Overexpression of prothymosin α accelerates proliferation and retards differentiation in HL-60 cells

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Prothymosin α (ProT α) is an acidic nuclear protein the expression of which is related to the proliferation and differentiation processes in mammalian cells. In the present study we have stably transfected HL-60 cells, a biological system that allows the study of both proliferation and differentiation, with recombinant vectors encoding sense and antisense ProT α mRNA. In the HL-60 cell clones overexpressing ProT α we observed an acceleration in the growth rate, whereas expression of the antisense orientation showed the opposite effect. Moreover, cell-cycle analysis demonstrated that the G₁-phase was shortened in the cells expressing the sense construct. Before studying how ProT α affects

differentiation, we showed that the down-regulation of ProT α gene during differentiation occurs in all mammalian cell lines (HL-60, K562, U937, MEL C88, N2A and PC12) analysed. The biological effect evoked by the induction of the ProT α sense vector was the retardation of cell differentiation, although expression of the antisense construct showed no effect on differentiation. In conclusion, our findings provide evidence that ProT α is directly implicated in cellular proliferation and that the maintenance of high levels of ProT α inside HL-60 cells is incompatible with their ability to differentiate.

INTRODUCTION

Prothymosin α (ProT α) is a 12.5 kDa acidic protein [1] originally regarded as a thymic hormone with a function in cellular immunity [2]. However, subsequent studies detected ProT α and its mRNA in a large variety of cell types, tissues and organisms [3–8], suggesting a more general role for this protein within the cell instead of playing a specific function in the immune system. The molecular analysis of ProT α cDNA [4,6,9–12] and genes [13] did not show any sequence encoding a hydrophobic signal which would direct the secretion of this protein. The finding of a nuclear signal at the C-terminus of ProT α led to the hypothesis of a nuclear location for this protein [14]. This hypothesis was first confirmed by microinjection into *Xenopus laevis* oocytes [15] and later on by gene-transfection experiments [16,17]. Furthermore, immunological studies using a polyclonal antibody against the N-terminal fragment of ProT α revealed that the cross-reacting material is in the nucleus associated with condensed chromatin [18]. An interesting finding was the demonstration of the existence in this protein of phosphorylation targets and that it becomes phosphorylated when the cells are stimulated to proliferate [19–21].

Until now the biological function of ProT α is unknown. Nevertheless, the bulk of experimental data points to an association of this protein with cell proliferation [4,6,18,22]. This relationship is supported by the correlation between ProT α (mRNA and protein) levels and the proliferative activity of the cells. Thus ProT α transcripts are induced upon growth stimulation of resting lymphocytes [4], thymocytes [22], NIH3T3 fibroblasts [4] and hepatocytes during liver regeneration [22].

Moreover, incubation of synchronized human myeloma cells with ProT α antisense oligonucleotides prevents cell division [23].

ProT α gene is highly activated during postnatal development at stages containing many proliferating and differentiating cells [24], raising the possibility that this protein is also related to the differentiation state of the cells. In this sense it was reported that ProT α gene expression is down-regulated in HL-60 cells that have received signals to terminally differentiate [25,26] and, more recently, it has been found that the ProT α gene is an oestrogen-responsive gene in neuroblastoma cells whose expression also decreases during the course of differentiation [27]. Previous work has shown that ProT α gene expression is regulated by *c-myc* [11], a gene that plays a crucial role in the control of mammalian cell proliferation. Furthermore, *trans*-activation from the E-box element in the rat ProT α intron enhancer is specific for Myc [28,29]. In agreement with this, it has been hypothesized that the control of ProT α gene expression by oestrogens could be dependent on Myc, because oestrogen was shown to regulate *c-myc* expression [27].

In the present study we have cloned ProT α cDNA in the mammalian expression vector pMAMneo in two orientations (sense and antisense). We achieved stable transfection of HL-60 cells with these constructs and we then analysed the biological effect caused by the induction of the ProT α sense vector in proliferation and differentiation. We found that overexpression of ProT α produced a shortening of the G₁ phase of the cell cycle, resulting in an acceleration of proliferation. Moreover, when the same cells were induced to differentiate, we observed a significant decrease in the number of differentiated cells, indicating that high levels of ProT α interfere with the differentiation process.

Abbreviations used: ProT α , Prothymosin α ; RA, retinoic acid; EGF, epidermal growth factor; BrdU, bromodeoxyuridine; RSV, rous sarcoma virus; LTR, long terminal repeat; MMTV, murine-mammary-tumour virus.

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EXPERIMENTAL

Cell cultures, proliferation and differentiation

HL-60 promyelocytic cells were grown in RPMI 1640 (Gibco), supplemented with 100 units/ml of penicillin and 100 µg/ml of streptomycin, containing 10% fetal-calf serum (Gibco) in a humidified atmosphere of air/CO₂ (19:1). The proliferation rate of untransfected and transfected HL-60 cells, incubated with 5 µM dexamethasone, was quantified by counting the viable cells after staining with Trypan Blue at intervals of 24 h. Labelling of cells with bromodeoxyuridine (BrdU) and quantification of BrdU incorporation were done according to the manufacturer's (Boehringer Mannheim) protocol ('Cell Proliferation ELISA, BrdU colorimetric').

