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Down-regulated *RPS3a/nbl* Expression during Retinoid-induced Differentiation of HL-60 Cells: A Close Association with Diminished Susceptibility to Actinomycin D-stimulated Apoptosis

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ABSTRACT. **The efficacy of anticancer agents significantly depends on the differential susceptibility of undifferentiated cancer cells and differentiated normal cells to undergo apoptosis. We previously found that enhanced expression of *RPS3a/nbl,* which apparently encodes a ribosomal protein, seems to prime cells for apoptosis, while suppressing such enhanced expression triggers cell death. The present study found that HL-60 cells induced to differentiate by all-*trans* retinoic acid did not undergo apoptosis following treatment with actinomycin D whereas undifferentiated HL-60 cells were highly apoptosis-susceptible, confirming earlier suggestions that differentiated cells have diminished apoptosis-susceptibility. Undifferentiated HL-60 cells highly expressed *RPS3a/nbl* whereas all-*trans* retinoic acid -induced differentiated cells exhibited markedly reduced levels, suggesting that apoptosis-resistance of differentiated cells could be due to low *RPS3a/nbl* expression. Down-regulation of enhanced *RPS3a/nbl* expression was also observed in cells induced to differentiate with the retinoid 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)-1-propenyl]benzoic acid without any significant induction of cell death. While down-regulation of *RPS3a/nbl* expression during differentiation did not apparently induce apoptosis, *RPS3a/nbl* antisense oligomers triggered death of undifferentiated HL-60 cells, but not of retinoid-induced differentiated cells. It therefore seems that while down-regulation of enhanced *RPS3a/nbl* expression can induce apoptosis in undifferentiated cells, down-regulation of enhanced *RPS3a/nbl* expression during differentiation occurs independently of apoptosis, and could be regarded as reverting the primed condition to the unprimed (low RPS3a/nbl) state.**

Key words: apoptosis/differentiation/retinoic acid/HL-60 cell/RPS3a/nbl/actinomycin D

Apoptosis is a physiological process that occurs during normal development and maturation of most tissues, and culminates in the elimination of cell fragments without significant burden to a multicellular organism (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Wyllie, 1992). Successful cancer chemotherapy largely depends on the innate and acquired propensities of target cancer cells to undergo such an apoptotic program in response to anticancer agents. The difference in susceptibility of undifferentiated cancer cells and differentiated normal cells to undergo apoptosis in response to anticancer agents is one of the most crucial aspects of clinical treatment that needs to be considered.

Retinoids efficiently activate differentiation and/or apoptotic processes by binding to specific nuclear retinoid receptors: the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Nagy *et al.*, 1995). In particular, all-*trans* retinoic acid (ATRA) has been shown to activate RARs and induce differentiation of leukaemic cells (Giguere *et al.*, 1987; Martin *et al.*, 1990). It has been observed that HL-60 cells treated with ATRA or other differentiation-inducers become increasingly less susceptible to apoptosis stimulated by a variety of anticancer agents which inhibit gene expression (Solary *et al.*, 1993; Del Bino *et al.*, 1994; McCarthy *et al.*, 1994; Terui *et al.*, 1995). However, this may not always imply that differentiated cells are less susceptible than parental undifferentiated cells to the anticancer agents tested. Although inhibitors of gene expression reportedly trigger apoptosis of undifferentiated HL-60 cells, but not of HL-60 cell-derived differentiated cells, the decreased susceptibility of differentiated cells has been correlated with

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Abbreviations: ATRA, all-*trans* retinoic acid; TTNPB, retinoid 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)-1-propenyl]benzoic acid; RARs, retinoic acid receptors; RXRs, retinoid X receptors; Act D, actinomycin D; MNC, mononuclear cell.

the cell cycle position of the target cells rather than the cell's differentiation state (Terui *et al*., 1995). The influence of gene expression inhibitors on ATRA-stimulated cells is complicated by the ability of ATRA to not only induce differentiation, but also to stimulate apoptosis which is believed to be a secondary effect (Nagy *et al*., 1995). Therefore, the susceptibility of ATRA-induced differentiated cells to apoptosis induced by gene expression inhibitors such as Act D requires further confirmation.

While the molecular mechanisms underlying differences in apoptosis-susceptibility of normal and cancer cells are as yet unclear, our previous studies indicate an important role of the expression level of *RPS3a/nbl*, which apparently encodes a ribosomal protein (Naora *et al*., 1995, 1996, 1998a 1998b; Naora and Naora, 1999). Enhanced *RPS3a/nbl* expression, either constitutive or transient, seems to be a prerequisite for "priming" a cell for apoptosis, while abrupt suppression of such enhanced expression leads to execution of apoptosis (Naora *et al*., 1998a, 1998b; Naora and Naora, 1999). Of particular interest is that many cancer cells, such as parental HL-60 cells, constitutively express *RPS3a/nbl* at high levels, whereas normal cells maintain low levels of *RPS3a/nbl* expression (Naora *et al*., 1995, 1996). This implies that cancer cells, by virtue of their high constitutive *RPS3a/nbl* expression, are already "primed" for apoptosis and thus show a high potential for apoptotic induction by gene expression inhibitors. In contrast, normal cells, e.g. thymocytes, appear to have to undergo an initial priming step with a transient enhancement of *RPS3a/nbl* expression and thus exhibit little or no apoptosis in response to various gene expression inhibitors (Naora *et al*., 1995; Naora and Naora, 1999).

Given the critical role which the level of *RPS3a/nbl* expression apparently plays in regulating apoptosis-susceptibility of cancer cf. normal cells, it was of interest in the present study to investigate its role in regulating apoptosis-susceptibility of undifferentiated cf. differentiated cells. Using ATRA-induced differentiation and Act D-induced apoptosis of HL-60 cells as a model system, we confirmed that during incubation with Act D apoptotic cells are exclusively derived from undifferentiated, not differentiated, HL-60 cells. More importantly, we also show that susceptibility to apoptosis induced by Act D is associated with the level of *RPS3a/nbl* expression in these cells. Finally, we observe that the primed cellular condition of undifferentiated cells with enhanced *RPS3a/nbl* expression can be reverted to the unprimed state, i.e. low levels of *RPS3a/nbl* expression, separately from the apoptotic process.

