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Control of lysozyme gene expression in differentiating HL-60 cells

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SUMMARY. We have investigated the control of lysozyme gene expression in HL-60 cells induced to differentiate into macrophage-like cells with phorbol myristate acetate (PMA). Differentiation, as evidenced by cellular adherence, and morphological changes corresponded temporally to an increase in nonspecific esterase activity. The lysozyme concentration in the medium of uninduced HL-60 cells was 10 $\mu g/10^7$ cells, increasing to a maximum of 46 $\mu g/10^7$ cells after 48 h incubation with PMA (16 nM). At 72 h the lysozyme concentration decreased to 16 $\mu g/10^7$ cells. Intracellular lysozyme activity remained constant throughout differentiation.

If HL-60 cells were exposed to PMA for 24 h, washed, then maintained in normal medium, they differentiated normally, confirming their irreversible commitment to differentiate. The increase in lysozyme secretion by these cells, however, is markedly blunted suggesting that continued PMA treatment of differentiated cells is required for their secretion of lysozyme. There is no change in the rate of extracellular degradation of lysozyme during differentiation.

The level of lysozyme mRNA does not correlate directly with the amount of lysozyme secreted into the medium. Hybridization of uninduced HL-60 cell RNA with a chicken lysozyme cDNA probe demonstrates moderate hybridization. There is a modest (five-fold) increase in lysozyme mRNA between 0 and 36 h of exposure to PMA, corresponding to the burst of lysozyme secretion by these cells. The lysozyme mRNA decreases to a level which is lower than the original baseline by 72 h, when the cells are still secreting substantial amounts of lysozyme.

These data suggest that both transcriptional and post-transcriptional controls are operative in the control of lysozyme gene expression during the differentiation of HL-60 cells. They also imply that lysozyme secretion is not a necessary component in the macrophage–monocyte differentiation of these cells.

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Lysozyme (muramidase) is secreted by both normal and leukaemic cells of the macrophage/monocyte lineage. We have studied the control of expression of the lysozyme gene in differentiating HL-60 cells (Collins *et al*, 1977; Gallagher *et al*, 1979), a promyelocytic leukaemia cell line which may be induced to differentiate into either granulocytes or macrophages (Collins *et al*, 1979, 1980; Tarella *et al*, 1982; Breitman *et al*, 1980, 1981; Olsson & Olofsson, 1981; Fontana *et al*, 1980; Olsson *et al*, 1982; Todd *et al*, 1981; Huberman *et al*, 1981; Abraham & Smiley, 1981; Rovera *et al*, 1979, 1980; Huberman & Callahan, 1979). Although the macrophage-like cells which are induced by induction with PMA are not functionally normal macrophages (Newburger *et al*, 1981), they do demonstrate increased secretion of lysozyme. Lysozyme is the predominant newly synthesized protein secreted activated macrophages (Gordon *et al*, 1974). The mechanism of this increase has been previously uninvestigated.

Secretion of large amounts of lysozyme by myelomonocytic leukaemia cells is reflected by the large quantities of lysozyme in the urine of these patients with myelomonocytic leukaemia (Osserman & Lawlor, 1966; Catovsky *et al*, 1971). Lysozyme may also be found in substantial quantities in normal granulocytes or CGL cells, but is not commonly secreted by these cells (Pinkus & Said, 1977).

We were able to take advantage of the sequence homology between chicken egg white lysozyme and human lysozyme (Dayhoff, 1973; Canfield, 1963) to use a chicken lysozyme cDNA plasmid (Plys 6) (Buell *et al*, 1979) to study expression of human lysozyme in differentiating HL-60 cells. We have shown that there is specific cross hybridization between the chicken lysozyme cDNA probe and the human lysozyme gene allowing us to utilize this probe to assay human lysozyme mRNA.

We have addressed the question of whether increased secretion of lysozyme is directly related to the differentiation of the HL-60 cells to macrophage-like cells or whether it is a distinct event. We have also investigated the question of whether increased secretion of lysozyme by these cells represents increased transcription of the lysozyme gene or whether post-transcriptional events may play a role as well.

