Investigations of Mechanisms of Drug-induced Changes in Gene Expression: \( N \)-Methylformamide-induced Changes in Synthesis of the \( M_r \) 72,000 Constitutive Heat Shock Protein during Commitment of HL-60 Cells to Granulocyte Differentiation

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N-Methylformamide-induced Changes in Synthesis of the Mr 72,000 Constitutive Heat Shock Protein during Commitment of HL-60 Cells to Granulocyte Differentiation

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ABSTRACT

HL-60 cells were treated with the differentiating agent N-methylformamide and early changes in gene expression and protein content were investigated. Analysis of protein synthesis had previously shown an early (<12 h) fall in the synthesis of Mr 70,000 heat shock proteins (F. M. Richards, A. Watson, and J. A. Hickman, Cancer Res., 48: 6715-6720, 1988). The changes have now been characterized in detail and their kinetics compared to those of the expression of the c-myc protein. Immunoblot analysis, using antibodies to either the stress-inducible heat shock protein hsp70 (4G4) or a pan-Mr 70,000 heat shock protein antibody (3A3), showed that there was a striking reduction in the levels of the constitutive heat shock protein hsc70 when cells were incubated continuously with 170 mM N-methylformamide. A reduction in the level of hsc70 RNA was observed within 3 h and continued thereafter. In contrast, transcription of the hsc70 gene was induced within 1–2 h, after which the rate returned to basal level. There were no significant changes in the rate of transcription of the stress-inducible heat shock proteins hsp70 or hsp90. When N-methylformamide was removed from the cells, prior to commitment to differentiation, the levels of hsc70 were reestablished, whereas after 36 h of treatment there was no recovery. Western blotting with an antibody to the c-myc protein showed this to fall to virtually undetectable levels by 3 h under the same conditions. The results suggest that the loss of hsc70, which may perform a protein chaperoning role, was mediated at both transcriptional and posttranscriptional levels of regulation and was an early event closely associated with the commitment of HL-60 cells to differentiation. The fall in hsc70 was not associated with alterations in the cell cycle, nor were the kinetics of the change suggestive of a relationship with the decrease in content of c-myc protein.

INTRODUCTION

Structurally and mechanistically disparate pharmacological agents are able to induce the terminal maturation of HL-60 promyelocytic leukemia cells along the granulocyte-neutrophil pathway (1). An interesting question regarding this response to such disparate agents concerns the nature of the mechanisms whereby interactions of drugs, at presumably different loci, are able to initiate the molecular events that lead to the differentiated state (2). Of particular interest are the conserved changes in gene expression of the “immediate-early” response genes, such as c-myc (3, 4). In previous studies, observations were made that the induction of differentiation of HL-60 cells took place when concentrations of agents were used which were marginally below those which induced cytotoxicity (5). We went on to suggest that the induction of terminal differentiation may involve a stress response but paradoxically we observed that one of the earliest changes in protein observed in HL-60 cells treated with NMF was a fall, rather than an increase, in the synthesis of the Mr 70,000 heat shock proteins (6). We were unable to identify which specific members of the Mr 70,000 heat shock proteins were being modulated.

Changes in the synthesis of heat shock proteins early during differentiation have been observed in Friend erythroleukemia cells treated with dimethyl sulfoxide (7), mouse embryonal cells treated with retinoic acid (8), HL-60 cells treated with phorbol esters (9, 10), THP-1 monocytic leukemia cells treated with γ-interferon or retinoic acid (10), K562 erythroleukemia cells treated with hemin (11), and in a variety of developmental processes (12, 13). The significance of these changes is not clear, and the qualitative, quantitative, and temporal aspects of them differ markedly according to cell type and conditions. Some of the changes appear to be associated with alterations in the proliferative status of the cells. The expression of the stress-inducible hsp70 has been shown to be cell cycle regulated (14).

The expression of the protooncogene c-myc is also associated with the proliferative capacity of a variety of cells (15) and a fall in expression has been considered to herald a withdrawal from the cell cycle (16). A fall in the expression of c-myc has also been observed to be a prelude to the terminal differentiation of HL-60 cells (3, 4). The kinetics of the change in c-myc expression was found to depend upon the nature of the inducer used and upon the lineage to which the cells become committed (17, 18). Because of a colocalization between c-myc protein and the stress-inducible hsp70 (19), we were interested in determining the precise characterization of the heat shock protein which changed as HL-60 cells were committed to differentiate, and whether there might be a relationship between the kinetics of this change and that of the expression of c-myc.

