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Co-ordinated downregulation of bcl-2 and bax expression during granulocytic and macrophage-like differentiation of the HL60 promyelocytic leukaemia cell line

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Abstract The bcl-2 protein suppresses apoptosis and the bax protein opposes the cytoprotective effect of bcl-2. A decrease in bcl-2 levels has been implicated in the induction of apoptosis during the terminal differentiation of HL60 myeloid leukaemia cells. We show here that bax protein also declined with a time course similar to the downregulation of bcl-2 following treatment of HL60 with phorbol myristate acetate (PMA), dimethyl sulphoxide (DMSO) or retinoic acid (RA). Decreased bcl-2 protein expression in induced cells was associated with down-regulation of its mRNA. By contrast, the decrease in bax occurred by a post-transcriptional mechanism. Co-ordinate downregulation of bcl-2 and bax proteins may fine-tune the induction of apoptosis during cellular differentiation.

Key words: Apoptosis; Proto-oncogene protein; Cell differentiation; Myeloid leukemia; HL60 cell

1. Introduction

Apoptosis is a form of physiological cell death regulated by endogenous cellular proteins including bcl-2, bcl-X and bax [1–3]. High bcl-2/bax ratios favour cell survival whereas low ratios promote apoptosis [4]. Therefore, regulation of the relative levels of bcl-2 and bax may play an important role in modulating the susceptibility of cells to apoptosis.

Bcl-2 levels decline following the induction of differentiation of HL60 myeloid leukaemia cells [5–7], a process which results in eventual apoptosis. Abrogation of differentiation-linked cell death by ectopic bcl-2 expression [6,7] suggests that the downregulation of this protein is an important determinant of apoptosis in this system. Because enforced expression of bcl-2 increases the stability of the bax protein [8], we carried out experiments to determine whether bax levels also changed during HL60 cell differentiation. We show here that bax protein levels decreased co-ordinately with bcl-2 when HL60 cells were induced to differentiate following treatment with phorbol myristate acetate (PMA), dimethyl sulphoxide (DMSO) or retinoic acid (RA).

2. Materials and methods

2.1. Cells and cell culture

HL60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and were induced to differentiate by the addition of 10^{-6} M RA, 1.4% DMSO or 100 nM PMA as described [9–11]. A proportion of the TPA-induced cells adhered to the culture flask. These cells were detached by incubation with 0.5 g l^{-1} trypsin. Data for adherent and non-adherent cells in TPA-treated cultures are therefore presented in the figures.

2.2. Detection of DNA strand breaks

The random oligonucleotide-primed synthesis (ROPS) method was used to estimate the induction of endonuclease-mediated strand breaks [12].

2.3. Antibodies and Western blotting

Bcl-2 and actin antibodies were from Oncogene Science, NY, USA. The P-19 bax antibody was from Santa Cruz Biotechnology, CA, USA. Western blotting was carried out essentially as described [13]. Thirty micrograms of protein were loaded in each lane of a 15% SDS-polyacrylamide gel. Following electrophoretic transfer to ECL Hybond (Amersham International, UK), the membrane was incubated sequentially with bcl-2, bax or actin monoclonal antibodies. The blots were detected by enhanced chemiluminescence (ECL System, Amersham International, UK). The bcl-2, bax and actin antibodies detected 26, 21 and 44 kDa proteins respectively.

2.4. RNA extraction and cDNA synthesis

RNA was extracted using the guanidium isothiocyanate/acid phenol procedure [14]. Complementary DNA (cDNA) was synthesised from 1 μg aliquots of each RNA preparation using random hexamer primers (Boehringer, UK) and Moloney murine leukaemia virus reverse transcriptase (Life Technologies, UK).