HL-60 cells were induced to differentiate to monocytes-macrophages with 10 ng/ml PMA ('TPA'; Sigma). Differentiation with either retinoic acid (RA) or DMSO was carried out as described elsewhere [25]. Differentiation was confirmed morphologically by microscopic examination. For PMA-induced differentiation, the number of viable differentiated cells was quantified at various times by counting the macrophage-like cells using an assay for non-specific esterase [30], a monocyte-macrophage-specific marker. For RA-induced differentiation, the quantification of differentiated cells was carried out by microscopic examination of cultures after staining with Wright-Giemsa stain. The differentiation of other cell types was carried out as follows. For macrophage differentiation, K562 (erythroblastic human leukaemia) and U937 cells (monocytic human leukaemia) were treated with 20 nM and 100 nM of PMA respectively. Erythroid differentiation was induced in MEL C88 cells (murine erythroleukaemia) by addition of 2% DMSO. N2A cells (mouse neuroblastoma) were induced to differentiate by serum deprivation and PC12 cells by treatment with 5 ng/ml of epidermal growth factor (EGF). The differentiation of K562, U937, N2A and PC12 cells was also confirmed by microscopic examination; the deep-red coloration of the cell pellet indicated the differentiation of MEL C88 cells.

Construction of ProTα sense and antisense vectors

For the transfection experiments, we chose the vector pMAMneo, a mammalian expression plasmid designed for high-level expression of eukaryotic genes [31,32]. It contains the rous sarcoma virus-long terminal repeat (RSV-LTR) enhancer linked to the dexamethasone-inducible murine-mammary-tumour virus (MMTV)-LTR promoter. This construct yields controlled, yet high-level, expression of cloned genes in cell lines. The *Escherichia coli neo* gene, driven by the simian-virus-40 early promoter, allows selection of transformants growing in media containing the antibiotic G418 (Geneticin). We have cloned a 342 bp fragment that contains the entire coding region of ProTα (short ProTα cDNA), within the *SalI* restriction site of the pMAMneo vector. This short cDNA was derived from the plasmid pTC31, which contains the human T-cell ProTα cDNA [6], after eliminating the 5' and 3' untranslated regions. This short ProTα cDNA was cloned into the plasmid pMAMneo in the sense and antisense orientations (Figure 1A below). The orientation of the inserts was established by digestions with *HindIII*, *XhoI* and *PvuII*. The pMAMneo vector without insert was used as a control.

Electroporation of HL-60 cells and cloning of transfected cells

The HL-60 cell line was stably transfected by electroporation at 200 V and 960 µF (Bio-Rad gene-pulser apparatus). Briefly, actively growing cells were harvested, resuspended in ice-cold

PBS without added Mg²⁺ or Ca²⁺, centrifuged and the pellet resuspended again in ice-cold PBS at a concentration of 12.5×10^6 cells/ml. Plasmid DNA (20 µg/ml) linearized by digestion with *NdeI* and carrier salmon sperm DNA (150 µg/ml) were added to the cell suspension. The DNA and cells were left for approx. 5 min at 0 °C in an electroporation cuvette and then the electric pulse was delivered. Three sets of electroporations were performed with 20 µg of pMAMneo vector, 20 µg of pMAMneo-short sense ProTα cDNA and 20 µg of pMAMneo-short antisense ProTα cDNA. After 48 h in RPMI medium, stable transformants were selected for their ability to grow in medium containing G418 (500 µg/ml). G418-resistant cell populations were observed after about 2 weeks. After selection of transfected cells, we isolated individual clones by serial dilutions on microtitre plates. Microscopic examination was used to confirm the presence of a single cell in each well. Cells were then allowed to grow, several clones of transfected HL-60 cells being obtained with each of the three plasmids employed. These clones were designated as follows: pMAMneo, HL-60 cells transfected with the pMAMneo vector; S,HL-60, cells transfected with the pMAMneo-short sense ProTα cDNA; A,HL-60, cells transfected with the pMAMneo-short antisense ProTα cDNA.

RNA preparation and Northern-blot hybridizations

Total RNA was isolated by the method of Chomczynski and Sacchi [33]. RNAs (10 µg) were fractionated by electrophoresis on a 6% (v/v) formaldehyde/1.2% agarose gel and transferred on to nylon membranes. Equal loading of RNAs in each lane was verified by visual analysis of ethidium bromide-stained rRNA bands. The Northern blot was hybridized with $(2-4) \times 10^6$ c.p.m./ml of the nick-translated ³²P-labelled plasmid pTC31 that contains a 1.15 kb *EcoRI*-cloned fragment of the human ProTα cDNA [6]. Hybridization reactions were carried out for 12-16 h at 42 °C in 50% (w/v) formamide/5 × SSPE [1 × SSPE is 0.18 M NaCl/10 mM NaH₂PO₄ (pH 7.4)/1 mM EDTA]/10% (v/v) dextran sulphate/2 × Denhardt's solution [1 × Denhardt's is 0.02% BSA/0.02% Ficoll 400/0.02% poly(vinylpyrrolidone)]/0.1% SDS/100 µg/ml denatured sonicated salmon sperm DNA. Filters were washed twice in 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS for 10 min at room temperature, then twice again in 1 × SSC/0.1% SDS for 10 min at 65 °C, once in 0.1 × SSC/0.1% SDS for 10 min at 65 °C and exposed to Hyperfilm β-max (Amersham) using intensifying screens. Filters were rehybridized with the β-actin probe [25], labelled by nick-translation under the same conditions as those used for pTC31. Hybridization with the β-actin probe was used as a standard internal control of RNA concentration.