Detailed descriptions of the regulation of the expression of some of the genes which encode the heat shock proteins have emerged recently, most particularly those of the Mr 70,000 family (20). Their role in protecting the cell from the effects of elevated temperatures (21) and their constitutive role as protein chaperones (22, 23) is the subject of intensive investigation. Because of this emerging knowledge of their molecular and cellular biology we consider study of changes in expression of the Mr 70,000 heat shock proteins may provide clues as to how a drug might bring about changes in gene transcription as well as perhaps clarifying what role these proteins may play in the process of differentiation. The solvent NMF was chosen as an archetypical differentiating agent (24) since it had been the subject of previous studies by us (5, 6, 25, 26).

MATERIALS AND METHODS

Materials. All materials were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom) unless otherwise stated.
CO2, 100% humidity. Cells were routinely maintained in logarithmic phase and subcultured by trypsin-EDTA removal from tissue culture flasks (Costar, High Wycombe, Buckinghamshire, United Kingdom) added directly to the cell cultures to give a concentration of 100,000 and cells were removed at predetermined time points for the assessment of differentiation and protein or RNA analyses.

**Assessment of Differentiation.** The percentage of differentiated HL-60 cells was assessed based on the ability to produce superoxide anion in response to TPA stimulation and reduce the dye nitroblue tetrazolium, as described in detail by us previously (5).

**Cell Cycle Kinetics.** Following drug treatment for up to 48 h, cells were resuspended in fixative (0.1% paraformaldehyde, 0.1% Triton) at a density of 1 x 10^6/ml for a minimum of 4 h at room temperature. Twenty μl propidium iodide solution (2.5 mg/ml) were added sequentially to each 500-μl sample, incubated for 5 min at room temperature, before cell cycle analysis using a Coulter EPICS Counter. The flow cytometer was set to excite at 400 nm with the 488 nm line and red fluorescence was collected through a 630 nm long pass filter. Cell cycle analysis was performed by using in-house software.

**Measurement of Gene Transcription Rates.** Isolation of nuclei and transcription run-on analysis were carried out according to previously published methods (11, 27). Briefly, isolated nuclei were labeled with [32P]UTP (Amersham International, Aylesbury, Buckinghamshire, United Kingdom) and the transcription reaction was arrested by the addition of 600 μl of a stop buffer and a further 2-h incubation at 45°C. After trichloroacetic acid precipitation of the labeled nascent transcripts, they were hybridized with plasmid DNA immobilized onto nitrocellulose Hybond-C (Amersham International; pH 2.3 (human hsp70) (28); pUC 801 (human hsp90a) (29); pUC 811 (human hsp90b) (29); pHG2 (human grp78) (30); pH7.6 (human hsc70) (24); pH-1 (human o-actin) (31) at 42°C for 72 h, in a buffer containing 50% formamide, 6 x SSC, 10 x Denhardt's, 0.2% SDS. Filters were then washed in a series of buffers: 6 x SSC/0.2% SDS, 1 x 15 min at room temperature; 2 x SSC/0.2% SDS, 2 x 30 min at 65°C; 0.2 x SSC/0.2% SDS, 2 x 30 min at 65°C and exposed to X-ray film (X-OMAT AR, Kodak, Hemel Hempstead, Hertfordshire, United Kingdom) at -80°C.

**Isolation of RNA and Western Blot Analysis.** Total cellular RNA was isolated by using a modified version of the guanidinium isothiocyanate method as described previously (32). Briefly, 5 x 10^6-1 x 10^7 cells were lysed in 4 μl guanidinium isothiocyanate solution, layered onto 5.7 M CsCl/0.1 M EDTA, and centrifuged overnight (35,000 x g, 4°C, 17 h). Following phenolic extraction and overnight ethanol precipitation, 20 μg purified RNA were fractionated on a denaturing 1% agarose-formaldehyde gel. Ethidium bromide (40 μg/ml) was included with the RNA to allow visualization of the loading of each gel. After overnight capillary transfer to Hybond-C, RNA was immobilized by UV irradiation and prehybridized in formamide containing buffer (50% formamide, 6 x SSC, 5 x Denhardt's, 0.1% SDS, 50 μg/ml tRNA), at 42°C for approximately 6 h. Filters were hybridized overnight to the 32P-random prime-labeled (Boehringer Mannheim, Lewes, East Sussex, United Kingdom) 600-bp base pair EcoRI fragment encoding the human 5' sequence of hsc70 (pha 7.6). The filters were then washed in a series of buffers, as described above for analysis of transcription.