MATERIALS AND METHODS

HL-60 cells were a gift of Dr Robert Gallo (Collins *et al*, 1977). They were grown to a concentration of 1×10^6 cells per ml in RPMI-1640 (Gibco) with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml).

PMA was added to a final concentration of 16 mM (10 μg/ml) in 0·01% dimethyl sulphoxide (DMSO), (a DMSO concentration lower than that required to induce differentiation of these cells). For experiments involving incubation with DMSO cells were incubated in a similar fashion with a final concentration of 2% DMSO.

For each experiment the cells were resuspended in fresh medium. Cells used for mRNA extraction were grown in 50 ml sterile polypropylene tubes to reduce adherence. Cells used for the adherence and lysozyme assay were grown in 16 mm diameter wells (Costar Culture Cluster Wells).

Lysozyme assay. Lysozyme activity was assayed by the procedure of Litwack (Solsted &

Martinez, 1978). The reaction mixture contained one-tenth volume cell medium in *Micrococcus lysodeikticuticus* solution (25 mg/ml *M. lysodeikticuticus*, 1% NaH_2Cl, 0·2 M $NaPO_4$, pH 6·2). Lysozyme activity was assayed as a decrease in optical density at 450 nm per minute from 30 to 90 s. Egg-white lysozyme was used as a standard.

These values were confirmed by the 'lyso-plate' lysozyme assay (Yam *et al*, 1971).

Cell adherence. Differentiation of HL-60 cells to macrophage-like cells was assayed by quantitating adherence to plastic tissue culture wells. Log phase cells were centrifuged and resuspended in fresh medium with 16 nM PMA. Cell suspensions were placed in wells at a concentration of 1×10^6 cell/ml. Nonadherent cells were counted in the supernatant after washing. Adherant cells were quantitated by lysis and quantitation of nuclei. The sum of adherent and nonadherent cells counted correlated well with the original number of cells placed in each well. Viability was documented by Trypan Blue staining.

Nonspecific esterase staining. Cells were also grown under identical conditions in tissue culture tubes to which they did not adhere. For each time point, cells were centrifuged and analysed for non-specific esterase (NSE) using alpha naphthyl butyrate as a substrate (Yam *et al*, 1971).

RNA extraction. RNA was extracted as previously described (Miller *et al*, 1978). Vanadium ribonucleoside was utilized as an RNAse inhibitor (Berger & Birkenmeier, 1979). Approximately 100 mg RNA was obtained from 10^8 cells.

Liquid RNA–cDNA hybridization. Liquid RNA–DNA hybridizations were performed as described by Benz *et al* (1977). Hybridizations were performed in 50% formamide, 0·05 M Hepes, pH 7·1, 5 mM EDTA, 0·5 M NaCl, 7 μg/ml *E. coli* DNA, and 0·05 μg/ml *E. coli* tRNA with 25 mg/160 μl total RNA and 0·51 pg/160μl ^{32}P nick translated plasmid (Plys 6 3×10^7 cpm/μg). Hybridization was quantitated by S_1 nuclease digestion. Lysozyme sequences were detected by hybridization to the Plys 6 chicken lysozyme cDNA probe kindly supplied by Dr Robert Schimke.

RESULTS

PMA induced differentiation of HL-60 cells was assayed by cellular adherence to plastic. The number of adherent cells correlated directly with the number of cells which had differentiated to macrophages morphologically. A slight increase in the number of adherent cells occurred within 12 h, with a rapid rise between 24 and 48 h. Maximum adherence occurred at 48 h and continued for approximately 24 h. Less than 3% of uninduced cells adhered to the plate (see Fig 1) throughout the experiment. Increase in nonspecific esterase staining correlated temporally with the increased adherence of the differentiating cells (data not shown). If the PMA was removed at 24 h and the incubation continued in the absence of PMA, differentiation continued at a rate not significantly different from that of cells with continuous exposure to PMA.

