Changes in the Translational Activity of Polyadenylated Messenger RNA of HL60 Promyelocytic Leukemia Cells Associated with Myeloid or Macrophage Differentiation

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ABSTRACT

In order to characterize the events which commit the HL60 human promyelocytic leukemia cell line to differentiate into macrophages or mature myeloid cells, we have analyzed the in vitro [35S]methionine-labeled translational products obtained from polyadenylated messenger RNA of the HL60 cells before and after exposure to: (a) dimethylformamide (DMF), an inducer of myeloid differentiation; (b) 12-O-tetradecanoylphorbol-13-acetate (TPA), an inducer of macrophage differentiation; or (c) a combination of the two inducers. Exposure of the HL60 cells to either TPA or DMF results in decreases in the relative abundancy of translational products with molecular weights of 20,000, 17,000, and 15,000. Exposure of the HL60 cells so as to generate macrophage differentiation results in elevations of the abundancy of translational products with molecular weights of 60,000, 47,000, 42,000, 35,000, 32,000, 27,000, 14,000, and 12,300, while DMF-induced myeloid differentiation is associated with increases in the abundancy of translational products with molecular weights of 60,000, 42,000, 35,000, 32,000, 27,000, 13,000 and 12,300. The addition of the macrophage inducer TPA to HL60 cells previously exposed to the myeloid inducer DMF results in changes in the relative abundancy of several translational products, yielding a pattern which differs quantitatively from that obtained from cells treated with DMF or TPA alone. These changes in the relative abundancies of the HL60 translatable products suggest that the steady state levels of several different populations of mRNA or the ability of these mRNAs to be translated are being modified during the induction of myeloid or macrophage differentiation in the HL60 promyelocytic leukemia cell line.

INTRODUCTION

Established cell lines have been used as model systems in which to study the genetic mechanisms which mediate differentiation in normal and leukemic hematopoietic cells. The HL60 human leukemia cell line, which was isolated by Collins et al. (2), was shown by these workers to resemble promyelocytes and to mature into metamyelocytes when exposed to DMF (2, 3). These and other workers subsequently demonstrated that exposure of these cells to the tumor promoter TPA resulted in a population of cells which contained the differentiated markers of macrophages (11–13). Studies with cloned populations of HL60 cells established that the HL60 cell line was composed of a single cell type which is competent to commit itself to macrophage as well as myeloid differentiation (4). The commitment of HL60 cells to a macrophage lineage by TPA is irreversible after 6 hr of treatment and is not affected by the subsequent addition of DMF to the culture. In contrast, the commitment of myeloid differentiation requires continuous exposure of the cells to DMF for 96 hr (4). Addition of TPA to cells undergoing DMF-induced myeloid maturation results in a population with a mixed myeloid-macrophage phenotype (4).

In this report, a quantitative analysis of the in vitro translational capacity of polysomal poly(A)+ mRNA extracted from HL60 cells treated for various periods of time in culture with either DMF or TPA is carried out. The pattern of translational products obtained with poly(A)+ mRNA extracted from HL60 cells sequentially treated with DMF and the TPA (mixed myeloid-macrophage phenotype) is shown to differ from that obtained with mRNA extracted from HL60 cells treated with either DMF or TPA alone.

MATERIALS AND METHODS

Maintenance of HL60 cells was carried out as described previously (4). Cells are passed at a cell concentration of 5 x 10⁵ cells/ml, and the inducers TPA (Sigma Chemical Co., St. Louis, Mo.) or DMF (Sigma) are added at a final concentration of 64 mw or 120 mw, respectively, 48 hr following passage, when the cell concentration approaches 2 x 10⁶ cells/ml. Cultures are intermittently fed and found to be free of Mycoplasma infection.

Polysomal poly(A)+ mRNA Extraction. Chemically treated or untreated cells were harvested by centrifugation, washed once in 0.9% NaCl solution, and pelleted again by centrifugation at 4°C. The cells are resuspended in 10 to 20 volumes of lysing buffer [0.14 M NaCl/0.05 M MgCl₂/10 mM Tris (pH 7.4)/0.25 mM sucrose] supplemented with RNA 50 μg/ml and 0.05 μM volume of 10% Nonidet P-40. The suspension is then subjected to 4 to 5 strokes in a loose Dounce homogenizer followed by centrifugation at 10,000 rpm for 10 min at 4°C. The resulting postmitochondri al supernatant is carefully layered over a 2.0 M sucrose cushion in lysing buffer, and the polysomes are pelleted by centrifugation at 45,000 rpm for 3.5 hr at 4°C. The resulting pellet is then dissolved in RNA extraction buffer [0.5% sodium dodecyl sulfate/0.075 M NaCl/0.025 mM EDTA/0.001 M Tris (pH 7.4)]. This solution is then subjected to 2 x 10⁶ cells/ml. Cultures are intermittently fed and found to be free of Mycoplasma infection.