Materials and Methods

Cell Culture

HL-60 cells were cultured in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY, USA) with 10% fetal bovine serum (GIBCO-BRL) (Naora and Naora, 1995). Cells were incubated at 2×10^5 cells/mL with either 1μM ATRA (all-*trans* retinoic acid) or 1μM TTNPB (4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)-1-propenyl]benzoic acid) (both from Sigma, St Louis, MO, USA) for 5 days unless otherwise stated. In some experiments, aliquots of 1×10^6 cells/mL were taken after the indicated length of incubation with or without the differentiating agents, and resuspended in fresh media for 6 hours with or without 1μg/mL Act D (Sigma, St Louis, MO, USA) (Naora *et al*., 1996) or 1μg/mL A23187 (Boehringer Mannheim GmbH, Mannheim, Germany) (Naora and Naora, 1995). Phosphorothioate oligomers used in the present experiments were the same as those used previously (Naora *et al*., 1998a). The *RPS3a/nbl* antisense oligomer contained the sequence complementary to the ATG initiation codon and the next four codons: 5'-CTTGCCAACCGCCAT-3'. A control oligomer contained random sequences of the same base composition as the *RPS3a/nbl* antisense oligomer; 5'-TCCAAGCCTTACGCC-3'. Oligomers were added at a concentration of 20μM to 1×10^4 cells in 96-well plates. Undifferentiated and differentiated cells were resuspended in 100μL fresh medium and incubated for 2 days with or without oligomers. ATRA was supplemented at 1μM to the fresh incubation medium for the differentiated cells. Mononuclear cells (MNC) were isolated from healthy human peripheral blood using LymphoSep Lymphocyte Separation Medium (ICN, NSW, Australia) according to manufacturer's instructions.

Morphological assessment of cell populations

We identified apoptotic and non-viable cells by the procedure of trypan blue dye exclusion using the criteria previously described (Naora *et al*., 1996). Briefly, viable cells were identified as those cells that excluded trypan blue dye. Apoptotic cells were identified as those cells which were refractile and showed membrane convolution but still excluded trypan blue dye, whereas those cells whose membrane integrity was compromised and failed to exclude trypan blue dye were considered non-viable (Wyllie *et al*., 1980; Naora *et al*., 1996, 1998a). In each experiment, a minimum of 400 cells were counted. This procedure was found to be more accurate for identifying the number of apoptotic and non-viable cells than the cytospinning-Giemsa staining method and flow cytometry. In the present studies, unless otherwise stated, the numbers of apoptotic and non-viable cells were pooled together and presented as "apoptotic/non-viable" cells.

Cytospins from aliquots of the cultures were prepared, fixed with methanol and stained with Giemsa stain (Histochrome, Medos Pty Ltd, Vic, Australia) (Naora *et al*., 1998a). Ratios of differentiated:undifferentiated cells in a population were determined by morphological criteria described elsewhere (Nagy *et al*., 1995). Cells showing morphological features of metamyelocytes and more matured forms were scored as "differentiated" cells as used by other researchers (Nagy *et al*., 1995). A minimum of 300 cells were scored.

Isolation and quantification of RNA

The *RPS3a/nbl* cDNA probe, isolation and Northern blot analysis

of RNA, and quantification of hybridised mRNA levels by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) analysis has been described elsewhere (Naora *et al*., 1995). The 0.95kb EcoRI fragment of pB4, containing the full length of human bcl-2 cDNA (kindly donated by Dr. Y. Tsujimoto, University of Osaka, Osaka, Japan) was used.

Flow cytometry

Quantitation of the immunostained protein contents per cell were performed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) using WinMDI software.

Immunostaining. For indirect staining with RPS3a/nbl antibody, cells were fixed in 70% ethanol overnight (Kimura *et al*., 1995) or 0.25% paraformaldehyde for 1 hour, followed by 70% methanol overnight at 4°C. Cells were then incubated with primary RPS3a/nbl antibody for 1 hour at 4°C, followed by washing and incubation with goat anti-rabbit-FITC antibody (Calbiochem-Novabiochem Corporation, SanDiego, CA, USA) for 30 minutes at 4°C. The polyclonal antibody raised in rabbits against a synthetic peptide compromising amino acid residues 241 to 264 of RPS3a/nbl was the same as that used in previous studies (Naora *et al*., 1998a). Cells fixed in 0.25% paraformaldehyde-methanol were also stained with bcl-2-FITC antibody clone 124 (DAKO, Carpinteria, CA, USA). In other experiments, cells were washed and then stained with CD11b-RPE antibody (Ancell Corporation, Bayport, MN, USA) or CD45-RPE antibody (Ditec, AS, Oslo, Norway), followed by washing and fixing in 0.25% paraformaldehyde-methanol. Cells were then stained with antibodies against either RPS3a/nbl or bcl-2 as described above. FITC-labeled mouse IgG1 (DAKO) served as a negative control.

Cytometric determination of apoptosis using propidium iodide. The percentage of cells containing fragmented DNA indicative of apoptosis was determined using propidium iodide staining of ethanol-fixed cells as previously described (Russell *et al*., 1998).

Sorting cell populations. Aliquots of parental HL-60 cells, Act D-incubated and ATRA-treated cells were fixed in 0.25% paraformaldehyde-methanol as described above. Populations of undifferentiated and differentiated cells, and of apoptotic cells were resolved using forward versus side light scatter parameters. Differentiated cells were collected from ATRA-treated cultures, undifferentiated cells collected from cultures of parental HL-60 cells, and apoptotic cells collected from Act D-incubated cultures. Giemsa-stained cytospin slides of sorted populations were microscopically examined.

Results

Morphological and molecular alterations during Act D-induced apoptosis of HL-60 cells

Following addition of Act D (1μg/mL) to HL-60 cell cultures, it has been observed that apoptotic cells, which were shrivelled but still excluded trypan blue dye, appeared rapidly and reached a maximum (approximately 40% of total cell population) at 6 hours (Naora *et al*., 1996). A rapid, early decrease in forward light scatter, i.e. 3 to 6 hours after Act D addition (Fig. 1), corresponded exactly to the time course of apoptotic morphological changes detected by trypan blue dye exclusion and also to DNA ladder formation (Naora *et al*., 1996). In contrast, changes in propidium iodide DNA-staining patterns continued up to 12 hours when smeared DNA fragmentation patterns, rather than distinct DNA ladders, were observed on agarose gels (Naora *et al*., 1996). We therefore used a 6 hour incubation with 1μg/mL Act D and the procedure of trypan blue dye exclusion as our routine assay system, and included propidium iodide and Giemsa staining for further confirmation of apoptosis induction.

