to be demonstrated.

Substructural analysis of hnRNP, based on differential sensitivity of the two types of subcomplexes (14, 18, 20, 35), further showed that the 6D12 antigens are mainly associated with the nuclear matrix subcomplex. Consistent with this is the presence of the antigens within purified nuclear matrices. Indeed, this subcomplex is known to be common to hnRNP and the internal nuclear matrix and thus links these two structures in situ (2, 15). In contrast to this series of experiments, in situ studies, performed either by immunocytofluorescence or immunoelectron microscopy, never revealed any signal in normal cells. The most likely explanation would be that the epitopes are masked due to other nuclear components or to a higher degree of folding or compactness of in situ hnRNP vs. isolated hnRNP. In fact, the first possibility seems to be excluded since in whole mount nuclear matrices the 6D12 antigens are still unavailable, though an important part of the nuclear components are extracted during the isolation procedure.

A heat shock at 45°C, as short as 5 or 10 min, is sufficient to modify the behavior of the 6D12 antigens since in situ it triggers the appearance of a strong nuclear signal. In spite of these modifications, it appears that the signal is still associated with hnRNP which, in situ, correspond to peri- and interchromatin fibrils (12, 13, 31, 32). However, additional studies carried out on isolated hnRNP showed that after heat shock the 6D12 antigens are in fact strongly associated with a particular subpopulation of hnRNP. These hnRNP are characterized by a slightly smaller average density, a larger average size, and an increased resistance to salt and RNase when compared with the bulk of preexisting hnRNP which still have the same general properties as the antigen-associated hnRNP from normal cells. This observation on the bulk of preexisting hnRNP is, in fact, a good argument against the possibility that these resistant hnRNP might, for instance, arise due to artefactual hnRNP aggregation. A second argument is that the effect of heat shock on the 6D12 antigens was demonstrated by two different approaches, based on detection in situ and after subcellular fractionation. Therefore, our data rather support the idea that, after the onset of heat shock, the 6D12 antigens rapidly leave the bulk of preexisting hnRNP and switch to this subpopulation of large and resistant hnRNP that mostly contain only one of the two hnRNP subcomplexes, namely the nuclear matrix fibril (14,

20). Since our immunocytofluorescence experiments show that this switch is complete in no more than 5 min after the onset of heat shock, it is likely that hnRNA from the large and resistant hnRNP is preexisting hnRNA. At this point, one might also think of another interpretation of the heat shock effect we describe that would not necessarily be based on an antigen switch. One would then have to assume that in normal cells part of the 6D12 antigens is already associated with the subpopulation of large hnRNP. Heat shock would then trigger structural alterations that allow them to react with the antibody, whereas the bulk of hnRNP would become nonreactive. However, such a possibility seems to be unlikely. Indeed, sucrose gradient fractionation of hnRNP from normal cells followed by protein separation in denaturing conditions and further immunodetection (Fig. 9, A and B) should not only reveal the 6D12 antigens associated with the bulk of hnRNP but also those associated with the subpopulation of large hnRNP, which is obviously not the case.

Although after heat shock the 6D12 antigens are redistributed within the hnRNP population, our data show that in normal and in stressed cells they are always found on the nuclear matrix hnRNP subcomplex and therefore also on the internal nuclear matrix (2, 15). The fact that the 6D12 antigens are associated in a much stronger manner with nuclear matrices isolated from stressed cells is consistent with other studies showing that after heat shock the internal nuclear matrix, as a whole, is stabilized or rigidified in the sense that it becomes more resistant to salt dissociation (28). One aspect of this phenomenon actually appears on phase-contrast images where whole mount nuclear matrices from stressed cells are highly contrasted, whereas those from normal cells are not (Fig. 12). However, according to our studies performed on hnRNP isolated from stressed cells, it seems that this stabilization effect is restricted to those hnRNP that also contain the 6D12 antigens. Indeed, the bulk of preexisting hnRNP, which do not contain the 6D12 antigens any more, still have the same general properties as the bulk of hnRNP in normal cells. Thus, early after the onset of heat shock the 6D12 antigens are only present on stabilized hnRNP.

Immunoelectron microscopy analysis of the loci of accumulation of PG brought the same complementary information on the behavior of the 6D12 antigens. Indeed, within these loci, which are only detected during the later phase of recovery, the 6D12 antigens are again exclusively associated with hnRNP, namely the contrasted fibrils and the PG which both contain newly synthesized hnRNA. Although the characteristics of the contrasted fibrils are still unknown, it is noteworthy that PG are also structures with high stability (31), a feature they share with the antigen-associated hnRNP detected early after the onset of heat shock. PG accumulations might actually account, at least in part, for the relatively high amount of unspliced pre-mRNA shown to accumulate in mammalian cells (5, 21) and that seem to be processed and transported into the cytoplasm later during recovery (21).

In a functional perspective, it has to be emphasized that in nonstressed cells, where splicing and nucleocytoplasmic transport are actively carried out, the 6D12 antigens are associated with the bulk of hnRNP. In contrast, after a heat shock in temperature conditions that are known to lead to the inhibition of splicing and transport (5, 21, 42), these antigens rapidly leave the bulk of hnRNP whose general properties

remain unaltered. Taken together, these results suggest that the 6D12 antigens might well be involved in the control of splicing and/or transport. Such a possibility is all the more likely since the 6D12 antigens are in fact always associated in some way with the internal nuclear matrix, which was shown to be actively involved in splicing (33, 43, 44). Furthermore, since stabilization of the nuclear matrix is thought to be part of the survival strategy of the cell (28), stabilization of hnRNP might possibly be a way to protect hnRNA from degradation and also from uncontrolled splicing and transport. Interestingly, during the early as well as during the later phase of recovery the 6D12 antigens are exclusively associated with such stabilized hnRNP whose RNA transcripts are likely to be spliced and transported into the cytoplasm once normal physiological conditions are restored (21).

Finally, it is interesting to mention that these 72.5–74-kD antigens and the previously described 35–37-kD antigens (26), which in normal cells belong to distinct hnRNP subcomplexes, behave in a similar way early after the onset of a severe heat shock. Having now characterized these two antigen doublets and further studied their behavior in both normal and stressed cells, investigations on their structure and function will be pursued. Therefore, we will use two complementary approaches based on cDNA cloning and direct use of the two selected monoclonal antibodies in appropriate *in vitro* and *in vivo* systems.

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