In Vitro Translation. The in vitro translation assay was carried out with a cleared reticulocyte lysate translation kit (New England Nuclear, Boston, Mass.). The magnesium and potassium ion concentrations were optimized to concentrations of 200 and 15 μM, respectively, for...
incorporation of [35S]methionine using HL60 mRNA. One μg of polysomal poly(A)+ mRNA was incubated in 25 μl of translation reaction mix at 37°C for 90 min. The reaction was terminated by the addition of EDTA to a final concentration of 4 mM, and pancreatic RNase (40 μg/ml) was added. The samples were allowed to incubate for an additional 20 min at 37°C prior to analysis by polyacrylamide gel electrophoresis.

**Electrophoretic Separation of Translational Products and Their Quantitation.** One-dimensional separation of translation products was carried out by polyacrylamide gel electrophoresis using a linear 10 to 18% gradient system with a 4% stacking gel and conditions similar to that described by O’Farrell (10) for molecular weight separation of 2-dimensional gels. Routinely, 2 to 4 μl of a translation mix (200,000 cpm) were added with an equal volume of 2× loading buffer [0.25 Tris (pH 6.8)/10% β-mercaptoethanol/2.0% sodium dodecyl sulfate/20% glycerol/bromophenol blue] and boiled for 4 min prior to loading. Electrophoresis was carried out under constant current (10 ma) for 12 to 16 hr. Following electrophoresis, the gels were fixed in 10% trichloroacetic acid for 20 min and treated for fluorography with EN3HANCE (New England Nuclear) as suggested by the company. The gels were then dried on an LKB Instruments gel-drying system and exposed to X-ray film (XRP-1; Kodak, Rochester, N. Y.) for various periods of time.

Two-dimensional polyacrylamide gel electrophoresis with equilibration isoelectric focusing was performed utilizing conditions similar to those described by O’Farrell (10) and with a 10 to 18% (w/v) gradient in the second dimension. Gels were loaded with approximately 1.2 × 10⁶ cpm of radioactive protein corresponding to a volume of 4 to 8 μl which were diluted to 50 μl with the electrophoretic buffer.

Quantitation of the relative density (relative abundance) of bands obtained in the fluorogram was carried out with a Kratos Model SD3000 spectrodensitometer equipped with a density computer and a Hewlett-Packard 3380A integrator (Palo Alto, Calif.). For each gel analyzed, long-, intermediate-, and short-exposure fluorograms were scanned at varied sensitivity and background reject settings.

**RESULTS**

Changes in Cell-free Translational Activity of HL60 Poly(a)+ mRNA following DMF and TPA Treatment. We studied the changes in the relative abundance of the mRNA populations present in HL60 at several points following exposure of the cells to DMF or TPA by utilizing *in vitro* translation, 10 to 18% gradient polyacrylamide gel electrophoresis, and fluorography. Polysomal poly(A)+ mRNA was extracted as described in "Materials and Methods" from untreated HL60 cells during an exponential phase of growth; from HL60 cells treated with DMF for 48, 96, and 120 hr; and from HL60 cells treated with TPA for 8, 18, and 48 hr. By 120 hr of treatment with DMF or 48 hr of treatment with TPA, greater than 90% of the cells can be shown to contain markers specific for myeloid or macrophage differentiation, respectively (4). We chose to study the translational products of poly(A)+ mRNA from HL60 cells treated with TPA for 8 and 18 hr, since these 2 time points permit characterization of changes in populations of mRNA during and immediately after the cells commit themselves to macrophage differentiation (4).

A typical fluorogram of [35S]-labeled translational products obtained with poly(A)+ mRNA of untreated (Fig. 1, Lane 4), DMF-treated (Fig. 1, Lanes 1, 2, and 3), and TPA-treated (Fig. 1, Lanes 5, 6, and 7) HL60 cells is presented in Fig. 1. The electrophoretic pattern of translational products is highly reproducible except for the products migrating at M, 23,000 and M, 25,000 in TPA-treated cells and for the M, 31,500 and M, 32,500 products in DMF-treated cells, for which variation in intensity in the fluorograms may be seen from run to run. As shown in Fig. 1, a M, 42,000 translational product clearly increases in intensity during the macrophage or myeloid differentiation induced by exposure of the cells to TPA or DMF, respectively, while a M, 17,000 band present in untreated HL60 cells clearly decreases in relative intensity following treatment of the cells to DMF or TPA.