To analyse molecular events occurring in HL-60 cells following Act D addition, *RPS3a/nbl* and *bcl-2* expression were examined. Rapid decay of *RPS3a/nbl* mRNA was observed (half life: 57 minutes), while the RPS3a/nbl level was down-regulated at a much slower rate (half life: 8.6 hours) (Fig. 2). The bcl-2 level decreased at a faster rate (half life: 3.7 hours) than that of RPS3a/nbl during the first 6 hours and thereafter was reduced at a slower rate (half

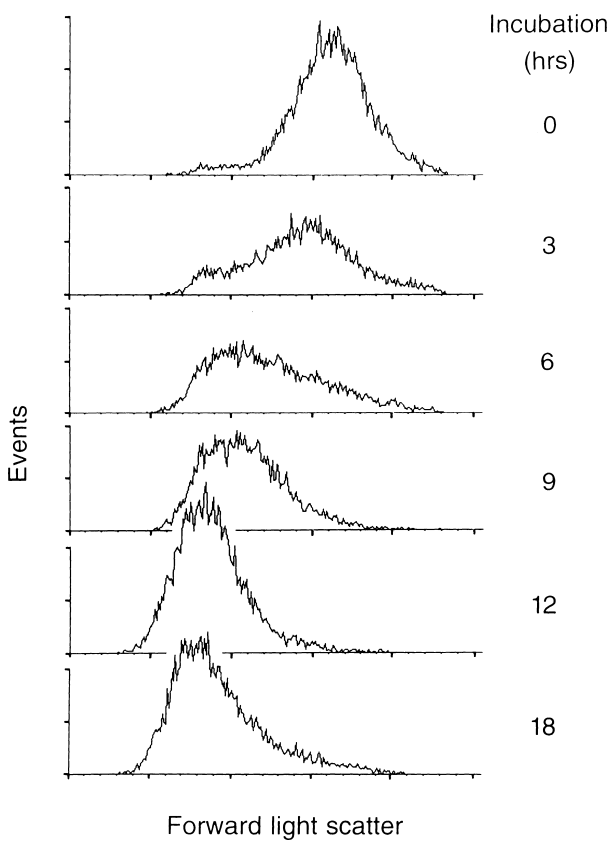

Fig. 1. Changes in forward light scatter patterns of HL-60 cells during Act D-induced apoptosis. HL-60 cells were incubated with 1μg/mL Act D for the indicated times and analysed by flow cytometry. Cell debris, identified on forward v side light scatter dotplots, were excluded from analysis.

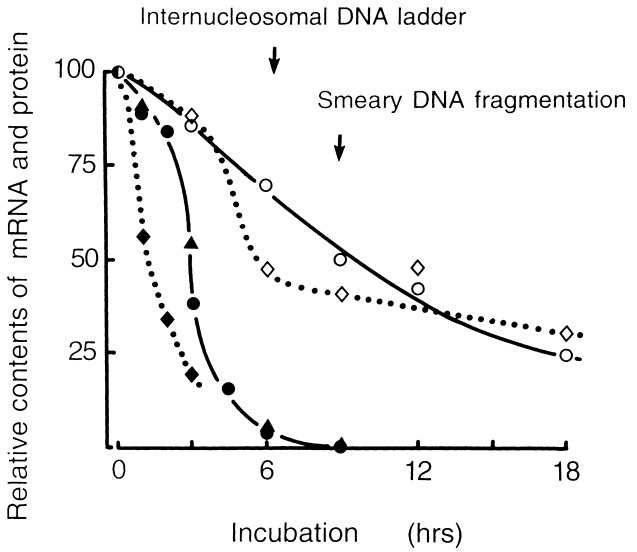

Fig. 2. Alterations in *RPS3a/nbl* and *bcl-2* mRNA and protein levels in HL-60 cells during Act D-induced apoptosis. HL-60 cells were incubated with 1μg/mL Act D, except at 5μg/mL (block triangle), for the indicated times. Levels of *RPS3a/nbl* mRNA (block circle/triangle), RPS3a/nbl (open circle), *bcl-2* mRNA (block diamond) and bcl-2 (open diamond) were quantified as described in the text. The measured levels were normalised such that the initial values prior to Act D addition equalled 100. The occurrence of major alterations in DNA integrity are indicated by arrows.

life: 18.5 hours). At 6 hours following Act D addition, the RPS3a/nbl content was approximately 70% of the original level, and apoptotic and non-viable cells comprised 33% and 4%, respectively, of the total cell population. It seems possible that a substantial amount of RPS3a/nbl was reduced in an apoptotic cell before the cell becomes unable to exclude trypan blue dye while non-apoptotic viable cells maintained their original RPS3a/nbl levels. Down-regulation of RPS3a/nbl levels was also observed when apoptosis was induced by the calcium ionophore A23187, suggesting that down-regulation of RPS3a/nbl levels was not a specific effect of Act D, but probably a conserved process in apoptosis.

Differentiated cells are insensitive to the induction of apoptosis by Act D

As mentioned earlier, ambiguity exists regarding the view that HL-60 cell-derived differentiated cells become insensitive to induction of apoptosis by various agents, e.g. Act D. We therefore carried out the following experiments. HL-60 cells were treated with 1μM ATRA for a period of 3 to 7 days. ATRA-treated HL-60 cells were then treated with 1μg/mL Act D for 6 hours. No alteration in total cell numbers was observed during the second incubation; the percentage of cells recovered after incubation with Act D being 104±7%. After incubation with Act D, three different cell types: undifferentiated, differentiated (as identified by the cytospinned-Giemsa staining procedure), and apoptotic/non-viable (as identified by the trypan blue dye exclusion procedure) were scored. The ratio of differentiated cells:undifferentiated cells in the total cell population increased with the length of ATRA stimulation and the net increment in the apoptotic/non-viable cell population following Act D induction inversely decreased with the length of prior ATRA stimulation. The size of the population of differentiated cells did not significantly alter during Act D treatment. The net increment in the apoptotic/non-viable cell population during Act D treatment was found to closely match the net decrement in the undifferentiated cell population. Figure 3 shows a summary of the observations made with increasing ratios of differentiated:undifferentiated cells. Extrapolation of increment and decrement lines reveals that both cross exactly at the point of 100 : 0 with equal rates of alteration, indicating that no apoptosis was induced by Act D if all cells had already been differentiated and that all of apoptotic/non-viable cells which were induced by Act D were directly derived from undifferentiated cells.