DNA extractions and Southern blot analysis

DNAs from untransfected and transfected HL-60 cells were isolated as described by Laird et al. [34] and digested with *HindIII* in the buffer recommended by the suppliers. Digested DNAs were separated by electrophoresis in 0.9% agarose gels and transferred by Southern blotting [35] on to QIABRANE membranes (QIAGEN). Blots were hybridized with the *E. coli neo* gene probe [$(2-4) \times 10^6$ c.p.m./ml] labelled by nick-translation using ³²P-labelled deoxynucleoside triphosphates. This probe consists of a 2616 bp fragment obtained by digestion of the pMAMneo plasmid with *BamHI*. Hybridization reactions, washing and exposure were carried out as described above.

Western-blot analysis

Cytoplasmic and nuclear extracts from HL-60 cells were obtained

following the method of Dignam et al. [36]. Briefly, cells were harvested by centrifugation, washed twice in ice-cold PBS, resuspended in 5 packed-cell vol. of hypo-osmotic buffer [10 mM Tris/HCl (pH 7.9)/1.5 mM MgCl₂/10 mM KCl/10 mM Na₃VO₄/20 μ g/ml Na₂P₂O₇/50 μ g/ml aprotinin/0.5 mM dithiothreitol/2 mM PMSF] and incubated for 10 min on ice. After centrifugation at 600 *g* cells were resuspended again in hypo-osmotic buffer and disrupted with several strokes in a Dounce homogenizer. The suspension was centrifuged at 2000 *g* for 10 min at 4 °C. The supernatant was made 10% (v/v) with respect to glycerol and stored as the cytoplasmic fraction. The nuclear pellet was resuspended in 1 vol. of hyperosmotic buffer [20 mM Hepes/KOH (pH 7.9)/500 mM NaCl/1.5 mM MgCl₂/0.2 mM EDTA/20% glycerol/20 μ g/ml Na₂P₂O₇/50 μ g/ml aprotinin/2 mM dithiothreitol/2 mM PMSF] and disrupted as described above. The suspension was cleared of debris by centrifugation at 15000 *g* for 10 min at 4 °C. Concentration of proteins in both fractions was determined using the Bio-Rad Protein Assay.

Whole-cell extracts from proliferating and differentiated cells were obtained by harvesting the cultures, washing with PBS and resuspending the cell pellets in 1 vol. of 160 mM Tris/HCl (pH 6.8)/4% (w/v) SDS/0.8% β -mercaptoethanol. The suspension was incubated at 95 °C for 5 min, and then lysed by freeze-thawing. The viscosity of the samples was reduced by sonication. Finally, lysates were clarified by centrifugation at 15000 *g* for 10 min at 4 °C.

Aliquots of 100 μ g of the HL-60 extracts were resolved by SDS/15%-PAGE. As ProT α does not bind to nitrocellulose filters [37], proteins were electrotransferred on to DEAE-cellulose paper (Schleicher and Schuell). Filters were blocked overnight at 42 °C in 3% gelatin/TNT [100 mM Tris/HCl (pH 7.4)/1 M NaCl/0.5% Triton X-100] buffer, washed five times with TNT buffer and incubated with 1 μ g/ml of anti-(thymosin α_1) IgG [38] in 0.3% gelatin/TNT buffer for 2 h. After five washes in TNT buffer, the filter was incubated with a secondary peroxidase-labelled anti-rabbit IgG antibody and developed with the ECL^R (enhanced chemiluminescence) system (Amersham).

Cell-cycle analysis

Cells were pelleted by centrifugation at 4 °C, washed in PBS, fixed in 1% paraformaldehyde for 10 min, then permeabilized with 1 ml of ice-cold 70% (v/v) ethanol for 10 min at -20 °C. The cells were pelleted twice in ice-cold PBS and resuspended in PBS containing 100 μ g/ml RNase A (Boehringer-Mannheim) and 20 μ g/ml of propidium iodide (Sigma). Cell-cycle analyses were performed on a Becton-Dickinson FACSCALIBUR fluorescence-activated cell sorter. A total of 20000 cells were employed for each assay, and the data were analysed using ModFit LT software for DNA analysis (Verity Software House, Topsham, ME, U.S.A.).