2.5. RT PCR and Southern blotting

PCR was performed in 25 μl reactions using Taq DNA polymerase (Life Technologies, UK). cDNA equivalent to 0.1 μg RNA was amplified in each reaction. Forward (F) and reverse (R) primers for quantitation of mRNA, designed to span at least one intron, were as follows: Actin F: TGC TAT CCA GGC TGT GCT AT (nts 443–462). Actin R: GAT GGA GTT GAA GGT AGT TT (nts 887–868 [15]). Bcl-2 F: ACA ACA TCG CCC TGT GGA TGA C (nts 512–533). Bcl-2 R: ATA GCT GAT TCG ACG TTT TGC C (nts 920–899; Fig. 3A of [16]). Bax F: TGA CGG CAA CTT AAC TGG G (nts 303–322). Bax R: TTC CAG ATG GTG AGC GAG G (nts 569–548 [3]). Primary (unspliced) bcl-2 transcripts were quantitated using the bcl-2 F primer in conjunction with a reverse primer located within the second intron of the bcl-2 gene (AGC CCA GAC TCA CAT CAC CAG ATG; nucleotides 616–593; Fig. 3B of [16]). PCR annealing temperatures of 54°C, 56°C and 59°C were used for amplification using actin, bcl-2 and bax primers respectively.

PCR products were electrophoresed in 2% w/v agarose gels, transferred to Hybond N membranes (Amersham International, UK) and hybridised to ^{32}P-labelled riboprobes generated by in vitro transcription of sequences cloned into the bluescript vector (Stratagene, Cambridge, UK). Autoradiographic bands were quantitated using a Molecular Dynamics Scanner. The cDNA inputs and cycle numbers used for PCR analysis were established in preliminary experiments such that the product band intensity was proportional to the cDNA input. Each PCR reaction was sampled following 20, 22, 24 and 26 cycles to ensure that reactions were in the exponential phase of amplification. For clarity, the bands resulting from 22 amplification cycles only have been excised for presentation (Fig. 4).

3. Results

Treatment of HL60 cells with PMA induces their differen-

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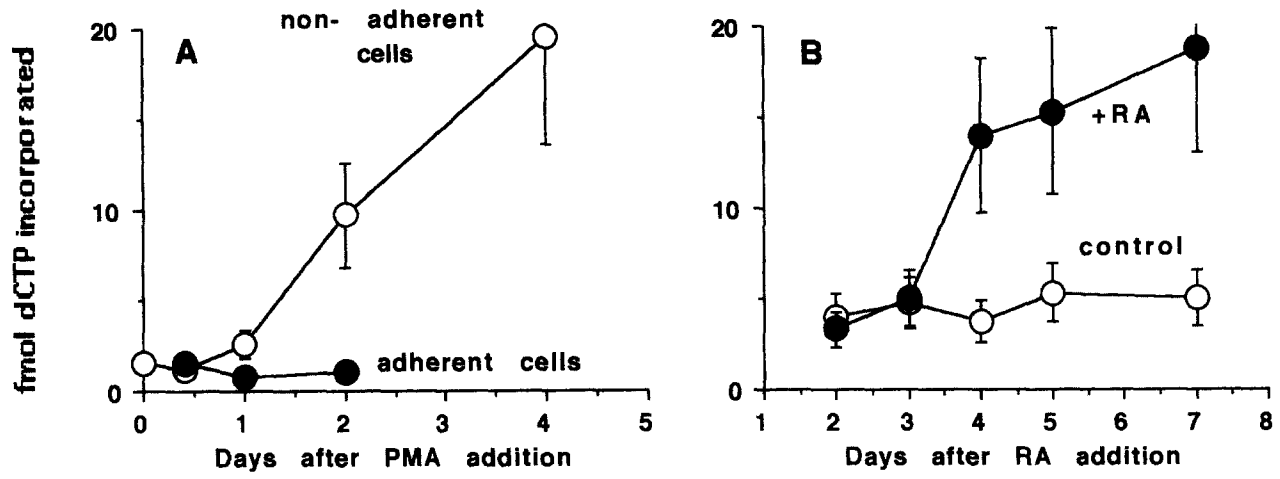

