BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 258–264 (1996)
ARTICLE NO. 1017

Constitutively Enhanced *nbl* Expression Is Associated with the Induction of Internucleosomal DNA Cleavage by Actinomycin D

Honami Naora,*[,1] Tsutomu Nishida,† Yutaka Shindo,†
Masakazu Adachi,† and Hiroto Naora*

**Research School of Biological Sciences, The Australian National University, G.P.O. Box 475, Canberra, ACT 2601, Australia; and †Cellular Technology Institute, Otsuka Pharmaceutical Co. Ltd, Tokushima, Japan*

Received May 21, 1996

Previous studies have found *nbl* expression to transiently rise and fall during glucocorticoid-induced thymic apoptosis. This induction of apoptosis is blocked by the transcriptional inhibitor actinomycin D. However, actinomycin D can trigger apoptosis in other cell types, e.g., HL-60 cells. This study found that internucleosomal DNA cleavage typical of apoptosis is induced by actinomycin D in cell lines such as HL-60 which constitutively express high levels of *nbl* above a certain ''threshold.'' In contrast, ''DNA ladder'' formation was not induced by actinomycin D in cell lines with low constitutive *nbl* expression. Enhanced *nbl* expression therefore appears to be associated with apoptosis which is either blocked or induced by actinomycin D.

Cell death via apoptosis appears to involve different pathways depending on the nature of the inducing stimulus and cell type. Transcriptional and translational inhibitors can block glucocorticoid-induced thymic apoptosis (1,2). However, death of target cells by cytotoxic T lymphocytes is unaffected by macromolecular synthesis inhibitors (3), while such inhibitors can themselves trigger apoptosis in several cell types such as the human promyelocytic leukemia cell line HL-60 (4,5). Key questions are whether the apparently different pathways involve independent sets of genes, and whether certain genes could be involved in more than one pathway.

The *nbl* gene was originally isolated by virtue of its abundance in a Namalwa Burkitt Lymphoma cDNA library (2). Our previous studies in mouse thymus *in vivo* found active gene expression to be a strictly controlled temporal requirement for glucocorticoid-induced apoptosis, involving a transient enhancement and subsequent halt in *nbl* expression prior to maximal formation of internucleosomal DNA cleavage (2). Given the association of *nbl* expression with an apoptotic process which is blocked by transcriptional inhibitors such as Act D, we wished to investigate *nbl* expression in apoptotic processes inducible by Act D. In this study, the susceptibility of cells to induction by Act D of internucleosomal DNA cleavage, a hallmark of apoptosis, was found to be associated with constitutively enhanced levels of *nbl* expression.

MATERIALS AND METHODS

Cell culture. The following human cell lines were used: Culture of HL-60 cells, the histiocytic lymphoma U937 and WISH amnion cells is described elsewhere (5). The T cell leukemia Jurkat was cultured as described for HL-60 cells with the inclusion of 0.05 mM β-mercaptoethanol. Chang liver cells were cultured as described for WISH cells. The bladder carcinoma 5637 and hepatoma HepG2 were cultured in MEM (GIBCO BRL) supplemented with 20% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, plus modified animo acids, non-essential

[1] Corresponding author. Fax: 61 62 473643.
Abbreviation: Act D, actinomycin D.

