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Letter to the Editor

Identification of two novel mRNA splice variants of *bax*

Dear Editor,

The ability of a cell to avoid apoptosis in response to genotoxic stress is of central importance in its progression to a malignant phenotype, and to the acquisition of drug resistance in cancer.[1] In the course of studies of the relationship between resistance to chemotherapeutic agents and expression of apoptosis-modulating genes, we used RT–PCR to examine *bax* mRNA in the non-small cell lung cancer cell line NCI-H460V. PCR primers based upon the published cDNA sequence for *bax* α[2] were designed to amplify *bax* sequences incorporating exons 1 to 6 (Figure 1A). Predictably a major band of approximately 580 bp consistent with *bax* α was observed, accompanied by a smaller product of approximately 430 bp, corresponding to the recently described *bax* δ.[3] However, we also detected PCR products of approximately 620 bp and 380 bp, which did not correspond to previously identified *bax* splice variants. The sequences of the two unidentified PCR products were consistent with novel splicing arrangements of the *bax* gene. The smaller of the two products appeared to be missing exons 2 and 3 but was otherwise identical to *bax* α. To provide continuity of nomenclature, we have named this variant *bax* ε. The larger ($\sim$620 bp) PCR product contained all six exons of the *bax* gene; however, the cDNA sequence indicated the presence of a 49 bp insert between exons 5 and 6 consistent with a fragment of intron 5 containing an unrecognised sequence. Further detailed PCR analysis suggested that this insert is derived from the 3′ end of intron 5. We named this new variant *bax* ϕ. Kyte-Doolittle analysis confirmed that Bax ϕ protein contains no hydrophobic regions that might correspond to a transmembrane domain.

In vitro transcription and translation was used to determine whether these novel sequences would generate proteins (Figure 1B). Full-length cDNAs corresponding to *bax* α, δ, ε and ϕ were generated by RT–PCR and cloned into the expression vector pCR3.1. The *bax* α construct gave rise to a radiolabelled product with a molecular weight of 21 kDa upon gel electrophoresis as expected, while *bax* ϕ gave a single predicted product of 24 kDa. No protein band was seen with the *bax* ε construct, which was predicted to produce a 1 kDa polypeptide. Western blotting of lysates prepared from the NCI-460V cell line with anti-Bax antibody N-20 (Santa Cruz Biotechnology, CA, USA) revealed protein bands of approximately 21 and 24 kDa (data not shown). The 24 kDa protein may correspond to Bax ϕ, but could also represent Bax β, which has the same predicted mass.[2]

Having demonstrated that *bax* ϕ could potentially give rise to a 24 kDa protein lacking a transmembrane domain, we carried out experiments to determine whether this protein has biological function. For this purpose, Bax ϕ protein was over-expressed in Jurkat cells using the pTRE Tet-off transient expression system (Clontech). Parallel sets of cells were transfected with a *bax* α construct for purposes of comparison. Over-expression of Bax α was confirmed by Western blotting at 4 h, and this was maintained for at least 44 h. The proportion of Jurkat cells undergoing apoptosis following transfection with empty vector (pTRE) or *bax* constructs (pTRE-*bax* α and pTRE-bax ϕ) was assessed by flow cytometry using Annexin V and propidium iodide (Figure 1C).[4] By 8 h post-transfection with *bax* α, approximately 12% of the Jurkat cells were apoptotic, 4% more than seen after transfection with empty vector. The apoptotic population increased to a plateau at about 14% (significantly different from empty vector; $P<0.05$) by 20 h, which was maintained until the last time-point at 44 h. Following transfection with *bax* ϕ, apoptosis was induced to levels comparable with *bax* α by 44 h, although the full effect was not apparent until 28 h, at which time-point the level of apoptosis in *bax* ϕ-transfected cells was not significantly different from *bax* α-transfected cells.

The significance of *bax* mRNA splice variants is unclear. A comprehensive immunohistochemical analysis[5] has suggested that Bax protein is present in a wide range of normal mouse tissues, in particular those characterised by a high apoptotic death rate. Furthermore, immunoblotting of these tissues shows that 21 kDa Bax α is the predominant protein product. Oltvai *et al*[2] also demonstrated a 24 kDa species on immunoblotting consistent with Bax β; however this translated product was not sequenced. As yet no convincing evidence exists for the translation *in vivo* of *bax* δ or γ.

One of the new isoforms we describe here appears to be identical to that described very recently by Zhou *et al*[6] and termed *bax* ω, which contains a 49 base insert from intron 5, between exons 5 and 6. Using ribonuclease protection assays, Zhou *et al*[6] demonstrated that *bax* ω is widely distributed in human heart, brain and liver. By RT–PCR, we have detected mRNAs for *bax* ϕ, as well as for ε and δ, in a wide range of cultured cells, both primary and established lines.