Immunofluorescence cytochemistry

HL-60 cells, induced to differentiate into monocytes-macrophages as described above, were seeded on coverslips and fixed in 3.7% phosphate-buffered paraformaldehyde for 10 min, whereas non-differentiated-proliferating HL-60 cells (untransfected as well as transfected HL-60 cell clones) were fixed in methanol for 10 min at -20 °C. After this time, cells were washed three times for 5 min in PBS at room temperature and incubated with blocking solution [1% horse serum/3% BSA in 100 mM Tris/HCl (pH 7.4)/1 M NaCl/0.5% Triton X-100] for 1 h at room temperature. Cells were then incubated with a 1:100

dilution of the anti-(thymosin α_1) serum [38] in blocking solution for 4 h in a humid chamber. After the removal of the primary antibody, coverslips were washed three times for 10 min in PBS. The cells were incubated with the secondary antibody (Lissamine Rhodamine-conjugated goat antirabbit IgG, F(ab')₂-fragment-specific, from Jackson Immuno Research Laboratories, Jackson Immuno Research Laboratories, West Grove, PA, U.S.A.) diluted 1:50 in blocking solution for 1 h in a dark and humid chamber. The coverslips were washed in PBS for 1 h and mounted on glass slides with Immunofluore mounting medium (ICN Biomedicals, Costa Mesa, CA, U.S.A.) and viewed and photographed with Kodak 400 TMY film in a Provis AX-70 fluorescence microscope (Olympus, Tokyo, Japan).

RESULTS

Stable transfection of HL-60 cells with pMAMneo-ProT α cDNA expressing vectors

We and others have previously demonstrated that HL-60 cells are a suitable biological system for studying the expression of

Figure 1 Schematic drawing of the expression vectors pMAMneo-short ProT α cDNA

(A) represents the main features of the recombinant plasmids obtained after cloning the short ProT α cDNA in the pMAMneo vector. MCS, multiple cloning site. RSV represents the RSV-LTR enhancer and MMTV-LTR is the promoter linked to this enhancer. The *E. coli neo* gene (represented as *neo*) is driven by the simian-virus-40 (SV40) early promoter. A pBR322 origin of replication (pBR322 ori) and ampicillin-resistance gene (*Amp*^r) allow propagation in the bacteria. This representation is not drawn to scale. In the cDNA insert the *Pvu*II sites are shown in the sense and antisense orientations. The sites corresponding to the restriction enzymes used for establishing the insert orientation (*Hind*III, *Xho*I, *Pvu*II) are also indicated. (B) Integration of transfected plasmids into the cell genome. DNAs from untransfected and transfected HL-60 cells were isolated, digested with *Hind*III, fractionated by electrophoresis, Southern-blotted and hybridized to the ³²P-labelled *E. coli neo* gene as described in the Experimental section. A band of approx. 2500 bp (arrow) was found in the transfected clones, but not in the untransfected HL-60 cells. Lane A, DNA of the A clone, HL-60 cells transfected with the antisense construct; lane S, DNA of the S clone, HL-60 cells transfected with the sense constructs; lane P, DNA of pMAMneo-transfected cells; lane C, DNA of untransfected HL-60 cells (negative control). MW, sizes of the molecular-mass marker (λ DNA digested with *Hind*III) in bp.

Figure 2 Western-blot analysis of ProT α in proliferating and differentiated HL-60 cells

(A) Aliquots of 60 μ g of cytoplasmic (C) or nuclear (N) extracts from HL-60 cells, obtained as indicated in the Experimental section, were separated by SDS/15% PAGE and electrotransferred on to DEAE-cellulose membranes, followed by immunodetection of ProT α as described in the Experimental section. (B) Whole-cell extracts from equal numbers (10^7) of proliferating (P) or PMA-differentiated (D) HL-60 cells were subjected to SDS/PAGE and Western-blot analysis as described in the Experimental section.

ProT α in both the proliferation and differentiation processes [25,26]. This system has the additional advantage of knowing the behaviour of the *c-myc* proto-oncogene, the transcriptional regulator of ProT α gene expression [11,27–30]. Moreover, although it has been shown that antisense oligonucleotides inhibit cell division [23], there is no clear experimental evidence of how overexpression of ProT α could affect either proliferation or differentiation. Therefore, to determine the biological consequences of ProT α overexpression, we established lines of HL-60

cells transfected with either ProT α -sense or ProT α -antisense expression plasmids (Figure 1A summarizes the main features of these recombinant plasmids). For this purpose, HL-60 cells were transfected by electroporation with the pMAMneo vector alone, the pMAMneo-short sense ProT α cDNA, which encodes for the same protein as the ProT α mRNA, and the pMAMneo-short antisense ProT α cDNA, which produces an RNA complementary to the ProT α mRNA. The stable transformants were selected for their ability to grow in a medium containing G418, and individual transfected clones were isolated as described in the Experimental section. The plasmid integration in the cellular genome was verified by Southern-blot analysis using as a probe the *E. coli neo* gene, a fragment only present in the pMAMneo vector. After DNA extraction, blotting and hybridization, a band of approx. 2500 bp was found in all transfected clones, but not in the untransfected HL-60 cells, demonstrating the integration of the plasmid vector (Figure 1B). Since the intensity of the hybridization was almost identical in the different blotted DNAs, we can conclude that the ProT α gene dosage is the same in all transfected clones.

We selected representative clones from each group and verified the presence of ProT α mRNA by Northern-blot analysis. As shown in Figure 6 (below), in the undifferentiated/proliferating HL-60 cell clones transfected either with the sense (lane 5) or antisense constructs (lane 9) the levels of ProT α mRNA were similar to the controls (untransfected and pMAMneo-transfected cells, lanes 1 and 3). When the sense and antisense clones were incubated in the presence of dexamethasone (lanes 7 and 11), the relative amount of ProT α mRNA in these clones was higher than both the controls and the same uninduced clones (lanes 5 and 9).

