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RESPONSE OF HIGH MOBILITY GROUP PROTEINS OF HUMAN KIDNEY T1 AND MURINE L 929 CELL LINES TO HEAT SHOCK

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Summary

High mobility group (HMG) proteins in human kidney T1 and murine L 929 cells have been investigated after exposure to heat shock at 41 °C and their influence on the organizational change of chromatin under heat shock condition has been examined. Results reveal that the two cell lines show differential response of the HMG proteins 1 & 2 and 14 & 17 to heat shock. Neither T1 nor L 929 cells show significant differences in response to heat shock with respect to the binding affinities of HMG proteins 1 & 2 or 14 & 17 to DNA, as revealed by DNase I sensitivity and chromatin reconstitution assays. Furthermore, the HMG proteins of both the non-heat shocked and the heat shocked T1 and L 929 cells can recover their chromatin activity following reconstitution. These findings suggest that although the HMG proteins might undergo some change in response to heat shock, their inherent potential of reassociation with DNA is still retained.

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Key Words: **T1 cells in vitro, L 929 cells in vitro, high mobility group proteins, heat shock, chromatin organization, DNase I**

The high mobility group (HMG) proteins are a class of non-histone chromosomal proteins having a critical role in the induction and regulation of gene expression, and in DNA replication (1-4). The HMG proteins bind with the nucleosome in either the core or the linker region and show strong affinities for both single and double stranded DNA in chromatin. After the depletion of HMG, the chromatin becomes resistant to DNase I, importantly reflecting their influence in the functional organization of chromatin (5). However, it is not clear which type of alteration is caused in the nucleosome or chromatin structures by the depletion of HMG (3,6).

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HMG proteins of various eukaryotic systems show considerable homology among the specific HMG types suggesting conservation of the proteins during evolution (3,7). Another well studied conserved protein family is that of heat shock proteins. If heat shock is given to cells, tissues, or organisms 7 to 9 different heat shock genes are activated while most other genes become silent (7-9). These facts raise the questions: (a) does heat shock induce the HMG protein genes to become silent and (b) does heat shock change the DNA-HMG interaction in the chromatin?

Here, the influence of heat shock on the major HMG proteins of T1 and L 929 cells has been investigated. The binding affinities of the extracted HMG proteins from normal and heat shocked cells (or nuclei) to HMG depleted chromatin have also been analyzed.

Materials and Methods

Cell culture

T1 cells (human kidney) and L 929 (murine fibroblast) were cultured in basal medium Eagle (BME) and Eagle's minimum essential medium (MEM), respectively, with 10 % fetal calf serum, 1 % glutamine and 10,000 units/l penicillin/streptomycin in 600 ml culture flasks at 37 °C in presence of 5 % CO_2. The cells attained confluence between days 5 and 6 without changing the growth medium. On day 6, the confluent monolayer (mainly G_0) was trypsinized (0.25 % trypsin) to prepare single cell suspension for use in these experiments (10).

Heat shock treatment

Cells (5 - 10 x 10^7) were incubated in growth medium either at 37 °C (without heat shock, HS^-) or at 41 °C (with heat shock, HS^+) for 30 min. The medium was removed by centrifugation (121 x *g* , 10 min, 4 °C) and the cell pellet was used for protein labeling or for isolation of nuclei.

Radioactive labeling of proteins

The tritium-labeled amino acids were purchased from Amersham, UK. After washing with fresh growth medium at 37 °C cells were pulsed with ^{3}H-aspartic acid (5.55 MBq/ml), ^{3}H-leucine (3.7 MBq/ml) or ^{3}H-lysine (37 MBq/ml) for 30 min either at 37 °C (HS^-) or at 41 °C (HS^+). Following the pulse the cells were washed with growth medium at 37 °C and then transferred into saline-EDTA (50 mM NaCl, 1 mM EDTA, pH 7.0) for isolation of nuclei.