Our data suggest that transient *bax* ϕ over-expression induces apoptosis in Jurkat cells at levels comparable to *bax* α, although the effect appears to be slightly delayed. Zhou *et al*[6] using a different system to functionally characterise *bax* ω, obtained broadly similar results. Interestingly their data for *bax* ω suggest that over-expression protects stably-transfected L929 cells from TNF-induced apoptosis; however transient over-expression of *bax* ω increases basal levels of cell death in monkey E5 cells to levels greater than those seen with *bax* α.[6] There may be problems inherent in the use of stable transfectants that could explain what appears to be an anomalous result. Stable transfectants of Jurkat cells over-expressing Bcl-2 contain markedly higher steady-state levels of endogenous Bax.[7] The inference of these data is that the clones that are

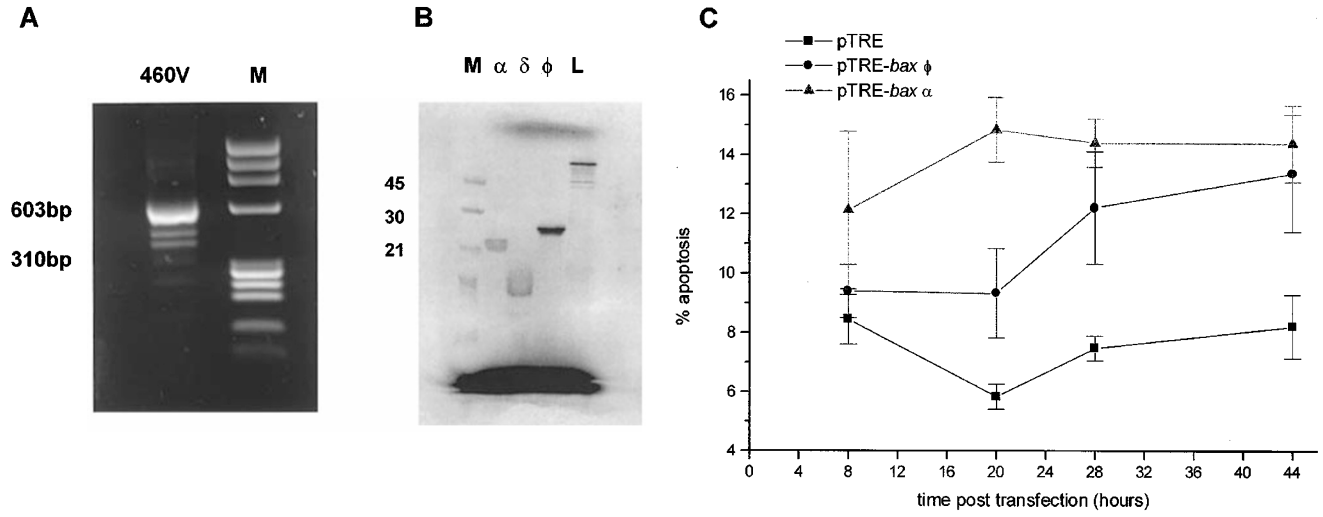

Figure 1 Characterisation of novel *bax* isoforms. (**A**) RT–PCR of cDNA from the NCI-H460V cell line, using primers for full-length *bax* α.[2] Total RNA was isolated from 10^6 cells using the PUREscript RNA isolation kit (Gentra Systems Inc, MN, USA). Separation of the products by electrophoresis reveals the presence of four major bands corresponding to the previously identified *bax* α (580 bp) and *bax* δ (430 bp), as well as two novel products of 620 bp and 380 bp. M, ϕX174/Hae III markers. (**B**) *In vitro* transcription-translation of pCR3.1 constructs containing full-length cDNAs for *bax* α, δ and ϕ and luciferase (control). ^{35}S-methionine-labelled translation products were generated using the TNT coupled reticulocyte lysate system (Promega, WI, USA). *Bax* α produced the expected 21 kDa product, while for *bax* ϕ the predicted product of 24 kDa is seen. The product for *bax* δ is smaller than expected at 12 kDa. L; luciferase control. M; ^{14}C-labelled protein markers. (**C**) Percentage apoptosis at various times following over-expression of *bax* α or *bax* ϕ in Jurkat cells. *Bax* α and ϕ constructs were sub-cloned from the expression plasmid pCR3.1 into the Tetracycline-off response plasmid pTRE (Clontech, Basingstoke, UK). 2×10^6 Jurkat Tet-off cells (Clontech) were transfected with *bax* cDNAs using DMRIE-C reagent (Gibco, Life Technologies, Paisley, UK). Percentage of apoptotic cells following induction of *bax* expression was assessed by FACScan (Becton Dickinson, San Jose, CA) analysis after labelling with Annexin V and propidium iodide[4] using the Annexin V Kit (Bender Medsystems, Vienna, Austria). The data demonstrate that comparable levels of apoptosis are induced with the two isoforms of *bax*. Numbers represent mean ($\pm$S.E.M.) of three individual experiments performed on different days

selected may contain other proteins that affect their response to apoptosis, and inadvertently a non-representative clone has been selected for.

In summary, we have demonstrated the occurrence of two novel splice variants of the *bax* gene, one of which lacks a transmembrane domain but retains pro-apoptotic function. While the physiological significance of this finding is unclear, a parallel situation is to be found in the existence of splice variants of the *Bcl-2* gene. One isoform of *Bcl-2* encodes a protein that lacks a transmembrane domain.[8] In two studies truncated forms of *Bcl-2* retain anti-apoptotic function,[2,9] while in another it is lost.[10]

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