Figure 3 Intracellular localization of ProT α by immunofluorescence analysis

Untransfected and transfected HL-60 cells, prepared for immunocytochemistry, were incubated with the antibody against thymosin α_1 (primary antibody) and with the secondary antibody (Lissamine Rhodamine-conjugated goat anti-rabbit IgG) as indicated in the Experimental section. When examined by fluorescence microscopy, a large accumulation of ProT α was detected within the nucleus. No ProT α was detected elsewhere in the cytoplasm. (A) Untransfected HL-60 cells; (B) HL-60 cells transfected with the plasmid expressing the sense ProT α cDNA; (C) HL-60 cells transfected with the plasmid expressing the antisense ProT α cDNA; (D), control HL-60 cells (in this case the primary antibody was omitted to show the background immunofluorescence). Photomicroscopy was carried out by using the same time exposure and film processing protocols to assess different levels of antigen expression. Controls were carried out by omitting the primary antibody.

The dexamethasone treatment of both non-transfected and pMAMneo-transfected HL-60 cells did not produce any effect on the stimulation of ProT α synthesis (results not shown).

Overexpression of ProT α produces accumulation of this protein inside the nucleus

The results obtained with the Northern blot indicated that cells transfected with the sense vector are overexpressing ProT α mRNA. To measure the relative abundance of this protein and, simultaneously, establish if this protein was indeed located in the nucleus, we used Western-blot analysis and immunocytochemical assays.

First, to verify if the anti-(thymosin α_1) IgG recognizes ProT α in HL-60 cell extracts and to determine the intracellular distribution of ProT α , we carried out a Western-blot analysis. As shown in Figure 2(A), the antibody against thymosin α_1 detects ProT α , and most of the immunoreactivity was mainly found in the nuclear fraction. Moreover, Western-blot analysis of proteins prepared from whole-cell extracts from both proliferating and differentiated HL-60 cells demonstrated that ProT α expression was clearly diminished in the differentiated HL-60 cells (Figure 2B).

Since ProT α is a nuclear protein [15–18], it became very important to find out if there was an effect of the recombinant pMAMneo-ProT α vectors on the presence of this protein inside the nucleus of transfected cells. Thus the subsequent experiments would be more biologically meaningful. To verify this, we carried out immunofluorescence analysis using the different HL-60 cell clones, cultured in the presence of dexamethasone and the antibody described above. The results of the immunofluorescence experiments are shown in Figure 3. In the HL-60 cells overexpressing ProT α , the presence of this protein was located inside the nucleus (Figure 3B). Furthermore, the highest concentration of ProT α , judged by the brightness of cell preparations after immunostaining, was found in the HL-60 cells transfected with the cDNA in the sense orientation compared with the controls (HL-60 cells transfected with the pMAMneo vector alone and untransfected cells; Figure 3A). Interestingly, the signal in the antisense clone was lower than in the controls (Figure 3C). The background in these experiments was very low, established by omitting the primary antibody (Figure 3D). Therefore the cells transfected with the pMAMneo-short sense ProT α cDNA contained the highest amount of this protein inside the cell nucleus.

Immunofluorescence analysis with differentiated cells showed a morphological change in the cells, demonstrating that differentiation took place. However, in the differentiated cells we could not detect changes in the levels of ProT α among all the clones used in our experiments (results not shown). The most probable explanation for this finding is that differentiated HL-60 cells contain very low levels of ProT α [25,26]. In fact, as we demonstrate in the present study, high levels of this protein in HL-60 cells interfere with their ability to differentiate (Figure 6 below).

Overexpression of ProT α accelerates the proliferation of HL-60 cells

Accumulating data correlate ProT α expression with alterations in the proliferative state of cells indicating a direct involvement of this protein in proliferation. Our first purpose was to find out if differences in growth kinetics could take place as a consequence of induced expression of ProT α sense and antisense vectors. For these experiments we used our stably transfected HL-60 cell clones and determined their growth rates by counting the number

Figure 4 Effect of ProT α overexpression on the proliferation rate and DNA synthesis

(A) Growth curves of the different HL-60 clones (identified in the Figure). Aliquots of 500 000 cells from each HL-60 cell clone were cultured as indicated in the Experimental section. Cells from triplicate plates were counted at the indicated times and the results shown represent the average for three experiments. (B) BrdU incorporation into DNA was quantified in multiple independent wells using a microplate reader at 450 nm with a reference wavelength of 690 nm. We carried out four sets of BrdU assays using different initial cell numbers (from 3000 to 24 000 cells/well). Error bars represent the S.D.

of viable cells at intervals of 24 h (Figure 4A). In preliminary experiments, we tested the effect of dexamethasone in the cell cultures and we found that this chemical did not alter their proliferation rate (results not shown).