Isolation of nuclei

Cells were washed with 5 ml of saline-EDTA, resuspended in TMN buffer (10 mM tris-HCl, pH 6.8, 5 mM $MgCl_2$, 10 mM NaCl) and centrifuged (190 x *g*, 5 min). To the pellet, 5 ml of TMN buffer, 1.7 ml of 1 M sucrose and 100 µl of 25 % solution of Triton® X-100 containing 0.1 mM phenyl-methyl-sulfonyl fluoride (PMSF) were added. The suspension was incubated on ice for 20 min with occasional shaking. Thereafter, cells were homogenized for 5 min on ice in a moderately tight teflon homogenizer. The suspension was centrifuged (1710 x *g*), the supernatant aspirated out, and the pellet (nuclei) washed once with 5 ml TMN buffer containing 0.1 mM PMSF.

Extraction of HMG proteins

After incubation of cells at 37 °C (HS^-) or 41 °C (HS^+), the isolated nuclei were used for the extraction of HMG proteins. The nuclei were dispersed in 5 ml of 5 % perchloric acid (PCA) containing 0.1 mM PMSF and centrifuged (427 x *g*, 10 min, 4 °C). The supernatant was mixed with 1.25 ml of 100 % of trichloroacetic acid (TCA) to a final concentration of 20 % and left on ice for 2.5 h. The TCA precipitate containing HMG and some histone proteins were collected by

centrifugation (800 x *g*, 10 min, 4 °C). The total time between termination of heat shock expo
and beginning of extraction was about 75 min.

Fractionation of HMG proteins by acetone-HCl and acetone
The 20 % TCA precipitable proteins were dispersed in 2.8 ml of a freshly prepared mixtu
acetone and 1 M HCl (5:2, v/v), kept on ice for 40 min and centrifuged (8000 x g, 10 min, 4
The pellet containing mainly HMG 1 & 2 was retained. The supernatant was mixed with
volumes of acetone and left for 2.5 h on ice to precipitate the HMG 14 & 17. The precipitate
collected by centrifugation (8,000 x g, 10 min, 4 °C). Both pellets were separately dissolve
TE buffer (10 mM tris-HCl, pH 7.4, 1 mM EDTA) containing 5 µl of 1 M dithiothreitol
stored at -80 °C.

Polyacrylamide gel electrophoresis
The purified proteins were analyzed by 12.5 % SDS-polyacrylamide gel electropho
following the method of Laemmli (11). The electrophoresis was carried out at 30 mA (stac
gel) and 50 mA (separating gel) with a circulating water cooling system at 0.9 °C. The gels were stained by coomassie brilliant blue.

Isolation of chromatin from native and HMG depleted nuclei
After the extraction of HMG proteins by 5 % PCA (see above) the nuclei were subjected to two more similar extractions to obtain the final HMG depleted nuclei. Non-extracted nuclei were the source of native chromatin. Both HMG depleted and native nuclei were separately lysed in TNES buffer (10 mM tris-HCl, pH 7.8, 75 mM NaCl, 1 mM EDTA, 0.5 % SDS) for 1 h. The viscous liquid was centrifuged (27,590 x *g*, 4 °C, 30 min). The pellet was suspended in 1.5 ml of 5 mM Na_3PO_4 and 2 mM EDTA (pH 7.4). The samples were stored at -80 °C.

Reconstitution of chromatin with HMG proteins
For the reconstitution of the HMG-depleted chromatin (chromatin^{-HMG}) with specific HMG fractions, the procedure of Weisbrod *et al.* (1) was essentially followed. To 500 µl of a chromatin suspension ($A_{260} \approx 1$) 100 µl of the desired HMG fraction and an equal volume of 0.8 M NaCl containing 0.1 mM PMSF were added and mixed. After an incubation for 40 min on ice the mixture was extensively dialysed against TMN buffer. The reconstituted chromatin was stored at -80 °C.

Chromatin activity assay by DNase I fragmentation
The native chromatin, the chromatin^{-HMG} or the reconstituted chromatin were subjected to the test of DNase I sensitivity according to Weisbrod and Weintraub (5) with minor modifications. In brief, chromatin preparations ($A_{260} = 0.1$) were mixed with 10 units/µl of DNase I and incubated for 20 min at 37 °C. The reaction was terminated by transferring the tubes to ice and adjusting EDTA to 2.5 mM. Finally, an equal volume of TES buffer (20 mM tris-HCl, pH 7.4, 2 mM EDTA, 1 % SDS) was added, mixed thoroughly and the tubes were kept for 30 min at room temperature and subsequently for 15 min on ice. The control samples (without DNase I) were mixed with the same volume of the solution containing 0.15 mM NaCl and 50 % glycerol and treated the same way. The samples were electrophoresed at 40 mA constant current on 1 % agarose gel using TBE electrode buffer (12). The gel was stained with 0.5 % ethidium bromide in TBE for 30 min and photographed with a UV transilluminator using Polaroid film 55.