**Immunoblot Analysis.** Cells (2 x 10^6) were washed in PBS, denatured by boiling in reducing sample buffer (2% SDS, 10% glycerol, 0.002% bromophenol blue, 40 μM Tris, pH 6.8), and total cellular protein was separated by SDS-polyacrylamide gel electrophoresis by using 10% polyacrylamide gels. Following the electrophoretic transfer of proteins to nitrocellulose Hybond-C Extra, the membranes were blocked overnight in 5 mg/ml BSA in PBS, and washed in 0.1% Tween in PBS. The gels were stained for residual protein with Coomassie blue to ensure equal loading and transfer. Membranes were then incubated with either anti-hsp70 specific (4G4), anti-hsp70/hsc70 (3A3), mouse monoclonal antibodies (gifts from Dr. Dawn Murphy, Northwestern University, Evanston, IL), or rabbit antisera pan-c-myc antibody (a gift from Dr. Gerard Evan, Imperial Cancer Research Fund, London) for 1-2 h (4G4 and 3A3 antibodies were diluted 1:100 and c-myc 1:1000 in 5 mg/ml BSA in PBS before use) and washed with 0.1% Tween in PBS. This was followed by an additional 1-h incubation with affinity purified goat anti-mouse IgG or goat anti-rabbit IgG conjugated to horse radish peroxidase (diluted 1:60000 in 5 mg/ml BSA in PBS before use) and repeated washings with 0.3 and 0.1% Tween 20 in PBS. The antibody-specific proteins were visualized by using the enhanced chemiluminescence detection system according to the recommended procedure (Amerham International). Filters that were to be probed with a different antibody were first incubated in stripping buffer (1× Tris, pH 6.8, 2% SDS, and 5% β-mercaptoethanol) for 30 min at 50°C. The immunoblotting procedure was then repeated as above.

**RESULTS**

**Characterization of NMF-induced Changes in Levels of the Mr 70,000 Heat Shock Proteins.** Immunoblot analysis showed that continuous treatment of HL-60 cells with 170 mM NMF induced a time-dependent decrease in the cellular amount of a heat shock protein with a molecular weight of approximately 70,000 (Fig. 1). Analysis by SDS-polyacrylamide gel electrophoresis of changes in protein synthesis, after labeling with [35S]methionine confirmed our previous findings (6) of a fall in the synthesis of a Mr 70,000 protein after approximately 6 h (data not shown). In order to identify which member of the hsp70 family changed, we compared the Western blot results by using two monoclonal antibodies, 3A3, which recognizes both the constitutive hsc70 and the inducible hsp70, and 4G4, which recognizes only hsp70. Protein samples from HL-60 cells exposed to NMF, and from heat-shocked HeLa cells, were analyzed by using the inducible hsp70-specific antibody, 4G4. The HeLa cells were used to provide a strongly inducible heat shock response, and therefore an increase in the level of the stress inducible hsp70 protein (Fig. 2A); Western blotting of HL-60 cells did not show a very significant change in the amount of this protein after heat shock, despite our previous finding of some change in synthesis (Fig. 1). The blot was then reprobed by using the antibody 3A3, which detects both the inducible and constitutive forms of hsp70. Both HL-60 and HeLa heat shock 70 proteins were immunologically reactive with 3A3 (Fig. 2B). Because of the slightly inducible nature of the constitutive heat shock protein, hsc70, and the severalfold induction of the stress inducible hsp70 protein following a heat shock, these two proteins from HeLa cells are not fully resolved. The detection of an additional upper band following incubation with 3A3 is, however, evident and is labeled accordingly (Fig. 2, Lane 6). This result suggested that there was a high basal expression of the constitutively expressed hsc70 protein which was reduced following NMF treatment. Although 1-dimensional analysis failed to detect the heat/stress-inducible protein hsp70 in HL-60 cells, small amounts of this protein were detected by using 2-dimensional
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Fig. 2. Detection of hsc70 protein expression in HL-60 cells using a combination of two mouse monoclonal antibodies specific for more than one member of the heat shock protein 70 family. Western blot analysis of total cellular protein from 2 × 10⁵ cells/lane was first carried out by using (A) the monoclonal antibody, 4G4, specific for the stress-inducible form of the Mr 70,000 heat shock protein, hsp70. A protein sample from heat-shocked (42°C, 1 h) HeLa cells was included as a positive control. The filter was stripped and reprobed (B) with the use of the monoclonal antibody 3A3 to detect both the constitutive (hsc) and inducible (hsp) forms of hsp70.