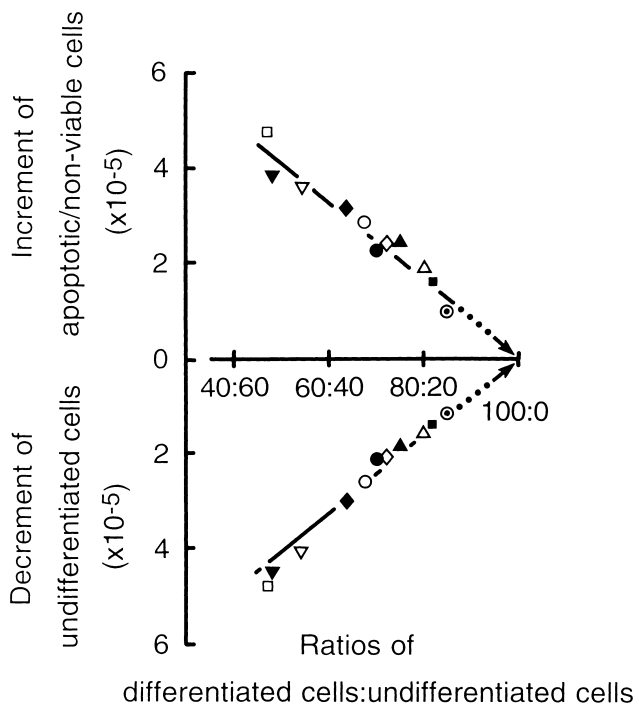

Fig. 3. Relationship between the appearance of apoptotic/non-viable cells and disappearance of undifferentiated cells during Act D-induced apoptosis. HL-60 cells (1×10^6 cells/mL) pre-stimulated with 1μM ATRA for 3 to 7 days were incubated for a further 6 hours with 1μg/mL Act D. The total number of undifferentiated and apoptotic/non-viable cells were counted before and after Act D addition. Numbers of these two cell types following Act D addition were expressed as a decrement below and an increment above the initial numbers of these cells prior to Act D addition. Each set of symbols represents the value of the decrement and increment, taken from numerous experiments, scored under a ratio of differentiated cells: undifferentiated cells.

Flow cytometric analysis of ATRA-treated and untreated HL-60 cells

Three distinct HL-60 cell populations were identifined by their light scatter patterns. The majority of ATRA-untreated cells were seen in the Group 2 (G2) cell population (high forward scatter) and some in the Group 1 (G1) cell population (low forward scatter) (Fig. 4a). Very few were seen in the Group 3 (G3) cell population (high side scatter). Following ATRA stimulation, a marked increase in the G3 cell population and concomitant decrease of the G2 cell population was observed (Fig. 4c), strongly suggesting that the G2 and G3 cell populations comprised undifferentiated and differentiated cells, respectively. This was verified by microscopic observations of cells sorted from the G2 and G3 populations.

When ATRA-untreated HL-60 cells were incubated with Act D for 6 hours, the cell population located between the G2 and G3 cell populations increased with a concomitant decrease in the G2 cell population (Fig. 4b). Since incubation with Act D induced apoptosis, it seems likely that these newly migrated cells were apoptotic. This was confirmed by microscopic examination of the cells sorted from this region. Apoptotic cells comprised only a minor proportion of the G2 and G3 cell populations. The cell population located between the G2 and G3 cell populations was considerably smaller in cultures of ATRA-stimulated cells following Act D incubation (compare Fig. 4d with Fig. 4b).

Expression patterns of various cell surface markers indicated that cells in the G3 population were differentiated, whereas those in the G2 population were undifferentiated HL-60 cells. ATRA-untreated HL-60 cells expressed CD11b, a widely used differentiation marker (Repo *et al.*, 1993; Park *et al.*, 1994; Kimura *et al.*, 1995; Kanatani *et al.*, 1997; Watson *et al.*, 1997), at very low or negligible levels. Increased CD11b expression was observed concomitantly with the increased population size of G3 following ATRA stimulation (Fig. 5a). A modest increase in CD45 expression was reproducibly seen following ATRA stimulation (Fig. 5b), as likewise observed by others in leukaemic cell undergoing differentiation (Taetle *et al.*, 1991; Buzzi *et al.*, 1992).