Fig. 1. Detection of DNA cleavage in HL60 cells treated with 100 nM PMA (A) or 10^{-6} M RA (B). The incorporation of α-[^{32}P]dCTP is proportional to DNA strand breaks [12]. The bars indicate the range of duplicate measurements.

tiation to cells resembling macrophages, a proportion of which adhere to the culture flask [11]. By contrast, DMSO and RA induce differentiation to cells resembling granulocytes [9,10]. Both these pathways result in eventual apoptosis [6,7,17]. We have confirmed that PMA (Fig. 1A) or RA (Fig. 1B) treatment induces DNA strand breakage, an early feature of apoptosis [12], in HL60 cells. In PMA-treated cells, induction of strand breaks was only detected in the non-adherent population, as previously reported [17]. We have also confirmed the induction of DNA strand breaks in DMSO-treated cells using the TUNEL assay [18] (data not shown).

Western blot analysis showed that the bcl-2 protein was downregulated in HL60 cells treated with PMA (Fig. 2A,), DMSO (Fig. 2B) or RA (Fig. 2C). In PMA-treated cultures bcl-2 downregulation was clearly evident in both the adherent and non-adherent cell populations (Fig. 2A). The downregulation of bcl-2 protein during HL60 differentiation was confirmed by densitometric scanning of the bands and their normalisation with respect to actin band intensities determined on the same blots (Fig. 3A,C,E). Bax protein expression was also strikingly downregulated following induction of differentiation by each of the agents tested (Fig. 2). Quantitation of the bax bands and normalisation with respect to actin showed that the time-courses of bax downregulation in HL60 cells induced to differentiate by each of the three agents tested were similar to that of the decline of bcl-2 expression (Fig. 3B,D,F).

To determine whether downregulation of bcl-2 and bax protein were mediated via modulation of their respective mRNAs, we analysed the expression of the transcripts encoding these species by semi-quantitative RT PCR procedures. The bcl-2 or bax bands generated by RT PCR were detected by Southern blotting using appropriate radiolabelled probes and were normalised with respect to actin RT PCR bands generated by amplification of the same cDNAs. Bcl-2 mRNA expression declined sharply in both adherent and non-adherent cells following PMA treatment (Fig. 4A, Fig. 5A) and in cultures induced with DMSO (Fig. 4B, Fig. 5C) or with RA (Fig. 4C, Fig. 5E). These observations suggest that the decline in bcl-2 protein results from a decrease in cellular levels of bcl-2 mRNA following the induction of differentiation by each of the agents tested and are compatible with a transcriptional mechanism of downregulation of bcl-2 expression.

The bcl-2 gene contains a large (>350 kb) intron. Because RA treatment modulates the splicing of some primary transcripts [19], we determined whether the decline in bcl-2 mRNA levels in RA-treated HL60 cells resulted from a decreased rate of excision of this intron from primary transcripts. Simultaneous RT PCR analyses were performed using primer pairs which amplified either the spliced or the unspliced bcl-2 transcript (Section 2.5). Both types of transcript declined in parallel following RA treatment (Fig. 6), showing that the RA-induced downregulation of bcl-2 mRNA was not