Lysozyme secretion, however, did not follow the same pattern (Fig 2). If the differentiating HL-60 cells were continuously exposed to PMA lysozyme secretion reached a maximum of 46 $\mu g/10^7$ cells at 48 h, then declined to 15 $\mu g/10^7$ cells at 70 h. This decrease was consistently observed, and most likely represents decreased synthesis of new lysozyme and degradation of

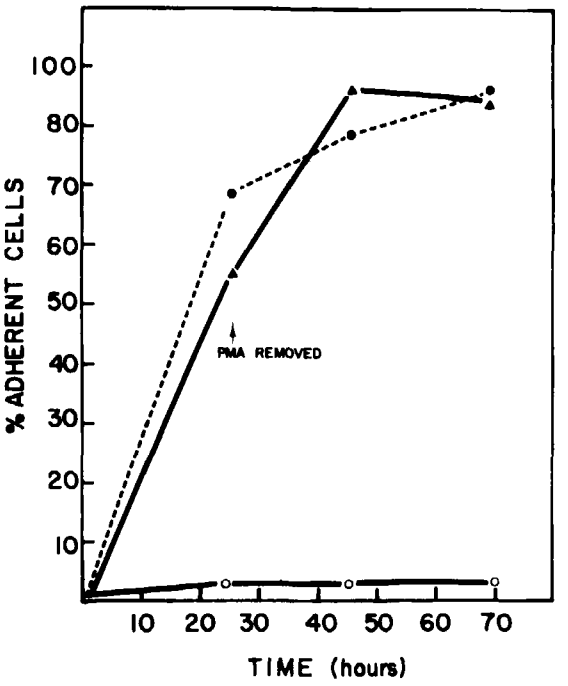

Fig 1. Macrophage differentiation of HL-60 cells treated with phorbol myristate acetate. Differentiation was quantitated as adherence to tissue culture plastic. ●, Continuous PMA treatment; ▲, 24 h PMA treatment; ○, no PMA.

previously secreted lysozyme. The maximal increase in lysozyme activity occurred 6–12 h after the maximal differentiation. The uninduced cells did not increase their level of intracellular lysozyme or the amount of lysozyme secreted into the medium. HL-60 cells which were exposed to PMA for only 24 h secreted much less lysozyme, reaching a peak of 21 $\mu g/10^7$ cells at 72 h. Intracellular lysozyme levels did not change significantly during either continuous or 24 h exposure. Cell number and viability were virtually identical for those cells treated continuously and those treated for only 24 h. Uninduced control cells demonstrated a constant level of lysozyme in the medium at 10 $\mu g/10^7$ cells.

To test for specific degradation of extracellular lysozyme by products of differentiating cells we measured the loss of exogenous lysozyme activity added to the medium of phorbol treated and untreated HL-60 cells after centrifugation and removal of the cells. As is obvious from Fig 3 the rate of degradation is identical in the medium of differentiated and nondifferentiated cells suggesting that enzymatic catabolism is not responsible for the altered level of lysozyme activity which we observe.

Considerable sequence homology exists between the chicken and human lysozyme molecules. The amino acid sequences of both of these are known (Dayhoff, 1973; Buell *et al*, 1979) allowing us to make a direct comparison. Seventy-eight of 130 amino acids (60%) are identical, indicating a moderate degree of homology. This predicts that specific cross hybridization might occur between the chicken and human lysozyme nucleic acid sequences.