A small fraction of ATRA-untreated HL-60 cells, show-

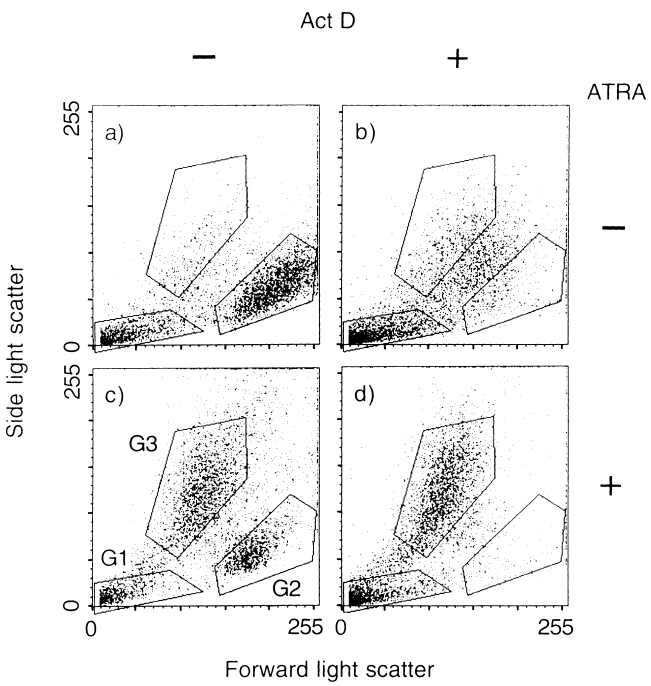

Fig. 4. Forward and side light scatter patterns of untreated and ATRA-treated HL-60 cells. HL-60 cells were incubated for 5 days with (c, d) or without (a, b) 1μM ATRA, followed by a further 6 hour incubation with (b, d) or without (a, c) 1μg/mL Act D. Different cell populations were identified by their distinct forward v side light scatter patterns. The G1 cell population includes cell debris. The G2 and G3 cell populations comprise undifferentiated and differentiated cells, respectively, while non-viable/apoptotic cells are primarily located in the region between the G2 and G3 populations. These populations were verified by the microscopic appearance of cells sorted from each population and their expression patterns of differentiation markers (see text).

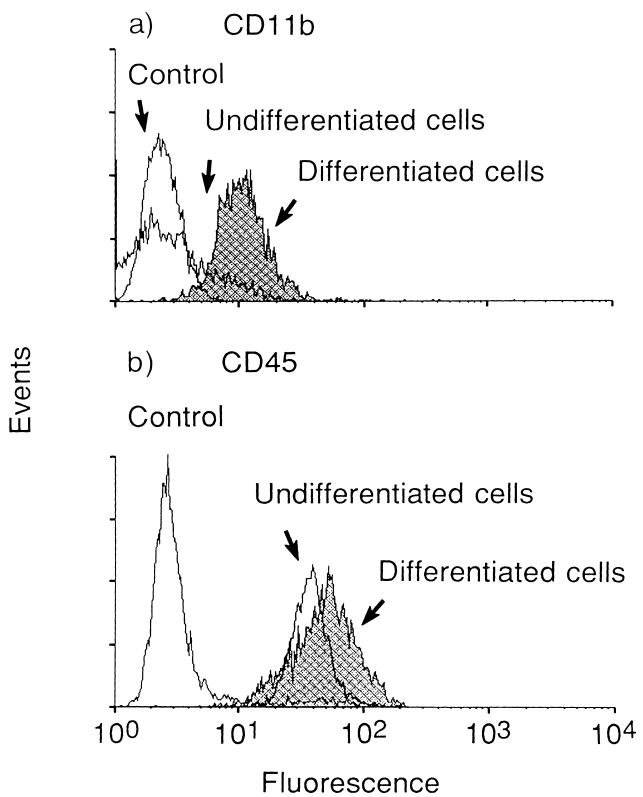

Fig. 5. Enhancement of CD11b and CD45 expression on ATRA-stimulated HL-60 cells. Changes in surface expression of (a) CD11b and (b) CD45 were assessed by immunofluorescence analysis of parental undifferentiated HL-60 cells (unshaded histograms), and HL-60 cells induced to differentiate by 1μM ATRA stimulation for 5 days (shaded histograms). Negative controls are also shown.

ing "granulocyte"-like morphology, were seen in the G3 cell population and showed increased CD11b and CD45 expression similar to cells of the G3 population in ATRA-stimulated cultures. Therefore, it seems likely that a small fraction of ATRA-untreated HL-60 cells spontaneously underwent differentiation. Also, cells that remained in the G2 cell population after 5 days ATRA stimulation showed virtually identical levels of CD11b and CD45 expression as the G2 population in ATRA-untreated cultures. Thus, it can be concluded that cells present in the G3 cell population were differentiated, whereas those in the G2 population were undifferentiated.

Changes in RPS3a/nbl levels during differentiation of HL-60 cells by ATRA

Since up-regulating *RPS3a/nbl* expression in cells which normally express *RPS3a/nbl* at low levels increases their susceptibility to Act D-induced apoptosis (Naora *et al.*, 1998b), we considered the possibility that the diminished apoptosis-susceptibility of ATRA-induced differentiated cells to Act D could be associated with down-regulated *RPS3a/nbl* expression. When HL-60 cells were induced to differentiate by treatment with ATRA for 5 days, *RPS3a/nbl* expression drastically decreased to 9.0±1.7% of the original level (Fig. 6a and b, Table I). The basal level of *RPS3a/nbl* expression in MNC isolated from healthy human blood was 10.4±0.4% of that of undifferentiated HL-60 cells (Table I), indicating that the reduced RPS3a/nbl content of ATRA-induced differentiated cells was at the level of normal *in vivo* differentiated cells. Induction of differentiation also led to a decrease in bcl-2 levels (Fig. 7), as likewise observed by others (Bradbury *et al.*, 1996; Mengubas *et al.*, 1996); bcl-2 levels decreased to 36.0±4.0% of the original level after 5 days of ATRA treatment. Down-regulated *RPS3a/nbl* expression was also observed in spontaneously differentiated cells in ATRA-untreated HL-60 cultures (Table I). Conversely, high *RPS3a/nbl* expression was maintained in HL-60 cells which remained undifferentiated following 5 days ATRA stimulation (Table I). These observations together suggest that down-regulation of *RPS3a/nbl* expression in differentiated cells is not a response peculiar to ATRA stimulation, but could be involved in the progression and/or maintenance of cell differentiation.

We also examined changes in RPS3a/nbl levels of undifferentiated and ATRA-induced differentiated cells following Act D treatment. Following incubation of parental HL-60 cells with Act D, we analysed the RPS3a/nbl content of these cells which were primarily found in the apoptotic cell population located between the G2 and G3 cell populations and in the G3 cell population (Fig.4b). As shown in Figure 6a, RPS3a/nbl levels significantly decreased during Act D-induced apoptosis of undifferentiated HL-60 cells (Fig. 6a, compare unshaded region with shaded region, +Act D). In contrast, little change was observed in the RPS3a/nbl distribution pattern of differentiated cells following Act D treatment (Fig. 6b); though a slightly elevated shoulder was noted (shaded region), which corresponded to the peak RPS3a/nbl level in apoptotic undifferentiated cells. Since a minor population in the ATRA-treated cultures remained undifferentiated, it seems likely that the residual undifferentiated cells become apoptotic during Act D treatment and the decreased RPS3a/nbl levels of such apoptotic cells were detected at the shoulder of the RPS3a/nbl distribution pattern. These results indicate a strong association between high resistance to apoptosis-induction by Act D and low levels of RPS3a/nbl. This implies that high *RPS3a/nbl*-expressing cells, i.e. undifferentiated cells, are readily susceptible to apoptosis induced by Act D, whereas cells in which *RPS3a/nbl* expression was down-regulated, i.e. differentiated cells, are no longer susceptible to Act D.