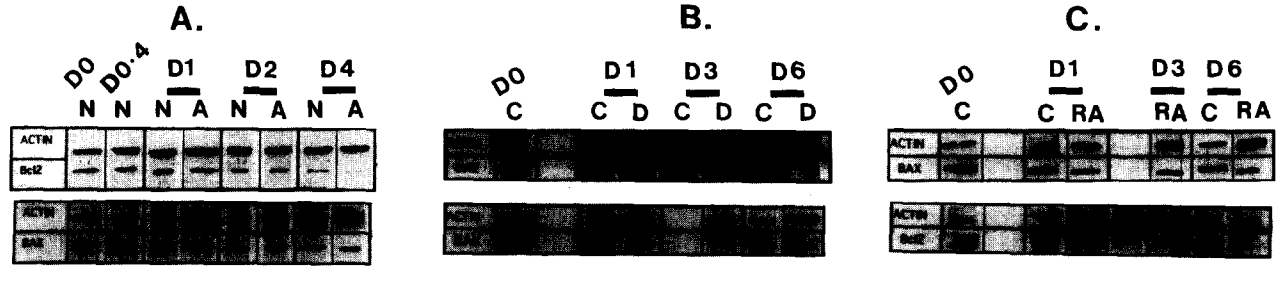

Fig. 2. Western blot analysis for actin, bcl-2 and bax proteins in HL60 cells treated with 100 nM PMA (A), 1.4% DMSO (B) or 10^{-6} M RA (C). The days after addition of inducers are indicated above the lanes. In (A), N and A denote non-adherent and adherent cells respectively. In (B), C and D denote control and DMSO-treated cells. In (C), C and RA denote control and RA-treated cells.