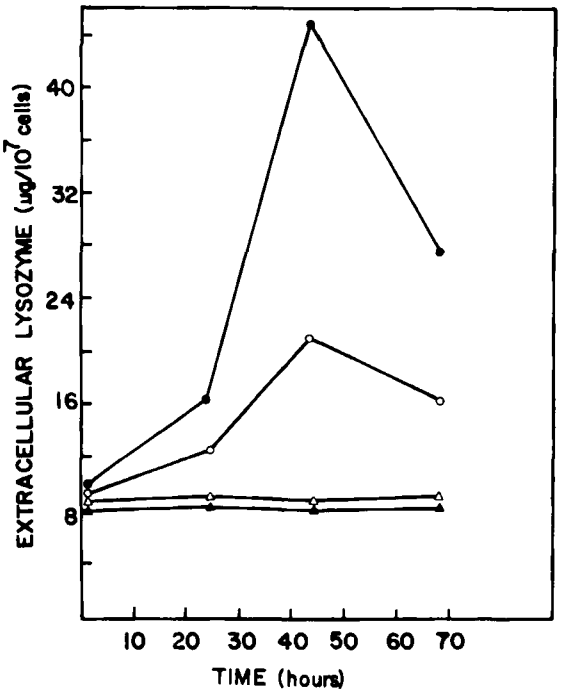

Fig 2. Lysozyme secretion by cells induced to differentiate by phorbol myristate acetate. Lysozyme was measured by turbidometric method as described in Methods and quantitated as chicken egg white lysozyme equivalents. ●, Continuous PMA treatment; ○, 24 h PMA treatment; △, no PMA; ▲, DMSO treatment.

We tested this prediction by performing melting curve analysis of hybrids between nick-translated Plys 6 and RNA from the cells of a patient with myelomonocytic leukaemia who was secreting large amounts of lysozyme. The melting curve analysis is shown in Fig 4. The Tm is 68°, exactly that of a globin cDNA–globin mRNA hybrid determined in this system in a parallel experiment. A plasmid–*E. coli* RNA hybridization revealed less than 5% reannealing at the time the melting curve was performed. The sharp melting curve documents specific cross hybridization and demonstrates the usefulness of this probe for our experiments.

Quantitative analysis of lysozyme mRNA was performed by liquid hybridization. Liquid RNA–cDNA plasmid hybridization demonstrated a moderate level of hybridization with total RNA extracted from uninduced cells, with a five-fold increase in hybridization with total RNA extracted from cells exposed to PMA for 32 h. A relatively low level of hybridization (one half that of uninduced cells) was observed with RNA from cells exposed to PMA for 72 h (see Fig 5). This indicated that the level of lysozyme-specific RNA is relatively high in uninduced cells, despite the fact that these cells are secreting little or no lysozyme. After treatment with PMA for 50 h the level of lysozyme-specific RNA increases five-fold, simultaneous with the increase of lysozyme secretion. At 72 h, however, the level of lysozyme RNA has decreased dramatically despite the fact that lysozyme continues to be secreted at relatively high levels.

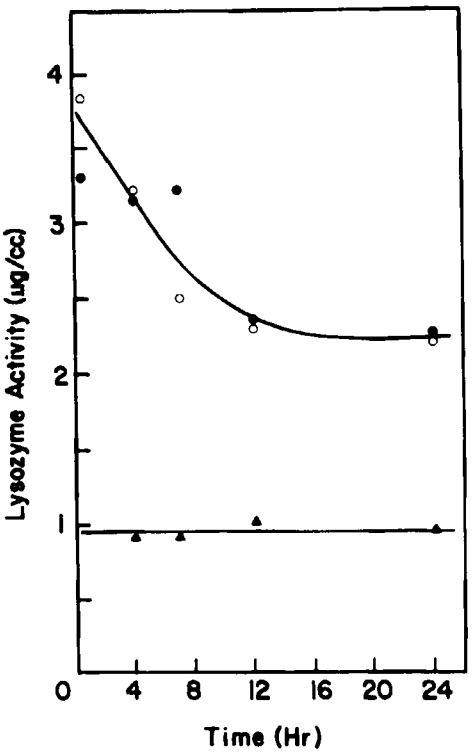

Fig 3. Enzyme activity of chicken egg white lysozyme added to medium of HL-60 cells treated with phorbol myristate acetate. Lysozyme (4 μg/ml) was added to medium from HL-60 cells treated with PMA after removal of all cells. Residual lysozyme activity was assayed with both the turbidometric and lyso-plate assays and expressed as chicken egg white lysozyme equivalents. ○, No PMA treatment; ●, 24 h PMA treatment; ▲, no PMA treatment; no added lysozyme.