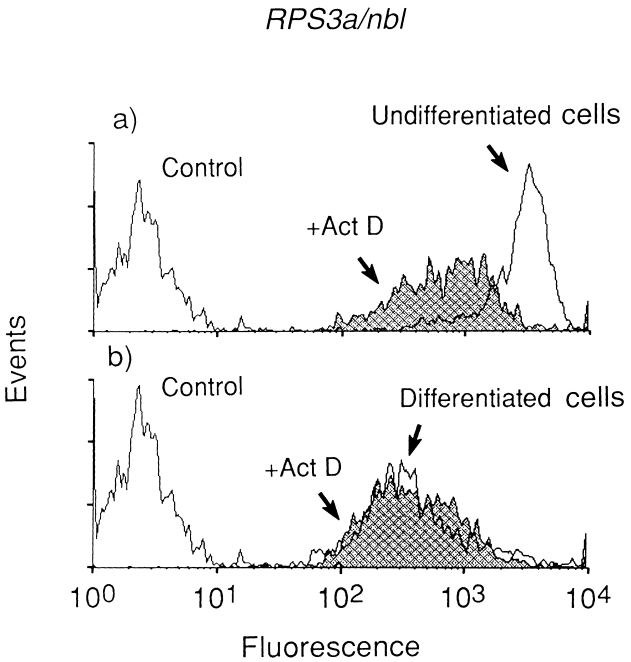

Fig. 6. Act D-induced changes in *RPS3a/nbl* expression in undifferentiated and ATRA-induced differentiated HL-60 cells. (a) Parental undifferentiated HL-60 cells and (b) HL-60 cells induced to differentiate by 1μM ATRA stimulation for 5 days were incubated with 1μg/mL Act D for a further 6 hours. Changes in RPS3a/nbl levels were assessed before (unshaded histogram) and after (shaded histogram) Act D addition. Negative controls are also shown. A direct comparison of (a) and (b) can be made.

RPS3a/nbl antisense oligomers induce apoptosis in undifferentiated cells, but not in differentiated cells

It has been shown that apoptosis can be induced in cells which express *RPS3a/nbl* at high but not at low levels by specifically inhibiting *RPS3a/nbl* expression (Naora *et al.*, 1998a). Since differentiated cells contain low RPS3a/nbl levels, we considered the possibility that ATRA-treated HL-

Table I. RELATIVE RPS3a/nbl CONTENTS OF DIFFERENTIATED AND UNIDIFFERENTIATED CELLS

Treatments of cells	Relative RPS3a/nbl contents[a]				Apoptotic/ non-viable cells in total call population[b] (%)	Ratios of differentiated: undifferentiated cells within non-apoptotic, viable cell population[b]
	in vivo differented cells	Spontaneously differentiated cells	Chemically induced differentiated cells	Chemically induced undifferentiated cells		
No treatment	——	0.098±0.026[c] (n=6)	——	——	10.1±2.7 (n=13)	7.5±2.8:92.5±2.8 (n=19)
ATRA (1μM, 5 days)	——	——	0.090±0.017[c] (n=6)	1.128±0.123 (n=7)	52.5±15.3 (n=7)	77.5±4.1:22.5±4.1 (n=5)
TTNPB (1μM, 5 days)	——	——	0.098±0.007[c] (n=6)	0.990±0.072 (n=6)	16.5±3.6 (n=5)	72.3±5.9:27.7±5.9 (n=5)
Human MNC	0.104±0.004[c] (n=2)	——	——	——	——	——

[a] Relative RPS3a/nbl contents are expressed as mean±S.E. of the ratios between the mean fluorescence intensities of the indicated cell population and the mean fluorescence intensities of untreated, undifferentiated HL-60 cells with the numbers (n) of experimental samples measured shown in parentheses below.

[b] Apoptotic/non-viable cells and the ratios of differentiated:undifferentiated cells were measured by microscopic observations as described in the Materials and Methods and expressed as mean±S.E.

[c] Differences between the value of human MNC and those of spontaneously or chemically induced differentiated cells are not statistically (*t* test) significant.

60 cells become resistant to apoptosis triggered by specifically inhibiting *RPS3a/nbl* expression with *RPS3a/nbl* antisense oligomers. Parental HL-60 cells were incubated with *RPS3a/nbl* antisense oligomers for 2 days at a final concentration of 20 μM, conditions under which significant inhibition of *RPS3a/nbl* expression has been previously reported (Naora *et al*., 1998a). Such cultures showed a marked increase in numbers of non-viable cells and of cells exhibiting typical features of apoptosis such as shrinkage, blebbing, chromatin condensation, nuclear and cell fragmentation as previously reported (Naora *et al*., 1998a) (Fig. 8). In contrast, oligomers comprising random sequences did not induce any apoptotic cell death, with there being no significant difference in viability from cells incubated without oligomers (Fig. 8). This result indicates that the cell death observed with antisense oligomers is not due to possible non-antisense-specific effects of phosphorothioate oligomers which have been observed in other experimental systems (Krieg, 1992; Vaerman *et al*., 1995). In contrast to undifferentiated cells, cultures of ATRA-induced differentiated cells showed no increase in numbers of apoptotic and non-viable cells following incubation with *RPS3a/nbl* antisense oligomers (Fig. 8). It seems therefore that undifferentiated HL-60 cells are highly susceptible to apoptotic induction by specific inhibitory *RPS3a/nbl* antisense oligomers, whereas HL-60 cells, once they are differentiated, are no longer susceptible.