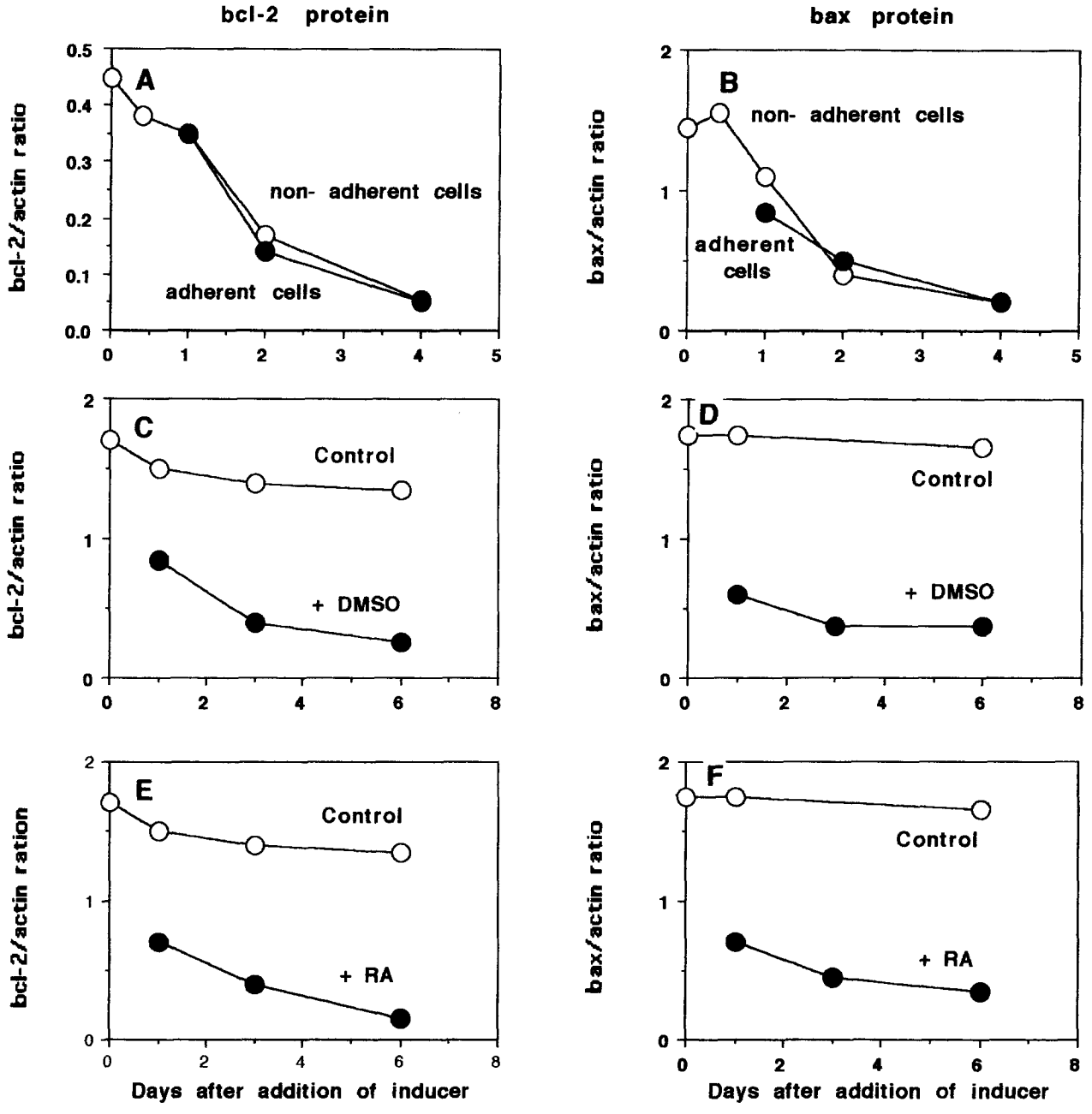

Fig. 3. Quantitation of expression of bcl-2 and bax proteins in HL60 cells treated with PMA (A,B), DMSO (C,D) or RA (E,F). The protein bands depicted in Fig. 2 were scanned. The intensities of bcl-2 and bax bands were normalised with respect to the intensities of actin bands detected on the same blots. Similar results were obtained in two independent experiments.

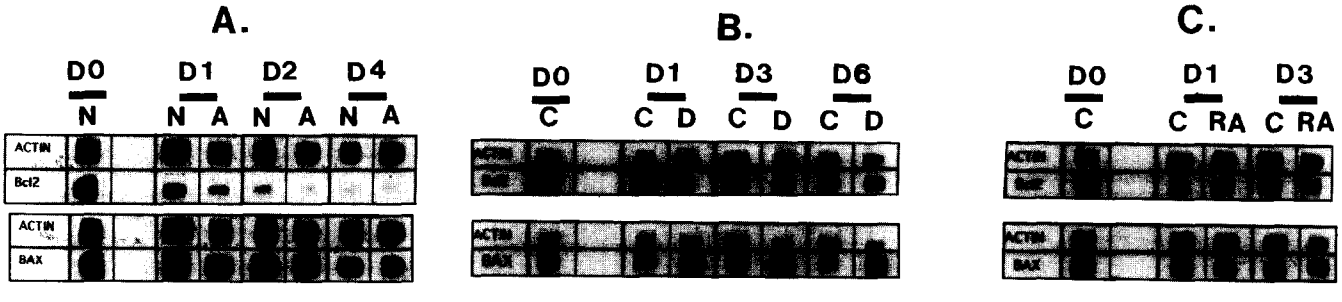

Fig. 4. RT PCR analysis for actin, bcl-2 and bax mRNA in HL60 cells treated with 100 nM PMA (A), 1.4% DMSO (B) or 10^{-6} M RA (C). Labelling of lanes is as in Fig. 2.