Results

Comparison of HMG proteins from human kidney T1 cells and murine L 929 cells before and after heat shock

Figs. 1 and 2 show the SDS-PAGE profiles of the isolated HMG proteins from T1 and L 929 cell lines, respectively. HMG proteins 1 and 2 (HMG 1 & 2) were mainly fractionated by acetone/HCl (lane 1) while HMG proteins 14 and 17 (HMG 14 & 17) exclusively by acetone (lane 2). In T1-cells, the HMG 1 & 2 (Fig. 1, lane 1) as well as HMG 14 & 17 (Fig. 1, lane 2) were more pronounced after heat shock than before. This is in contrast to the HMG profiles from L 929 cells (Fig. 2). These results are representative of 6 and 10 independent experiments with T1 and L 929 cells, respectively. The HMG bands were identical to those of the purified HMG proteins (through the courtesy of G. H. Goodwin) and those reported earlier (13).

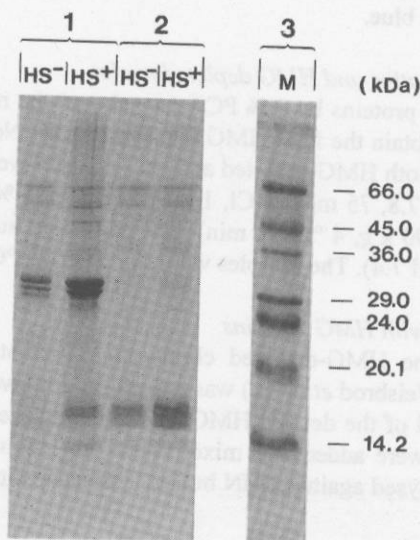

Fig. 1

SDS-polyacrylamide gel (12.5 %) electropherogram of HMG proteins of T1 cells. Lanes 1 and 2 represent acetone/HCl and acetone fractions, respectively from cells without (HS^-) and with heat shock (HS^+). Lane 3 shows molecular size marker (M).

Pattern of synthesis of HMG proteins in T1 and L 929 cells before and after heat shock

Whether HMG proteins were synthesized *de novo* after heat shock treatment or not has been examined by extracting the proteins after labeling with ^{3}H-aspartic acid, ^{3}H-leucine or ^{3}H-lysine at 37 °C (HS^-) or at 41 °C (HS^+). The syntheses of HMG 1 & 2 as well as HMG 14 & 17 were enhanced after heat shock in T1 cells while in L 929 cells syntheses of all HMG proteins were inhibited as compared with the unheated samples.

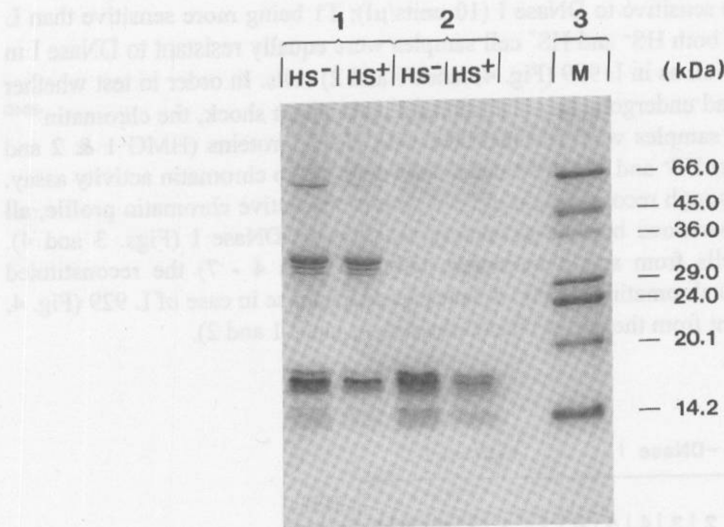

Fig. 2

SDS-polyacrylamide gel (12.5 %) electropherogram of HMG proteins of L 929 cells. Lanes 1 and 2 represent acetone/HCl and acetone fractions, respectively, from cells without (HS^-) and with heat shock (HS^+). Lane 3 shows molecular size marker (M).