In control clones (untransfected- and pMAMneo-transfected HL-60 cells) the proliferation rate was very similar, whereas the HL-60 cells transfected with the sense vector (sense clone) showed a higher multiplication rate. In contrast, the HL-60 cells expressing the antisense vector (antisense clone) exhibited a growth rate lower than that of controls. To verify the results obtained with cell number counting, we studied the rate of DNA synthesis in the four cell clones, in the presence of dexamethasone, by measuring the incorporation of BrdU into chromosomal DNA. As shown in Figure 4(B), the sense clone showed a rate of DNA synthesis higher than that observed with the controls (untransfected and pMAMneo-transfected cells) as well as with the antisense clone. The appearance that the antisense construct has a greater effect on proliferation rate (Figure 4A) than on DNA synthesis (Figure 4B), is due to the time differences in both

Figure 5 Effect of ProT α on the cell-cycle distribution of asynchronously growing HL-60 cell cultures

Aliquots (5×10^6) of exponentially growing cultures, incubated for 48 h in the presence of dexamethasone, were analysed by flow cytometry as described in the Experimental section. The histograms show the proportion of cells in each phase of the cell cycle for every clone analysed (untransfected and transfected HL-60 cells are identified on the top of the Figure). Data shown are representative of three experiments, each done in triplicate. S.D. values are shown by thin error bars.

types of experiments. The data shown in Fig. 4(A) were obtained throughout 5 days of culture, whereas those shown in Figure 4(B) were obtained after an incubation of 16 h in the presence of BrdU. Together these findings prove that overexpression of ProT α results in a significant increase in the rate of proliferation of HL-60 cells, suggesting a direct involvement of this protein in the events leading to cell proliferation.

High levels of ProT α decreases the G₁-phase of cell cycle

The results presented above indicated that, in the clones transfected with the vectors expressing ProT α , the length of the cell cycle should be affected. Therefore, to determine the basis for the differences in the growth rates among the four cell clones used in our experiments, we carried out a study of their cell-cycle distribution. From the growth curves for each of the clones (Figure 4A), we estimated an average cell-cycle length of 24 h for the control clones, 21 h for the clones expressing the sense construct and about 25 h for the antisense clone.

To analyse further the influence of high levels of ProT α on cell cycle progression, we performed fluorescence-activated cell-cycle analysis on asynchronously growing cell populations. The results of these experiments are shown in Figure 5. In the HL-60 clones expressing the sense ProT α , the fraction of G₀/G₁ cells was decreased on average by 27% compared with the controls and the fraction of S-phase cells was increased accordingly, whereas the fraction of cells in G₂/M was slightly increased. In the cells expressing the antisense ProT α cDNA, we observed a small increase (approx. 11%) in the fraction of G₀/G₁ cells and a concomitant decrease in the amount of cells in S-phase when compared with the controls; the percentage of cells in G₂/M remained unchanged. Since in a non-synchronized cell population the proportion of cells in a phase of the cell cycle is proportional to the relative length of this phase, our data suggest that the length of G₁ phase was decreased in the sense clone and increased in the antisense clone. Taken together, these results (Figures 4 and 5) indicate that the proliferation rate of the clone overexpressing ProT α was higher probably because the duration of the G₁-phase was lower.

Down-regulation of ProT α mRNA during mammalian cell differentiation

We and others have reported a decrease in the levels of ProT α mRNA during the differentiation of HL-60 cells to granulocytes or macrophages [25,26]. To establish if this down-regulation occurs in other cell types, we analysed the expression of the ProT α gene during differentiation of several mammalian cell lines of myeloid and non-myeloid origin. After incubation with the appropriate inducers, total RNA was extracted from cell cultures and mRNA levels evaluated by Northern-blot analysis. We found that differentiation of HL-60 cells to granulocytes (cells were treated with DMSO or RA) or to macrophages (the differentiation agent was PMA) produced a large decrease in the amount of ProT α transcript (results not shown).

To determine whether down-regulation of the ProT α gene is a general characteristic of the differentiation process, we studied other cell lines. Monocytic differentiation of human myeloid K562 and U937 cells also produced a progressive decrease (over 99% 24 h after induction) in the levels of this mRNA (results not shown). Therefore the myeloid macrophage-monocytic pathway is always accompanied by a marked reduction in the level of ProT α mRNA. Likewise, when the murine erythroleukaemia MEL C88 cell line was induced to erythroid differentiation, the expression of ProT α gene was again down-regulated and the mRNA levels decreased about 80% after 4 days of incubation in the presence of inducer (results not shown). To assess if the down-regulation of the ProT α gene observed in myeloid cells was peculiar to this kind of differentiation, we analysed other cell lines of neuronal origin: N2A, a mouse neuroblastoma, and PC12, a rat pheochromocytoma. When N2A cells were serum-deprived, stimulating neurite outgrowth, a 90% decrease in the levels of ProT α mRNA was observed. Moreover, incubation of PC12 cells with EGF, which also promotes neuronal phenotype under low-serum conditions [39], induced a significant decrease in the amount of ProT α transcript (results not shown). In conclusion, the results obtained with several cell lines and diverse inducers of differentiation strongly suggested that the expression of the ProT α gene is down-regulated during differentiation of myeloid and non-myeloid cells.