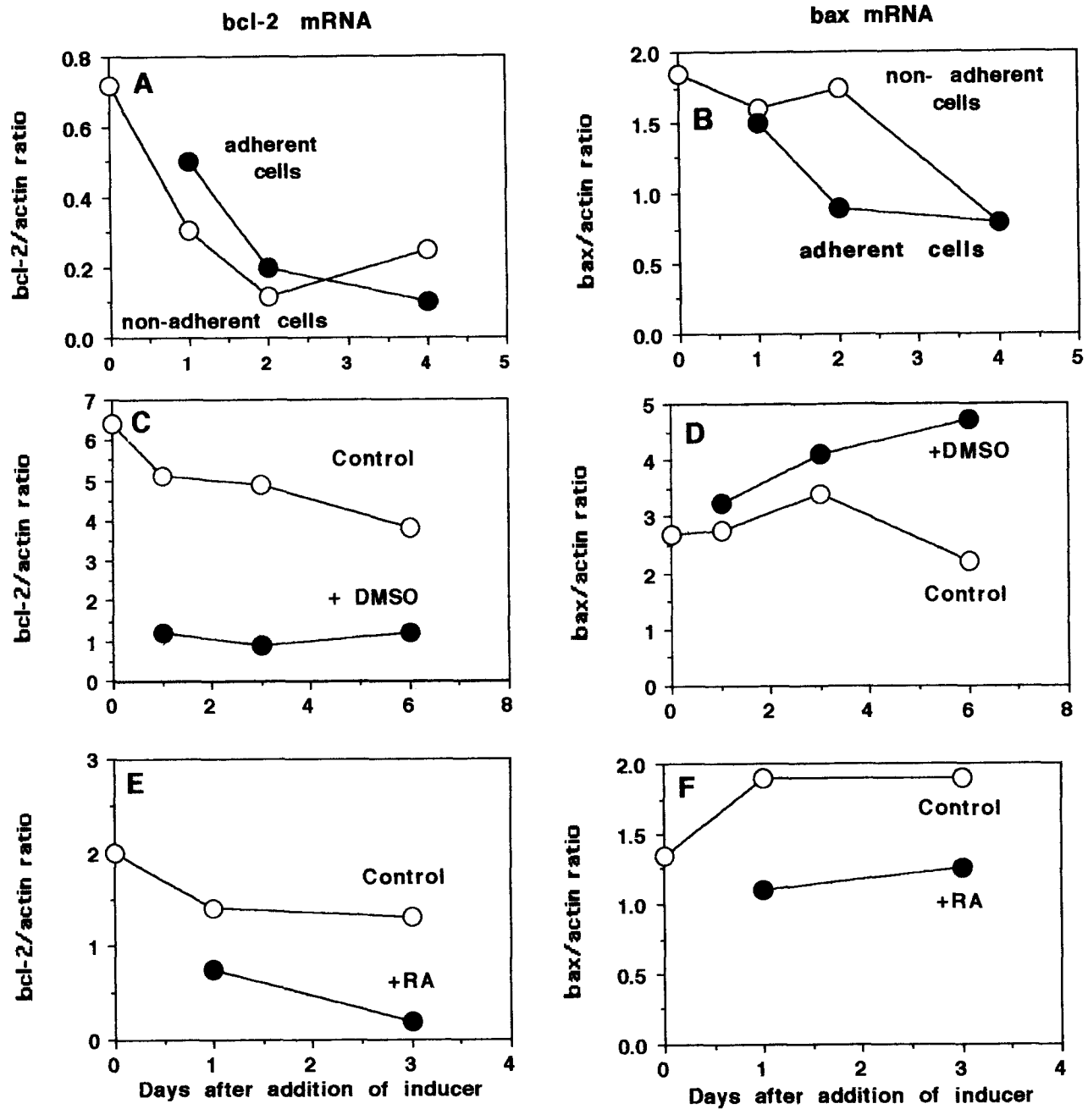

Fig. 5. Quantitation of expression of bcl-2 and bax mRNA in HL60 cells treated with PMA (A,B), DMSO (C,D) or RA (E,F). The RT PCR product bands depicted in Fig. 4 were scanned. The intensities of bcl-2 and bax bands in each cDNA sample were normalised with respect to the intensities of actin bands detected by PCR amplification of the same cDNA. Similar results were obtained in three independent experiments.

the result of impaired splicing. Instead, the data are compatible with the RA-induced downregulation of generation of primary bcl-2 transcripts.

By contrast, bax protein expression (Fig. 3B) declined before substantial downregulation of bax mRNA levels were detected in either adherent or non-adherent PMA-treated HL60 cells (Fig. 4A, Fig. 5B). Bax mRNA also remained elevated in cells induced to differentiate by treatment with either DMSO (Fig. 5D) or with RA (Fig. 5F). These observations show clearly that downregulation of bax protein expression in differentiating HL60 cells (Fig. 3B,D,F) is mediated by a post-transcriptional mechanism.