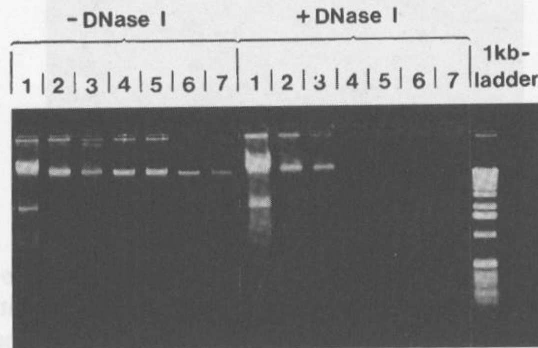

Fig. 3

Agarose gel (1 %) electropherogram of T1 cell chromatin exposed to DNase I (+) and compared with that in its absence (-): lane 1 = chromatin without heat shock (HS^-); lane 2 = HMG depleted chromatin ($chromatin^{-HMG}$) from HS^- cells; lane 3 = $chromatin^{-HMG}$ from heat shocked (HS^+) cells; lane 4 = reconstituted chromatin: $chromatin^{-HMG}$ from HS^- cells and HMG from HS^- cells; lane 5 = reconstituted chromatin: $chromatin^{-HMG}$ from HS^- cells and HMG from HS^+ cells; lane 6 = reconstituted chromatin: $chromatin^{-HMG}$ from HS^+ cells and HMG from HS^- cells; lane 7 = reconstituted chromatin: $chromatin^{-HMG}$ from HS^+ cells and HMG from HS^+ cells. A 1 kb ladder has been used as molecular size marker.

Organization of native and HMG-depleted chromatin (chromatin^{-HMG}) in T1 and L 929 cells
The chromatin activity assay revealed that the native chromatin of T1 (Fig. 3, lane 1) as well as L 929 (results not shown) were sensitive to DNase I (10 units/µl); T1 being more sensitive than L 929. The chromatin^{-HMG} from both HS$^-$ and HS$^+$ cell samples were equally resistant to DNase I in T1 (Fig.3, lanes 2 and 3) as well as in L 929 (Fig. 4, lanes 1 and 2) cells. In order to test whether the chromatin organization had undergone any alteration following heat shock, the chromatin^{-HMG} from both the HS$^-$ and HS$^+$ samples were reconstituted with HMG proteins (HMG 1 & 2 and HMG 14 & 17) extracted after HS$^-$ and HS$^+$ treatments and subjected to chromatin activity assay. The results show that even though reconstitution did not restore the native chromatin profile, all reconstituted chromatin preparations became extremely sensitive to DNase I (Figs. 3 and 4). While in the case of T1 cells from any combination (Fig. 3, lanes 4 - 7) the reconstituted chromatin appeared similar to chromatin^{-HMG} (Fig. 3, lanes 2 and 3), those in case of L 929 (Fig. 4, lanes 3 - 6) were very different from the native chromatin (Fig. 4, lanes 1 and 2).