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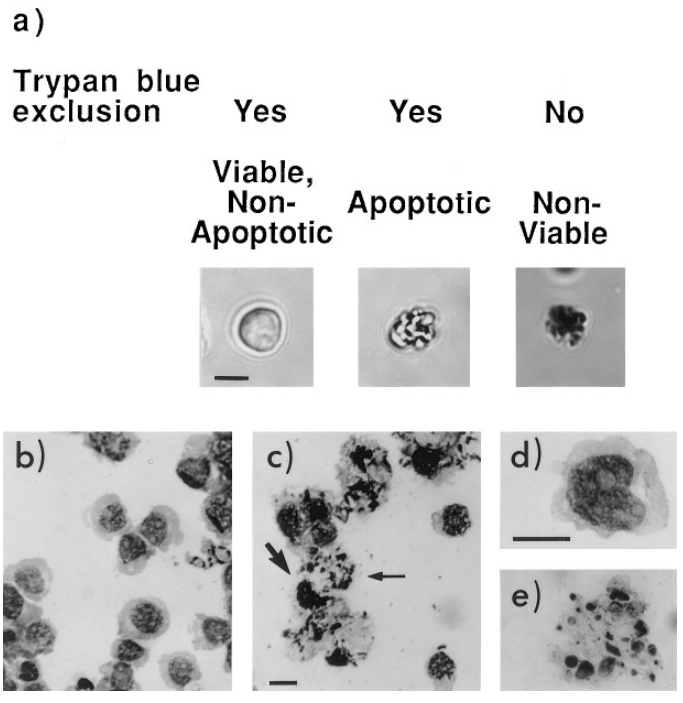

FIG. 1. Apoptotic morphology of HL-60 cells. **(a)** Trypan blue-stained cultures. Approximately 95% of untreated cells were viable and non-apoptotic. Apoptotic cells, seen in Act D-treated cultures, were shrivelled and refractile, with protuberances on their surface. Apoptotic cells excluded trypan blue dye, in contrast to non-viable cells. Bar, 10 μm. (**b–e**) Giemsa-stained cell preparations. Chromatin condensation to the periphery of the nuclear membrane (thick arrow) and nuclear fragmentation (thin arrow) were seen in many cells incubated with Act D (1 μg/ml) for 3 h (c), but only in very few untreated cells (b). Bar, 20 μm (shown in (c), also applies to (b)). Typical examples of an untreated cell (d) and nuclear fragmentation induced by 6 h Act D treatment (e) are shown at higher magnification. Bar, 20 μm (shown in (d), also applies to (e)).

amino acids and modified vitamins (ICN). Culture of the mouse T lymphoma cell line EL4.23 is described elsewhere (6). Prior to experimental use, suspension cells were resuspended in fresh medium at 5×10^5 cells/ml, while fresh medium was added to adherent cells at a confluency of 70-80%. After incubation (with or without the addition of Act D (Sigma)), suspension cells were collected by centrifugation. Adherent cells were pooled from the trypsinized monolayer and the culture medium. Cell viability and morphology was assessed as described previously (5).

cDNA probes, Isolation and analysis of RNA and DNA. RNA isolation and Northern blot analysis using the *nbl* cDNA probe, and the quantitation of hybridization signals by PhosphorImager analysis has been described elsewhere (2). The *myc* probe was a 1.3 kb *Cla* I/*Eco* RI fragment derived from the 9 kb human *myc* genomic clone (Oncor Inc.). The 18S ribosomal DNA probe was a 0.97 kb *Pst* I fragment derived from plasmid pX1r14F (7). The ubiquitin probe was a 1.04 kb *Dra* I/*Bam* HI fragment of the human Ub B gene (8). Isolation and electrophoresis of genomic DNA and the quantitation of DNA fragmentation was carried out as previously described (2).

RESULTS

Optimal conditions for apoptotic induction by Act D were initially investigated by microscopic examination of trypan blue-stained HL-60 cultures. Nearly half the cell population was shrivelled and refractile in appearance at 6 h after addition of Act D (1 μg/ml) (Figs. 1a,2). Such cells excluded trypan blue dye (Fig. 1a), in agreement with the known ability of apoptotic cells to maintain membrane integrity until relatively late in the apoptotic process (4). Protuberances on the cell surface, which appeared to be typical apoptotic bodies, and chromatin condensation in Giemsa-stained cell preparations were seen (Figs. 1a,c,e), as observed in other studies (9,10). The kinetics of induction of apoptotic morphology also correlated with those