Quantitation of changes in relative intensity of several predominant bands was carried out by scanning densitometry of the fluorograms. The relative density of each band was graphically displayed, and the area under each peak was integrated. Each lane in Fig. 1 was scanned at 3 or more different intensity settings on both short- and long-exposure fluorograms. The scanning profiles for each lane in Fig. 1 developed by this method are presented in Chart 1, and the relative abundance (the percentage of the total integrated absorbance on each fluorogram lane) for 25 of 45 total resolvable bands is presented in Table 1. Analysis of the data presented in Chart 1 and Table 1 shows that the transition from promyelocytes to either metamyelocytes or macrophages induced by exposure of HL60 cells to DMF or TPA results in increases in the relative intensities of the translational products migrating at the M, 60,000, M, 42,000, M, 32,000, M, 27,000, and M, 12,300 positions. Exposure of the HL60 cells to TPA results in unique increases in the species migrating at M, 47,000, and M, 14,000, while DMF-induced myeloid differentiation is associated with unique increase in the products migrating at the M, 35,000 and M, 13,000 positions.

Commitment and differentiation from promyelocytes to metamyelocytes or macrophages result in decreases in the intensity of the M, 20,000, M, 17,000, and M, 15,000 translational products in HL60 cells. When one follows the relative abundance of each translational product throughout the full 120 hr of DMF and 48 hr of TPA induction, transitory increases and decreases in intensity of these species may be seen.
decreases are observed to accompany DMF (e.g., M, 47,000, M, 38,000, and M, 32,500 bands) and TPA (e.g., M, 38,000 and M, 23,000 bands) treatment. The patterns observed for poly(A)+ mRNA at the end of DMF and TPA treatment are similar with major quantitative differences being observed with Poly(A)+ mRNA of HL60 Promyelocytic Leukemia Cells

Changes in Cell-free Translational Products Generated in Vitro from Poly(A)+ mRNA in HL60 following Combined TPA and DMF Treatment. In a previous publication (4), we demonstrated that TPA-treated HL60 cells which have committed to the macrophage differentiation pathway are not phenotypically affected by subsequent withdrawal of TPA and exposure to DMF (4). In contrast, cells treated for 96 to 120 hr with DMF followed by 24 hr of incubation in medium supplemented with TPA change from suspension to adherent cell growth and display both macrophage- and myeloid-specific markers (4). It was, therefore, of interest to perform analyses similar to those described above with polysomal poly(A)+ mRNA from HL60 cells treated for 96 hr with DMF followed by 24 hr of treatment with TPA. For comparative purposes, polysomal poly(A)+ mRNA from HL60 cells treated for 24 hr with TPA or for 96 hr with DMF were translated in vitro and analyzed in parallel. The resulting polyacrylamide gel fluorogram, densitometry scans, and relative abundancies of translational products for these 3 mRNA preparations are presented in Fig. 2, Chart 2, and Table 2, respectively.

As indicated in Table 2, the patterns of the translational products obtained with polysomal poly(A)+ mRNA from cells treated for 96 hr with DMF or 24 hr with TPA show differences in the relative abundance of translational products which migrate at M, 47,000, M, 21,000, M, 35,000, M, 13,000, and M, 12,300. A comparison of the translational products obtained with poly(A)+ mRNA from HL60 cells sequentially treated with DMF and TPA with those obtained with either inducer alone shows, however, that the relative abundance of most of the rest of the bands observed in cells exposed to the combination of DMF and TPA are quantitatively similar to values obtained with DMF and TPA poly(A)+ mRNA. However, significant differences in the relative abundance of specific translational products are also noted.

The relative abundance of the M, 35,000 and M, 12,300 translation products.