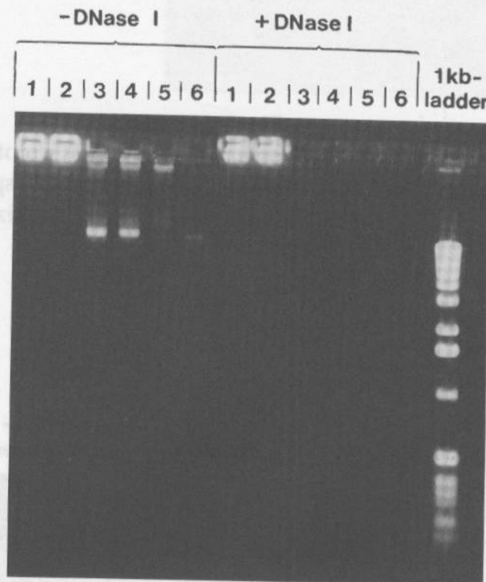

Fig. 4
Agarose gel (1 %) electropherogram of L 929 chromatin exposed to DNase I (+) and compared with that in its absence (-): lane 1 = HMG depleted chromatin (chromatin^{-HMG}) from cells without heat shock (HS$^-$); lane 2 = chromatin^{-HMG} from heat shocked (HS$^+$) cells; lane 3 = reconstituted chromatin: chromatin^{-HMG} from HS$^-$ cells and HMG from HS$^-$ cells; lane 4 = reconstituted chromatin: chromatin^{-HMG} from HS$^-$ cells and HMG from HS$^+$ cells; lane 5 = reconstituted chromatin: chromatin^{-HMG} from HS$^+$ cells and HMG from HS$^-$ cells; lane 6 = reconstituted chromatin: chromatin^{-HMG} from HS$^+$ cells and HMG from HS$^+$ cells. A 1 kb ladder has been used as molecular size marker.

Discussion

HMG 1 & 2 and 14 & 17 represent two sub-classes of a family of proteins affecting chromatin structure. Significant work on the nucleotide sequences of the genes of these HMG proteins, their

amino acid sequences and evolutionary conservation have been reported (3, 4). In this present study, the influence of the HMG protein family on chromatin organization constituted the focus of the study. The effects of heat shock on HMG protein function, its implications for understanding modifications in protein structure and its continued ability to reconstitute chromatin have been critically assessed. Since heat shock effect is not reversed until after 90 min (14,15) the total time between termination of heat shock exposure and the beginning of extraction was kept within 75 min.

The results (Figs. 1 and 2) reveal that the HMG proteins from the two cell lines, viz. human kidney T1 cells and murine L 929, are inherently different from each other with respect to their responses to heat shock. Compared with the controls, heat shock induced higher amounts of HMG 1 & 2 and HMG 14 & 17 in T1 cells (Fig. 1) and lower in L 929 cells (Fig. 2). This finding is supported by observation of similar differences in the *de novo* synthetic pattern of HMG proteins. The reasons for this difference between T1 and L 929 cells are not clearly understood and may be due to inherent differences between the two systems. The possibility that L 929, a murine cell line, is relatively thermotolerant can not be ruled out. Nonetheless, the reduction of HMG proteins after heat shock to L 929 cells (Fig. 2) and inhibited rate of their synthesis show that heat shock treatment was affecting the murine cell line.

The chromatin activity assay (Figs. 3 and 4) shows that chromatin^{-HMG} becomes resistant to cleavage by DNase I indicating organizational alterations. Heat shock did not reverse this situation. When HMG depleted chromatin from cells, which were not heat shocked or were subjected to heat shock, were reconstituted with HMG proteins (extracted either from HS^- or from HS^+ cells) the chromatin became hypersensitive to DNase I (Figs. 3 and 4). It was notable that the reconstitution in all combinations yielded identical hypersensitivities. Heat shock did not appear to play any role in this phenomenon. Thomas and Elgin (16) have shown that the hypersensitivity of the active genes might be due to the binding of HMG proteins at non-coding regions. It is possible that in T1 and L 929 cells the HMG proteins were also binding to non-coding regions during reconstitution. Here, results support this contention as the reconstituted chromatin preparations were hypersensitive to DNase I but did not restore the native chromatin profiles (Figs. 3 and 4). The hypersensitivity towards DNase I indicates that the ability of the HMG proteins to interact with DNA was not influenced by either HMG extraction or heat shock. However, the specificity of interaction of HMG to DNA nucleotide sequence may have been altered which, in part, may be responsible for shut down of normal gene expression following heat shock (9). Further investigation is required to elucidate mechanisms by which HMG genes in T1 cells (Fig. 1) remain open after heat shock.

In conclusion, this report shows that heat shock did not close HMG genes in T1 cells as it did in L 929 cells. In both cell lines heat shock did not alter the ability of reassociation of HMG proteins to DNA and the reconstituted chromatin preparations became hypersensitive to DNase I. The presence of HMG proteins was essential for chromatin organization as their depletion leads to extreme resistance to DNase I.

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