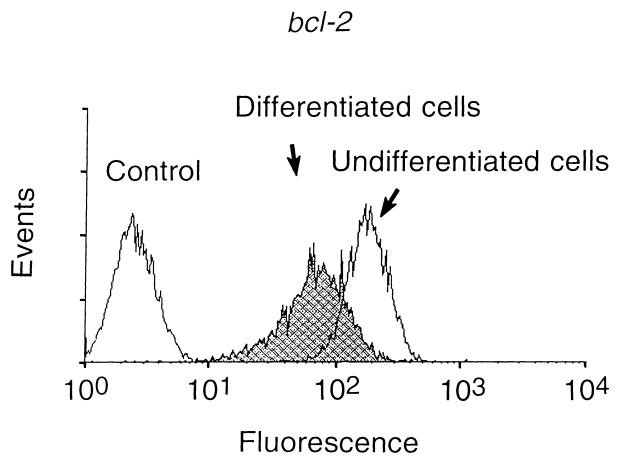

Fig. 7. Down-regulation of *bcl-2* expression in ATRA-induced differentiated cells. Changes in *bcl-2* expression were assessed by immunofluorescence analysis of parental undifferentiated HL-60 cells (unshaded region), and HL-60 cells induced to differentiate by 1μM ATRA stimulation for 5 days (shaded region).

Down-regulation of RPS3a/nbl levels during ATRA-induced differentiation is involved in the differentiation process, independently of apoptosis

It has been reported that cellular differentiation proceeds independently of apoptosis through ligand activation of RARs (Park *et al*., 1994; Nagy *et al*., 1995). While ATRA preferentially binds to RARs and induces differentiation (Nagy *et al*., 1995), the population of apoptotic cells seen in ATRA-treated cultures was higher than the small pool of spontaneously apoptotic cells observed in untreated parental HL-60 cultures (Table I). Apoptosis following ATRA treatment of

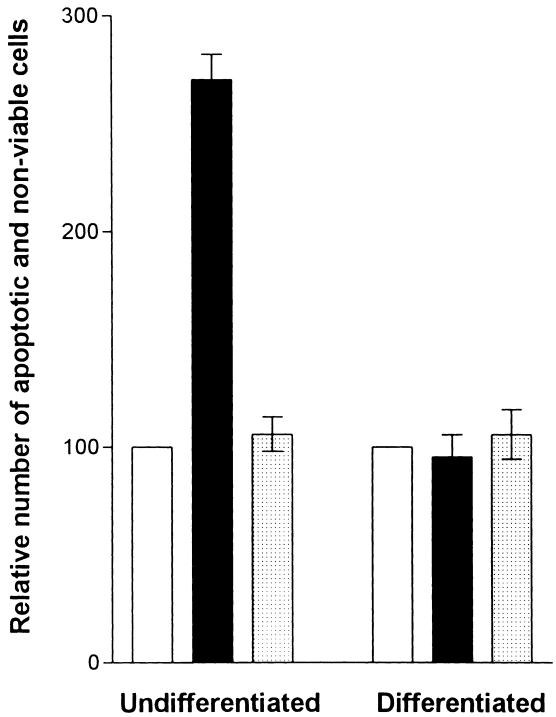

Fig. 8. Effect of *RPS3a/nbl* antisense oligomers on undifferentiated and ATRA-induced differentiated HL-60 cells. Parental HL-60 cells and HL-60 cells pre-stimulated with ATRA for 5 days were incubated with no oligomers (open bar), *RPS3a/nbl* antisense oligomers (solid bar) or random oligomers (dotted bar). The number of apoptotic/non-viable cells per well were scored as described in the *Materials and Methods*. Results were normalised such that the values observed with no oligomer equalled 100. Values represent mean ± S.E. of duplicates.

HL-60 cells has been observed by others and has been attributed to *in vitro* isomerization of ATRA to 9-*cis* retinoic acid which activates RXRs and triggers apoptosis (Nagy *et al.*, 1995). To assess whether down-regulation of RPS3a/nbl levels during ATRA-induced differentiation is involved in the differentiation process *per se*, we used the retinoic acid analog TTNPB, which is highly specific for RARs and preferentially induces differentiation but not apoptosis (Sheikh *et al.*, 1994; Nagy *et al.*, 1995). As shown in Table I, the population size of apoptotic/non-viable cells in TTNPB-treated cultures was markedly smaller than that seen in ATRA-treated cultures and only marginally higher than that observed in untreated parental cultures. Cells in TTNPB-treated cultures exhibited typical "granulocyte" like morphology, were primarily located in the G3 cell population and showed increased CD11b and CD45 expression, as likewise observed in ATRA-treated cultures.

RPS3a/nbl levels in TTNPB-induced differentiated cells were found to be almost identical to those in ATRA-induced differentiated cells (Table I), indicating that RPS3a/nbl levels were down-regulated in differentiated cells even under conditions in which the apoptotic process was not specifically activated. bcl-2 levels also decreased during TTNPB-induced differentiation. High levels of RPS3a/nbl, identical to those of untreated, undifferentiated HL-60 cells, were maintained in cells which remained undifferentiated following TTNPB treatment (Table I). It therefore seems that TTNPB did not down-regulate *RPS3a/nbl* expression unless a cell was differentiated or in progress towards differentiation. These observations indicate that down-regulation of RPS3a/nbl levels observed during ATRA-treatment is not involved as part of the apoptotic process, but occurs as part of the differentiation process.