Fig. 3. In vitro transcription in nuclei of HL-60 cells isolated during NMF treatment, and after a heat shock of 42°C for 1 h. Following isolation of nuclei and in vitro transcription reactions, [³²P]UTP-labeled transcripts were hybridized to filter bound DNA: pH 2.3 (human hsp70); pUC 801 (human hsp90α); pUC 811 (human hsp90β); pHG2 (human grp78) pH7.6 (human hsc70); pHFA-1 (human α-actin).

Western analysis, and the levels remained essentially unchanged throughout NMF treatment (data not shown). The onset of the selective decrease in the constitutive protein hsc70 was evident after 12 h of NMF exposure. The level of hsc70 fell to almost undetectable levels by 36 h, concomitant with the irreversible commitment of the cells to terminal differentiation (see below).

Changes in Heat Shock Gene Expression. Exposure to 170 mM NMF consistently induced the transient and selective increase in the transcription rate of the hsc70 gene within 1–2 h (Fig. 3). Significantly, the transcription of its stress-inducible counterpart, hsp70, and another heat shock gene hsp90, were unaffected. Following the transient induction of hsc70 transcription, there was a return to control levels by 4–8 h. Fig. 3 shows that heat and NMF treatment of HL-60 cells induced transcriptional changes of the α-actin gene, so that the basal and steady state levels of transcription of the other heat shock genes were taken to represent steady state levels of transcription. The expression of the hsc70 gene was also examined by Northern blot analysis. It was observed that hsc70 message levels were significantly reduced by 3 h (Fig. 4), despite the maintenance of basal levels of transcription (Fig. 3). Ethidium bromide staining of the gel prior to transfer (shown in Fig. 4, bottom) ensured equal loading of RNA. Significant levels of protein were detectable (Fig. 1) up until after 12 h, suggesting either that the protein had a significantly longer half-life than its message or that its half-life was extended in the presence of NMF.

Relationship between Changes in hsc70, c-myc Levels, Growth Arrest, and Commitment to Differentiation. The conditions for optimal HL-60 differentiation have been described previously (5): a 96-h continuous incubation with 170 mM NMF induced maximum differentiation to granulocyte-like cells. These parameters were reinvestigated for the purpose of current experiments, and furthered to determine the time after which the cells became irreversibly committed to a terminally differentiated phenotype, the so-called commitment time. It was found that a minimum exposure time of between 36 and 48 h to NMF was required to commit cells, as assessed by nitroblue tetrazolium reduction (Fig. 5A). Flow cytometric analysis, during the first 48 h of NMF exposure, showed that there was no significant change in the distribution of cells in the cell cycle, at which time the cells were committed. Analysis of cell numbers confirmed this (Table 1). Interestingly, cell cycle progression of HL-60 cells occurred independently of much reduced levels of c-myc protein. We observed a rapid decline in the levels of c-myc protein well before proliferation ceased (Fig. 5B). Although appreciable levels of c-myc were undetectable by approximately 3 h, cells were still capable of initiating another full round of cell division (Table 1).

Immunoblot analysis of proteins isolated from cells which had been incubated for increasing times with 170 mM NMF, then washed before a 24-h drug-free recovery period, showed that the effects of NMF to suppress hsc levels were reversible up to, but not beyond the point of

Fig. 4. Northern blot analysis of hsc70 gene expression during NMF treatment (0–6 h). Total cellular RNA was isolated at the indicated times using the guanidinium isothiocyanate/CsCl method. Twenty μg RNA were separated on a denaturing formaldehyde gel, transferred to nitrocellulose, and hybridized to a ³²P-labeled 600-base pair DNA fragment of hsc70 (pHA7.6).
commitment to granulocytic differentiation (Fig. 6). The production of hsc70 protein content after a 36-h exposure to NMF was not restored to control level after a 24-h recovery period.