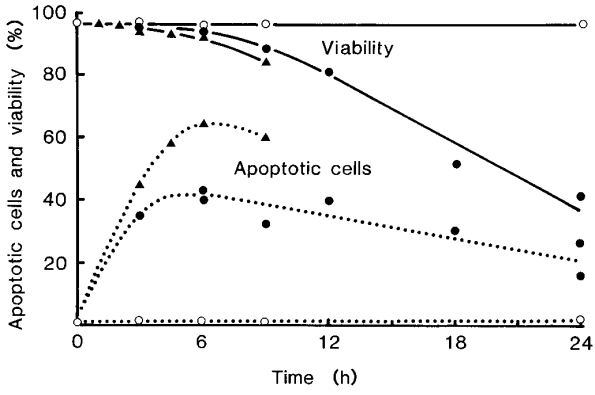

FIG. 2. HL-60 cells were left untreated (○), or incubated with Act D at 1 μg/ml (●) and 5 μg/ml (▲) for the indicated periods. Cell viability (unbroken lines) was assessed by the ability to exclude trypan blue dye. The proportion of apoptotic cells (dotted lines) was assessed by features described in Fig. 1a.

of internucleosomal DNA cleavage. Internucleosomal DNA cleavage was maximally induced at 6 h after Act D addition (Fig. 3). Extension of treatment time beyond 6 h led to decreases in integrity of the ''DNA ladder'' pattern and in cell viability (Figs. 2,3). Increasing the Act D concentration to 5 μg/ml modestly increased the degree of ''DNA ladder'' formation (data not shown) and the proportion of cells with apoptotic morphology, without significantly altering the kinetics of their induction (Fig. 2). Treatment for 6 h with Act D at 1 μg/ml was therefore used as standard assay conditions in subsequent experiments.

Treatment of Jurkat and U937 cells with Act D for 6 h induced substantial internucleosomal DNA cleavage, though to a lesser extent than in HL-60 cells, without significantly affecting cell viability (Figs. 4,5a). These cells exhibited morphology similar to that of Act D-treated HL-60 cells. 5637 and Chang cells also contained ''DNA ladders'' at 6 h after

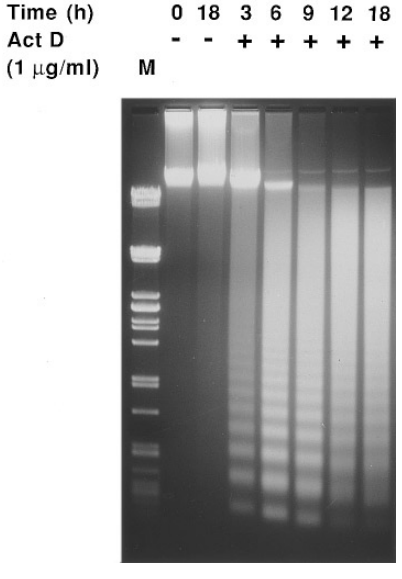

FIG. 3. DNA was isolated from HL-60 cells incubated with Act D (1 μg/ml) for the indicated periods and electrophoresed on 1.0% agarose gels with *Pst* I-digested λ DNA as size markers (M).