RNA dot blot hybridization confirmed this result demonstrating a low level of hybridization of the chicken lysozyme cDNA probe to total RNA extracted from HL-60 cells after 0 and 74 h of exposure to PMA. Total RNA from cells at 54 h of exposure to PMA demonstrated considerable hybridization to the chicken lysozyme cDNA probe.

The relationship between lysozyme RNA concentration and lysozyme gene expression is documented in Table I. Clearly the ratio of lysozyme RNA to lysozyme remains constant during differentiation of these cells, implying strict transcriptional control at this stage. Following differentiation, however, the level of lysozyme secretion falls more slowly than lysozyme mRNA implicating post-transcriptional control of expression. There is no major increase in the level of lysozyme mRNA which might explain its prolonged secretion.

DISCUSSION

HL-60 cells treated with PMA differentiate to morphologically normal macrophages (Todd *et al*, 1981). Numerous enzymatic and metabolic alterations have been noted during this

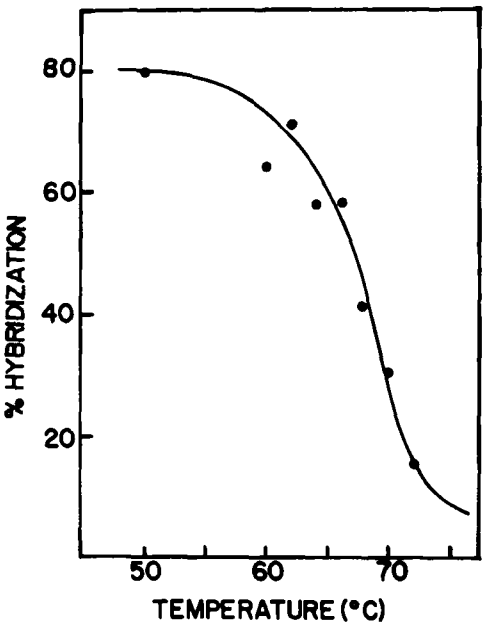

Fig 4. Melting curve of Plys 6 plasmid–myelomonocytic leukaemia RNA hybrids. Hybrids were formed for 48 h then temperature elevated at a rate of 1°/30 min. Per cent hybridization was assayed by S_1 digestion.

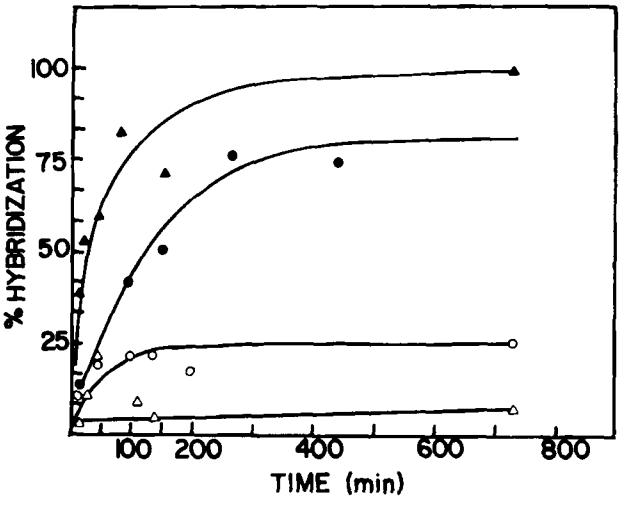

Fig 5. Hybridization of nick-translated plasmid Plys 6 to HL-60 RNA isolated at various times during macrophage differentiation. Nick-translated (8×10^7 cpm/mg) probe was hybridized to HL-60 RNA. Hybridization was assayed by S_1 digestion. ●, ○ h PMA treatment; ▲, 32 h PMA treatment; ○, 72 h PMA treatment; △, tRNA.