DISCUSSION

HL-60 cells treated with NMF underwent an early fall in cellular levels of one of the members of the M, 70,000 heat shock family. This corresponded to our previous observations regarding the fall in synthesis of heat shock proteins (6). Immunoblot analysis using two antibodies, which discriminated in the pattern of recognition of the members of the M, 70,000 hsp, suggested, by elimination, that the heat shock protein which was reduced in amount was the constitutive hsc70. Analysis of the proteins by two-dimensional electrophoresis followed by immunoblotting, showed that there was no change in the stress/heat-inducible hsp70 (data not shown). The disparity observed between transcription rate and the levels of RNA and protein suggest that the transcripts detected by nuclear run-on analysis may be incomplete and therefore not able to be fully processed to produce mature mRNA to be used for translation. This does not eliminate the possibility of the involvement of changes in the rate of transcription in some regulatory mechanism. The transcription rate of hsc70 was low (Fig. 3), but the constitutive levels of mRNA appeared to be considerable (Fig. 4), which is suggestive of the production of a stable message. The reduction in the amount of hsc70 mRNA to a level below that of control, under conditions of continued basal transcription, therefore suggests that NMF may affect message stability. Previous reports have shown that heat-inducible hsp70 expression is regulated at both transcriptional and posttranscriptional levels (40). Although down-regulation of hsc70 protein levels were also observed, these occurred at a time after the changes in RNA levels (compare Figs. 1 and 4). This suggests that either the stability of the protein is greater, or that RNA stability was changed after NMF treatment. This is under investigation.

The temporal change in hsc70 level induced by NMF was strongly suggestive of it playing a role in the maintenance of the block in differentiation which characterizes leukemic HL-60 cells. A comparison of the kinetics of the reduction in hsc70 protein levels with those of commitment (Fig. 5A) showed that as levels fell below those detectable by immunoblotting, cells were becoming committed to


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differentiate. Moreover, the fall in hsc70 levels was reversible only until 36 h of NMF treatment, a time which corresponded to the irreversible commitment to terminal differentiation (Fig. 6).

Early changes in transcription and in mRNA were followed by a fall in hsc70 protein levels by about 12 h. We were concerned that these falls in hsc70 levels might simply reflect accumulating numbers of cells moving out the cell cycle and to growth arrest, since the stress-inducible hsp70 had been shown to be cell cycle regulated (41). Analysis of the cell cycle and measurement of cell numbers did not support this hypothesis (Table 1). Additional experiments, not reported here, have shown that hsc70 protein levels remained unchanged when HL-60 cells were treated with 300 μM NMF, which induced a rapid cytostasis. Recently, it was reported that TPA induced an increase in the stress-inducible hsp70 mRNA in HL-60 cells committed to monocyte differentiation (9). These cells were rapidly growth arrested and it is possible that these changes may reflect changes in cell cycle rather than differentiation.

The loss of proliferative capacity of NMF-treated HL-60 cells coincided with their commitment to differentiation, which has been related to changes in the expression of the protooncogene c-myc (3; 4). We were interested in the temporal relationship, if any, between changes in the nuclear c-myc protein and hsc70, because hsc70 has been implicated in the translocation of proteins from the cytoplasm to the nucleus (36). Additionally, there has been an association reported between a heat shock protein and c-myc (19; 42). This raised the possibility that the fall in hsc70 might be associated with changes in c-myc expression in differentiating HL-60 cells. Immunoblotting showed that the fall in cellular levels of c-myc after NMF treatment occurred within 2 h (Fig. 5B), whereas changes in hsc70 levels did not occur until after 12 h (Fig. 1). This disparity of kinetic changes does not support the idea that hsc70 may be involved with changes in c-myc levels.

Interestingly, the rapid fall in c-myc protein levels occurred well before any changes were observed in growth or differentiation of the HL-60 cells. Furthermore, the cells initiated another full round of division in the absence of detectable levels of c-myc protein. Similar observations have been reported by other workers who have suggested that the kinetics of the decline in c-myc varies both with the nature of the inducing agent and the lineage to which the cells become committed (16; 17; 43).

The loss of a constitutively expressed protein, which has a role as a molecular chaperone, from cells committed to differentiate by NMF raises a number of important questions. How NMF brings about this process is a molecular curtain, from cells committed to differentiate by NMF to the inducing agent and the lineage to which the cells become committed (16; 17; 43). Additional observations have been reported by other workers who have suggested that the kinetics of the decline in c-myc varies both with the nature of the inducing agent and the lineage to which the cells become committed (16; 17; 43).

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