Table 1

<table>
<thead>
<tr>
<th>M. (x 10^-3)</th>
<th>Relative % abundance (% of total absorbance)</th>
</tr>
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<tbody>
<tr>
<td>Uninduced</td>
<td>8 hr TPA</td>
</tr>
<tr>
<td>85.0</td>
<td>0.81</td>
</tr>
<tr>
<td>78.0</td>
<td>0.83</td>
</tr>
<tr>
<td>65.0</td>
<td>2.06</td>
</tr>
<tr>
<td>62.0</td>
<td>0.90</td>
</tr>
<tr>
<td>47.0</td>
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</tr>
<tr>
<td>45.0</td>
<td>6.44</td>
</tr>
<tr>
<td>43.5</td>
<td>6.31</td>
</tr>
<tr>
<td>40.0</td>
<td>2.03</td>
</tr>
<tr>
<td>37.0</td>
<td>2.26</td>
</tr>
<tr>
<td>36.0</td>
<td>&lt;0.50</td>
</tr>
<tr>
<td>35.0</td>
<td>1.08</td>
</tr>
<tr>
<td>32.5</td>
<td>3.40</td>
</tr>
<tr>
<td>31.5</td>
<td>3.05</td>
</tr>
<tr>
<td>27.0</td>
<td>&lt;0.50</td>
</tr>
<tr>
<td>25.0</td>
<td>1.99</td>
</tr>
<tr>
<td>23.0</td>
<td>2.08</td>
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<tr>
<td>21.0</td>
<td>1.02</td>
</tr>
<tr>
<td>20.0</td>
<td>0.99</td>
</tr>
<tr>
<td>18.5</td>
<td>1.17</td>
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<tr>
<td>17.0</td>
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<tr>
<td>15.5</td>
<td>5.18</td>
</tr>
<tr>
<td>14.0</td>
<td>3.45</td>
</tr>
<tr>
<td>13.0</td>
<td>2.35</td>
</tr>
<tr>
<td>12.3</td>
<td>2.21</td>
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</table>

* Values indicated are for a double band. No attempt has been made to resolve the components.
translational products obtained with the combination of DMF and TPA are significantly higher than that observed with HL60 cells treated with either inducer alone. In contrast, the M, 27,000, M, 20,000, and M, 17,000 products observed in cells exposed to the combination of DMF and TPA were lower than values obtained in cells treated with either inducer alone. In addition, the M, 47,000 translational product of DMF and TPA combination appears to have a relative abundance value which is intermediate between the values obtained with poly(A)+ mRNA preparations from DMF- and TPA-treated cells, suggesting some form of balanced modulation of the abundance of the mRNA coding for the M, 47,000 translational product with DMF + TPA treatment.

**Analysis of Actin-like Proteins in DMF-, TPA-, and DMF plus TPA-induced HL60 Cells by 2-Dimensional Gel Electrophoresis.** One-dimensional gel electrophoresis of DMF, TPA, and DMF plus TPA revealed a marked increase in a protein species with a molecular weight of approximately 42,000 over that seen in the uninduced cells (Figs. 1 and 2; Charts 1 and 2). When 2-dimensional gel electrophoresis and fluorography in conjunction with in vitro translation was utilized to study the changes in the relative abundance of the mRNA populations in HL60 exposed to the various inducers for different time intervals, a marked increase over time in a translation product with a molecular weight of 42,000 and pi 5.3 was observed (Fig. 3). This protein species has a molecular weight and pi identical to those of actin (8). Hoffman-Leiberman and Sachs (7) have reported a marked increase in actin production during induced differentiation of mouse myeloid leukemic cells to mature macrophages and have found actin to be the major cytoplasmic protein in normal mouse granulocytes. Hartwig and Stossel (5, 14) have reported that actin makes up about 12% of the protein of normal macrophages. Therefore, a marked increase in this actin-like protein during the induced differentiation of HL60 is certainly compatible with the acquisition of the mature myeloid-macrophage phenotype.

**DISCUSSION**

As a first step in the analysis of the changes which occur in the steady state levels of mRNA populations during commitment to and expression of myeloid or macrophage programs of hematopoietic differentiation, we have quantitatively analyzed the translational capacity of polysomal poly(A)+ mRNA from HL60 cells before and after exposure to the inducers TPA or DMF. The relative levels of 3 translational products with
molecular weights of 20,000, 17,000, and 15,000, are seen to decrease, while products with molecular weights of 60,000, 42,000, 32,000, 27,000, and 12,300, are seen to increase in abundance when untreated HL60 promyelocytic leukemia cells are induced to either myeloid or macrophage differentiation. The levels of an additional 2 species with molecular weights of 47,000 and 17,000 appear to increase more following exposure of the HL60 cells to TPA, while 2 others with molecular weights of 36,000 and 13,000 appear to increase more following exposure to DMF. Transitory changes in the levels of a few translational products are also observed suggesting their importance during early phases of myeloid or macrophage differentiation. Finally, we demonstrate that sequential treatment of HL60 cells in culture with DMF and TPA directly affects the relative abundance of different mRNAs in both a synergistic and antagonistic manner. We cannot discern whether these latter changes in mRNA levels occur in all cells or in subsets of the total cell population. However, due to the variety of cellular functions affected by these compounds, it is possible that each agent exerts an effect at different points in a common pathway regulating steady state levels of specific mRNAs, leading to a dampening or potentiation of the final regulatory signal.