Table I. Relationship between lysozyme secretion and relative lysozyme mRNA concentration during differentiation of HL-60 cells

	Time of induction (h)		
	0	36	72
Secreted lysozyme ($\mu g/5 \times 10^6$ cells)	5·0	23·2	7·0
Intracellular lysozyme ($\mu g/5 \times 10^6$ cells)	<2·0	<2·0	<2·0
Relative lysozyme mRNA concentration*	1·00	5·9	0·5
Ratio of secreted lysozyme to relative lysozyme mRNA level	5·0	3·9	13·5

* The relative lysozyme mRNA concentration was calculated from the ratio of initial slopes of the hybridization curves.

differentiation. Increased nonspecific esterase activity, increased cellular adherence, increased responsiveness to phagocytic stimuli, as well as increased secretion of lysozyme accompany HL-60 cell differentiation. The mechanism of these alterations is unclear.

We have investigated the mechanism of one of these changes, the increased secretion of lysozyme during PMA-induced differentiation of HL-60 cells. We were particularly interested in the question of whether lysozyme secretion is a function of the macrophage–monocyte differentiation of these cells or whether it results from continued stimulation of differentiated cells. We were also interested in whether increased expression of the lysozyme gene (as evidenced by increased lysozyme secretion by the differentiated cells) was mediated at the transcriptional or post-transcriptional level.

HL-60 cells treated with PMA for 24 h, or less, are clearly irreversibly committed to differentiate. This has been demonstrated for other inducing agents, as well. We confirmed this observation by documenting that removal of PMA at 24 h did not influence differentiation of these cells. Peak stimulation of lysozyme secretion, however, was markedly decreased. This result implies that increased secretion of lysozyme by the differentiated HL-60 cells is dependent on continued stimulation of the cells by PMA, analogous to activation of mature macrophages by PMA. It also implies that lysozyme secretion is a marker for differentiated cells rather than a necessary component of differentiation. This conclusion is supported by the observation that the increase in lysozyme secretion occurs 12–24 h after the increase in adherence of these cells. The increase in nonspecific esterase, on the other hand, occurs simultaneously with the differentiation of the cells, implying that this alteration is a function of differentiation. Thus, there may be two sets of altered gene functions in cells induced to differentiate with PMA, those occurring as a direct result of differentiation, and those occurring as a result of continued stimulation of differentiated cells.

Our data suggest that the initial increase of lysozyme expression is directly related to increased lysozyme gene transcription with the ratio lysozyme mRNA to extracellular lysozyme activity remaining constant during a five-fold increase of both. The significant level of lysozyme mRNA in the uninduced cells is not surprising considering the baseline level of intracellular lysozyme and the low level of lysozyme secretion by uninduced cells.

More surprising, however, is the extremely low level of lysozyme mRNA (less than half that of uninduced cells) in cells treated with PMA for 72 h. This result suggests that PMA induces an initial burst of lysozyme mRNA synthesis in differentiated cells which lasts less than 24 h, followed by a very low level of lysozyme transcription. Increased secretion must also occur after a short delay. Pulse-chase experiments (now in progress) should elucidate the relative importance of mRNA stability in these alterations.

The persistence of significant lysozyme secretion in terminally differentiated cells with very low levels of lysozyme mRNA may reflect continued translation of low levels of lysozyme mRNA by these cells. It is unlikely to reflect increased stability of secreted lysozyme since degradation of lysozyme activity occurs at a similar rate in medium from differentiated and nondifferentiated cells.

PMA may have two sites of action. The transcriptional alterations of the lysozyme gene imply that it alters transcription of the lysozyme (and probably many other) genes. It must also act at the cell membrane, where it is specifically bound (Shoyab & Todaro, 1980; Sando *et al*, 1981; Driedger & Blumberg, 1980) altering secretion of lysozyme and other molecules. This mixed transcriptional/post-transcriptional mechanism of gene control may be more common than is currently suspected. It may play an important role in altered expression of 'tumour marker' genes in malignant cells.

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