Effect of ProT α sense and antisense RNA expression on the differentiation of HL-60 cells

Our next purpose was to establish if the overexpression of ProT α mRNA could repress the process of differentiation in the HL-60 cells. The changes in the relative levels of ProT α mRNA during differentiation in the several HL-60 clones employed in the present study was analysed by Northern blotting. All clones (HL-60 cells, pMAMneo, S and A clones) were cultured in the absence and in the presence of dexamethasone. We always found that the induction of differentiation with PMA produced a pattern of expression of the ProT α gene in agreement with the behaviour previously observed [25,26], namely the mRNA levels were always lower in differentiated cells. However, when the sense clone was cultured in the presence of dexamethasone, we observed at the beginning a remarkable increase in the levels of ProT α mRNA in the undifferentiated cells (Figure 6, lane 7). Similarly, in the antisense clone we detected a strongly hybridizing band, probably the consequence of antisense RNA expression (Figure 6, lane 11).

Once this *in vitro* system was tested, we stimulated the differentiation of the following HL-60 cell clones: untransfected HL-60 cells and HL-60 cells transfected with pMAMneo (controls), HL-60 cells transfected with the pMAMneo-short sense ProT α cDNA (clone S) and HL-60 cells transfected with the

Figure 6 Effect of ProT α on sense and antisense RNA expression on the rate of differentiation in HL-60 cells

Bars display the number of differentiated HL-60 cells at days 3, 4 and 5 after the addition of the differentiating agent (PMA). The quantification of viable differentiated cells was carried out as described in the Experimental section. Values represent means for duplicate samples $\pm$ S.D. in three different experiments. The continuous line shows the results obtained when the HL-60 cells (untransfected or transfected) were differentiated in the absence of dexamethasone and the discontinuous line shows analogous results but in the presence of 5 μ M dexamethasone. (A) Untransfected HL-60 cells; (B) pMAMneo transfected HL-60 cells; (C) HL-60 cell clone transfected with the vector expressing the sense ProT α cDNA; (D), HL-60 cells transfected with the vector expressing the antisense ProT α cDNA. Inside each panel there is an inset showing a Northern-blot analysis of total RNA prepared from undifferentiated (day 0) and differentiated (5 days after incubation in the presence of PMA) as indicated in the Experimental section. Insets: HL-60, non-transfected HL-60 cells; pMAMneo, HL-60 cells transfected with pMAMneo; CLONE S, HL-60 cells transfected with the plasmid expressing the sense ProT α cDNA; CLONE A, HL-60 cells transfected with the plasmid carrying the antisense ProT α cDNA. Lanes: 1, 3, 5 and 9, undifferentiated and uninduced; 7 and 11, undifferentiated and induced with dexamethasone; 2, 4, 6 and 10, differentiated in the absence of dexamethasone; 8 and 12, differentiated in the presence of dexamethasone. The number below the blots indicates the ProT α / β -actin ratio determined by densitometric scanning of autoradiograms.

pMAMneo-short antisense ProT α cDNA (clone A). To study how overexpression of ProT α RNA could affect differentiation, we set up two conditions with the S and A clones: in the absence of dexamethasone, a chemical that promotes the expression of the exogenous ProT α cDNA, or in the presence of 5 μ M dexamethasone. The transfected cells were seeded at an initial concentration of 0.83×10^6 cells/ml in fresh medium and induced to differentiate to macrophage-like cells with 10 ng/ml of PMA and to granulocytes with 1 μ M of RA. The number of viable differentiated cells was quantified as described in the Experimental section. As an additional control, we verified that the differentiation process of untransfected HL-60 cells and pMAMneo clone was not affected by the presence of dexamethasone in the cultures.

The results of the experiments carried out with PMA are represented in Figure 6. In the cultures differentiated without dexamethasone (continuous line), the sense clone (Figure 6C) showed a similar behaviour to that observed in both untransfected (Figure 6A) and pMAMneo-transfected (Figure 6B) HL-60 cells, indicating that the integration of the different constructs did not affect the down-regulation of ProT α mRNA. However, when the overexpression of ProT α sense RNA was induced by treatment with dexamethasone, we observed a significant in-

hibition of the differentiation process (Figure 6C, broken line). This inhibition was more than 50% compared with the values obtained without dexamethasone and with the controls. The overexpression of ProT α mRNA in the sense clone treated with dexamethasone together with the higher levels of ProT α observed in the differentiating sense clone, provides an explanation for the inhibition in the differentiation ability of sense clone and strongly suggests that the maintenance of relatively high levels of ProT α in HL-60 cells retards or prevents cell differentiation. In contrast, when the expression of ProT α antisense RNA was activated by incubation in the presence of dexamethasone, the number of differentiated cells was only slightly higher (Figure 6D). Similar results were obtained using other sense and antisense transfected clones as well as when the differentiation agent was RA (results not shown). Therefore the expression of ProT α antisense RNA did not increase the rate of differentiation, at least in our *in vitro* system.