Hoffman-Liebermann et al. (6) have recently demonstrated that the combined treatment of mouse myeloid leukemia cells with TPA and a macrophage-granulocyte inducing protein, both potent inducers of the mouse myeloid leukemia cell to the mature macrophage state, results in a marked synergistic response in gene expression by both increasing new mRNA production and translating preexisting mRNA.

Several features of this system make the analyses that we have conducted only approximations of the steady state levels of the mRNAs which are the templates of the translational products we have discussed above. The one-dimensional gel system in which densitometer profiles were utilized to quantitate the various protein species cannot distinguish between an increase in the relative abundance of one species and changes in multiple translational components. Additional technical obstacles in these analyses which prevent precision in the estimation of the relative abundance of translational products by peak area integration include changes of peak height due to peak clustering, variations in background absorption in the fluorogram, and poor resolution of closely migrating bands. Although we have tried to overcome these limitations by measuring major band intensities in fluorograms exposed for shorter and longer periods and densitometric scanning at varying sensitivity settings and speeds, the development of specific hybridization probes by molecular cloning techniques as well as densitometer analysis of 2-dimensional gel electrophoretic patterns of these translational products will be necessary to obtain truly quantitative estimates of changing mRNA levels during commitment and differentiation of the HL60 cells.

Utilizing 2-dimensional gel electrophoresis of HL60 cells which were labeled with [35S]methionine following the addition of either dimethyl sulfoxide, macrophage-granulocyte inducer protein, or TPA, Lieberman et al. (9) demonstrated a greater than 50% change in the qualitative protein patterns. These results differ significantly from ours. These authors utilized pulse-labeled whole cells, and thus their results are tempered by the variability in pool sizes and availability of the various pools during the differentiation process as well as degradation and variations in the half-lives of proteins. Our investigation was concerned with characterization of the translational activity of unique poly(A)+ polysome-bound mRNA generated during the differentiation process. This approach eliminated the aforementioned difficulties associated with pulse-labeled whole cells, and we think that it is a more direct approach to delineating those genetic mechanisms involved in HL60 differentiation. Two-dimensional gel electrophoresis studies utilizing polysome-bound poly(A)+ mRNA are in progress.

In spite of these limitations, our analyses of HL60 cells have clearly shown that the relative abundances of specific poly(A)+ mRNAs are either increasing or decreasing in a noncoordinate fashion during DMF or TPA induction of myeloid or macrophage differentiation. The increase in levels of mRNAs coding for the M, 42,000 and M, 12,300 species during DMF-induced myeloid differentiation and the increases in the mRNAs coding for M, 42,000 and M, 31,500 peptides during TPA-induced macrophage differentiation could be a function of activation of transcription of developmentally related families of genes or could reflect differential rates of mRNA processing or stability of gene transcription. Hoffman-Liebermann et al. (6) have documented that both of these processes take place during TPA- or macrophage-granulocyte inducer protein-induced differentiation of the mouse leukemia cell. The identification of the changes occurring in the translational products of these mRNA populations which we have conducted is a necessary first step in the development of the molecular tools which will be required to resolve these alternatives. It is through these latter studies that we hope will emerge a clearer idea of the molecular nature of the final regulatory signals which mediate the changes in the steady state levels of specific mRNA which we have shown to accompany the switch from myeloid to macrophage differentiation in the HL60 promyelocytic leukemia cell lines.

REFERENCES

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Fig. 3. Two-dimensional gel electrophoretic fractionation of HL60 translation products before and after exposure to DMF, TPA, and DMF plus TPA. Growth of the cells, concentration of the inducers, extraction of the mRNA, and 2-dimensional gel electrophoresis were performed as described in “Materials and Methods.” Arrows, translation product M, 42,000 and pI 5.3.
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