Discussion

Our previous studies have supported a model in which enhanced *RPS3a/nbl* expression could be regarded as priming a cell for apoptosis, the execution of which requires abrupt suppression of such enhanced expression (Naora *et al.*, 1996, 1998a, 1998b; Naora and Naora, 1999). The present study found undifferentiated HL-60 cells to be highly susceptible to Act D-induced apoptosis, whereas ATRA-induced differentiated cells are resistant. While undifferentiated HL-60 cells highly expressed *RPS3a/nbl*, it appears that the apoptosis-resistance observed in differentiated cells could be due to their markedly reduced levels of RPS3a/nbl which were similar to those of normal *in vivo* differentiated MNC. It was revealed, furthermore, that down-regulation of *RPS3a/nbl* expression during ATRA treatment was not directly linked to the apoptotic process triggered via RXRs activation, as down-regulation of enhanced *RPS3a/nbl* expression was also observed in HL-60 cells induced to differentiate with the highly RAR-specific retinoid TTNPB without any significant induction of cell death. While down-regulation of enhanced *RPS3a/nbl* expression during differentiation did not apparently induce cell death, apoptosis could be actively triggered by inhibiting enhanced *RPS3a/nbl* expression in undifferentiated cells since, at least under the experimental conditions used, incubation with *RPS3a/nbl* antisense oligomers triggered death of parental HL-60 cells. It therefore appears that down-regulation of enhanced *RPS3a/nbl* expression during differentiation takes place independently of apoptosis which also requires down-regulation of enhanced *RPS3a/nbl* expression. This notion is consistent with the general conclusion drawn from various studies that differentiation and apoptosis of retinoid-treated cells, while closely linked, proceed independently via distinct pathways (Park *et al.*, 1994; Nagy *et al.*, 1995, 1998). While down-regulation of the high RPS3a/nbl levels in undifferentiated cells apparently triggers cell death, down-regulation of RPS3a/nbl levels during retinoid-induced differentiation could be regarded as unpriming the cellular apoptotic machinery; in other words, safely reverting the primed apoptotic condition of undifferentiated cells to the unprimed state.

Various mechanisms could explain how down-regulation

of enhanced *RPS3a/nbl* expression occurs during differentiation without triggering cell death. The rate of *RPS3a/nbl* down-regulation during differentiation could play a vital role in avoiding apoptosis. While *RPS3a/nbl* down-regulation during apoptosis has been in general abrupt, in the order of hours both *in vitro* and *in vivo* experiments (Naora *et al*., 1995, 1996, 1998b), retinoid-induced differentiation gradually proceeded over several days. Since apoptosis and differentiation are multi-gene processes, other molecules could be involved in protecting cells during reversion from the primed (high RPS3a/nbl) to unprimed (low RPS3a/nbl) state. Such a mechanism could be built in the system required for the differentiation process or maintenance of the differentiated state, and could explain how all normal differentiated cells studied so far maintain *RPS3a/nbl* expression at low constitutive levels without their survival being adversely compromised. It is unlikely that bcl-2 *per se* is directly involved in preventing cell death from occurring during *RPS3a/nbl* down-regulation in cells undergoing differentiation, since we find that bcl-2 levels are also down-regulated. From similar observations made in earlier studies, it was concluded that differential apoptosis-susceptibility of undifferentiated and differentiated cells could be regulated by mechanisms independent of bcl-2 (Bradbury *et al.*, 1996; Mengubas *et al*., 1996). However, various members of the bcl-2 family could be cooperatively involved in the mechanism in which differential apoptosis-susceptibility at low and high levels of *RPS3a/nbl* expression is regulated. Cooperative involvement could include direct protein-protein interactions, as various individual ribosomal proteins can interact with, and modulate the activities of, growth regulatory factors. For example, ribosomal protein L34 has recently been reported to modulate the activities of cyclin dependent kinases 4 and 5 via protein-protein interactions (Moorthamer and Chaudhuri, 1999), while the role of the pituitary tumor-transforming gene product in male germ cell differentiation could involve its interaction with ribosomal protein S10 (Pei, 1999). A few possible models of the direct or indirect interactions of RPS3a/nbl with bcl-2 family members are attractive, but are purely speculative at present and thus await experimental confirmation.

The observation that retinoid-induced differentiated cells contain markedly reduced levels of RPS3a/nbl similar to those in normal *in vivo* differentiated MNC raises the question as to the role and biological significance of low *RPS3a/nbl* expression in differentiated cells. The observed parallel down-regulation of RPS3a/nbl and bcl-2 levels during differentiation could play a significant role in the limited life span of differentiated cells. While our observations indicate that undifferentiated cancer cells undergo apoptosis more readily than differentiated cells, it should be noted that differentiated cells do not proliferate and ultimately die under *in vitro* and *in vivo* conditions. Differentiated cells would presumably undergo apoptosis if cells are reprimed for apoptosis by genuine inducers. Such repriming steps could include transient enhancement of *RPS3a/nbl* expression as observed in thymocytes (Naora *et al*., 1995). Act D is clearly not the genuine apoptosis-inducer for differentiated cells; rather it suppresses the priming responses required for apoptosis thereby blocking the apoptotic process in differentiated cells.

Since RPS3a/nbl is an important ribosomal component (Naora and Naora, 1999), down-regulation of its levels could disrupt ribosome formation and thereby protein synthesis. Disrupted protein synthesis could in turn trigger apoptosis by reducing levels of labile survival/anti-apoptotic factors (Steller, 1995; Kho *et al*., 1996). While increased ribosomal protein expression and ribosome biogenesis occur in actively proliferating cells (Tushinski and Warner, 1982), down-regulated expression of various ribosomal protein genes has been observed during differentiation of leukaemic cells (Lin *et al*., 1994). It is possible that safe conversion of the apoptotic primed state to the unprimed state which occurs during differentiation is associated with down-regulated ribosome biogenesis and/or reduced protein synthetic activity. However, a role of RPS3a/nbl in translation *per se* cannot fully account for the prerequisite of RPS3a/nbl levels to be enhanced for apoptotic priming. Many individual ribosomal proteins can apparently function in a capacity independent of and mechanistically distinct from that in translation (reviewed in Wool, 1996; Naora and Naora, 1999). Various genes that have been identified as encoding presumptive ribosomal proteins were originally cloned in a search for non-ribosomal functions. For example, an endonuclease involved in DNA repair is apparently identical to ribosomal protein S3 (Kim *et al*., 1995), while a monocyte chemotactic factor has been identified as a homodimer of ribosomal protein S19 (Horino *et al*., 1998). In fact, *RPS3a/nbl* was originally isolated by virtue of its abundance in a Namalwa Burkitt Lymphoma cDNA library (Naora *et al*., 1995) and found to encode sequences of a clone isolated by screening an expression library with a RPS3a antibody (Metspalu *et al*., 1992). Since the notions of "gene recruitment" and "one gene-dual function" have become increasingly accepted (see Wistow, 1995), it is quite possible that RPS3a/nbl plays a crucial role in apoptosis in an extraribosomal capacity.

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