DISCUSSION

ProT α is expressed in a wide variety of mammalian tissues and its expression is more pronounced in proliferating cells (see the Introduction). The results presented here show that enhanced expression of ProT α accelerates the proliferation rate of HL-60 cells, decreasing the G₁ phase of the cell cycle. Until now, few genes have been described as candidates for a direct regulation by the Myc protein, a transcription factor that has a key role in the regulation of mammalian cell proliferation [40,41], ProT α being one of the clearest Myc targets [11,28–29,42]. The increase in both DNA synthesis and proliferation rate as a consequence of ProT α overexpression (Figure 3) adds to previous results a new finding, that ProT α is not only related to proliferation, but is directly involved in that process probably as an early component in the proliferation events triggered by the *myc* genes. Interestingly, two recent reports point towards the same conclusion. Thus it has been reported that conditional expression of N-*myc* in human neuroblastoma cells increases the expression of ProT α accelerating progression into S-phase [42] and that overexpression of ProT α , concomitant with c-*myc*, is related to rat hepatic carcinogenesis [43]. On the other hand, it was reported that treatment with ProT α antisense oligonucleotides induced the opposite effect, namely the retardation of cell division [23]. In our *in vitro* system the expression of the vector encoding the antisense RNA also produced a decrease in the proliferation rate. Therefore all the experimental data provide strong evidence leading to the conclusion that ProT α is critical for cell proliferation and participates directly in this biological process.

Several studies have shown that ProT α mRNA is continuously present throughout the cell cycle [22,44]. Nevertheless, there is controversy as to in which phase of the cell cycle ProT α mRNA is induced. Thus, by examining the time between exposure to the signal (mitogens, serum starvation or hepatectomy) and the induction of ProT α mRNA, various reports suggested a maximum accumulation of this transcript at different phases of the cell cycle, including G₁ [18,45], G₁/S [22], S/G₂ [46] and G₂/M [23]. The discrepancy in these results is probably due to both the different biological systems and the proliferative signals employed. In the present work we chose a distinct and novel experimental approach (the overexpression of ProT α mRNA) to find out how ProT α affects the cell cycle. Our results provide evidence that high levels of ProT α produce a decrease in the length of the cell cycle, accelerating the entry into the S-phase because the duration of G₁-phase was shorter. This is consistent with a recent report [42] that showed that induction of N-*myc* increased the expression of ProT α and accelerated progression

into S-phase shortening the G₁-phase of the cell cycle. According to all these findings it is reasonable to believe that, in the acceleration of the cell cycle induced by Myc, ProTα plays an important role, since it is one of the most well-described targets for *myc* gene products [11,28,29,42,43].

Cellular differentiation is usually linked to the inhibition of mitosis [47], and it is difficult to ascertain whether the expression of a particular gene is associated with differentiation or with the simultaneous blockade of proliferation. We have previously demonstrated that inhibition of HL-60 cell proliferation by serum withdrawal did not affect the ProTα mRNA expression, suggesting that the decrease in levels of ProTα mRNA during HL-60 differentiation was associated with this process rather than with the arrest of proliferation [25]. In contrast, other authors [26] reported that the treatment of HL-60 cells with interferon γ, inducing monocytic differentiation without diminishing the rate of proliferation, did not result in any reduction in ProTα mRNA. According to this finding, the expression of ProTα would be related to proliferation events instead of the differentiation state of the cells. This apparent discrepancy with our results could be caused by the short period of incubation with interferon. In fact, after 2 days of incubation in the presence of interferon, HL-60 cells were still proliferating and they were not yet fully differentiated; only the HLA-DR marker is induced and the macrophage differentiation markers (apolipoprotein E or tumour-necrosis factor) are expressed only after 5–7 days of incubation [48]. Furthermore, in these cells the *c-myc* expression did not change after 2 days of incubation with interferon, but it was highly repressed after 3 days of incubation when cells were still proliferating but showed a high degree of differentiation.

The transcriptional activation of many genes appears to be implicated in cell division. Among these we can include the ProTα gene, since its expression is closely related to cell proliferation [4,6,18,22]. During cell differentiation the expression of some of these genes should be inhibited and at the same time the expression of some other genes has to be turned on. Our results indicate that ProTα belongs to the group of genes whose expression has to be switched off when the cells are committed to differentiate because, when the ProTα levels remain elevated, as a consequence of the expression of the sense vector, the cells cannot differentiate. Down-regulation of *c-myc* gene expression is necessary for the induction of proliferation arrest and, consequently, terminal differentiation [49]. However, it is not known whether this effect takes place through direct interaction with the differentiation machinery or whether it is the result of continued Myc-stimulated cell-cycle progression of cells that must undergo growth arrest for terminal differentiation. Perhaps one of the pathways employed for the cell to undergo terminal differentiation begins with the blockade of *c-myc* gene expression which, in turn, results in the inhibition of ProTα gene expression, facilitating in this manner the onset of differentiation. The present results strongly support an inverse relationship between ProTα levels and the extent of the differentiation, although future work will be required to establish the exact function of ProTα in the events leading to cell differentiation.

In spite of the important amount of experimental data about ProTα, until now the biochemical function of this abundant Myc-regulated nuclear protein remains unknown. However, it is becoming more clear that the role of ProTα could be related to nucleosome structure or chromatin remodelling, probably through its interaction with histones [50,51]. The results presented here not only provide new insights into the participation of ProTα in proliferation and differentiation, but also describe a biological system which may be very useful in the discovery of the function of this elusive protein.

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