4. Discussion

The downregulation of the anti-apoptotic bcl-2 protein during myeloid differentiation is well established [5–7]. Here we show for the first time that expression of the pro-apoptotic bax protein also declined when HL60 cells were induced to differentiate following treatment with three different agents. The decrease in bcl-2 and bax expression occurred in the same time-frame as did the generation of DNA strand breaks, an early event in the commitment of cells to apoptosis [12]. Circumstantial evidence suggests that the decrease in bcl-2 expression during HL60 differentiation may play an impor-

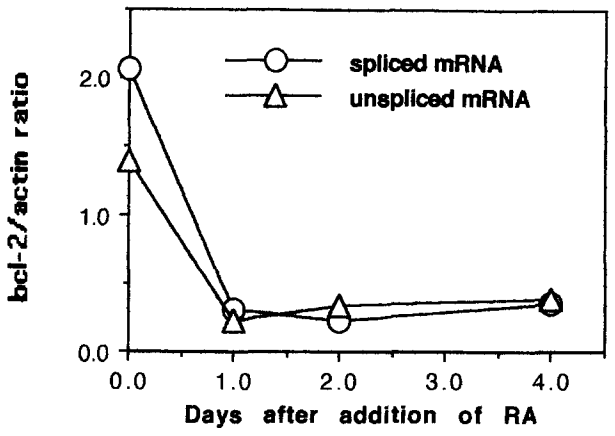

Fig. 6. Quantitation of spliced bcl-2 mRNA and unspliced bcl-2 transcripts in RA-treated HL60 cells. Spliced and unspliced transcripts were quantitated by RT PCR using the primer pairs described (Section 2.5). Densities of Southern-blotted PCR bands were normalised with respect to actin RT PCR bands determined using the same samples.

tant role in triggering the eventual death of differentiating HL60 cells [6,7]. Because the susceptibility of cells to apoptosis is determined in part by the ratio of bcl-2 to bax protein, the data here are compatible with the hypothesis that the downregulation of bax which accompanies the decline in bcl-2 expression may play a role in fine-tuning the induction of cell death during myeloid differentiation.

In PMA-treated HL60 cells, the downregulation of bcl-2 and bax proteins was evident in both the adherent and non-adherent populations. DNA strand breakage, however, was only detected in the non-adherent cells. A possible explanation for this apparent discrepancy is that adherent cells which become committed to cell death detach from the culture flask at an early stage of apoptosis which precedes the appearance of DNA scissions.

Comparison of changes in bcl-2 mRNA and protein levels during HL60 cell differentiation suggest that the fall in bcl-2 protein expression was modulated via a decrease in bcl-2 mRNA. In RA-induced cells, the decrease in bcl-2 mRNA apparently resulted from diminished levels of the primary transcript, consistent with transcriptional downregulation. By striking contrast, bax protein levels in differentiating HL60 cells declined before a substantial decrease in bax mRNA was detectable, strongly suggesting that bax expression decreased via a post-transcriptional control mechanism. Because bcl-2 protein stabilises bax protein [8], our observations are consistent with a model whereby the differentiation-linked decrease in bcl-2 results in a de-stabilisation of bax and a consequent decline in steady-state bax levels.

In summary, the data here are compatible with, but do not prove, the hypothesis that co-ordinate downregulation of bcl-2 and bax proteins play a role in the subtle regulation of the onset of apoptosis during the granulocytic and macrophage-like differentiation of HL60 cells. However, several additional bcl-2-related [2,20,21] and bcl-2-unrelated [22] proteins have recently been implicated in the regulation of apoptosis. Complete characterisation of the control of apoptosis during myeloid cell differentiation will require further studies on the regulation of these novel regulators of cell death.

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