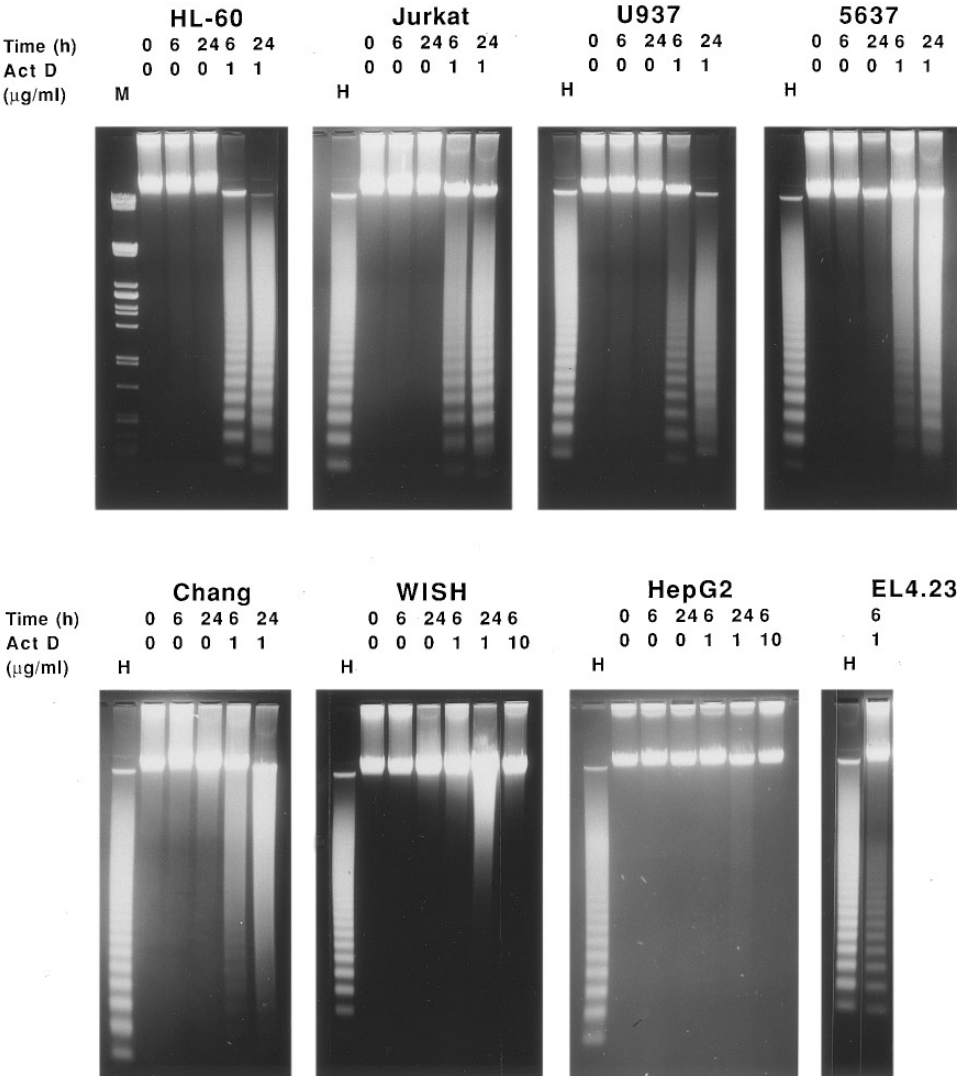

FIG. 4. Analysis of genomic DNA isolated from various cell lines incubated for 0, 6 and 24 h in the absence (0) and presence of Act D at 1 μg/ml or 10 μg/ml as indicated. *Pst* I-digested λ DNA was included as size markers (M) in the electrophoresis of HL-60 DNA. DNA, isolated from HL-60 cells incubated for 6 h with Act D (1 μg/ml), was included as a control (H) in electrophoresis of DNA of other cell lines. HL-60, U937 and WISH DNA gel figures are reproduced with modifications from Naora & Naora (5) with permission of Academic Press.

Act D addition (Fig. 4), and exhibited ''rounded'' morphology and increased detachment, as seen in other adherent cell types undergoing apoptosis (11). In contrast, no ''DNA ladders'' were observed in WISH and HepG2 cells at 6 h after Act D addition even at a ten-fold higher concentration (Fig. 4). These cells exhibited little change in morphology from their untreated controls. At 24 h after Act D addition, ''smeared'' DNA patterns, but no ''DNA ladders'', were observed in WISH and HepG2 cells (Fig. 4). The different responses to Act D do not appear to be due to possible variations in the ability of Act D to effectively penetrate different cell types, since levels of the primary ubiquitin transcript declined at similar rates in Act D-sensitive and -insensitive cell lines, as did *myc* mRNA levels (data

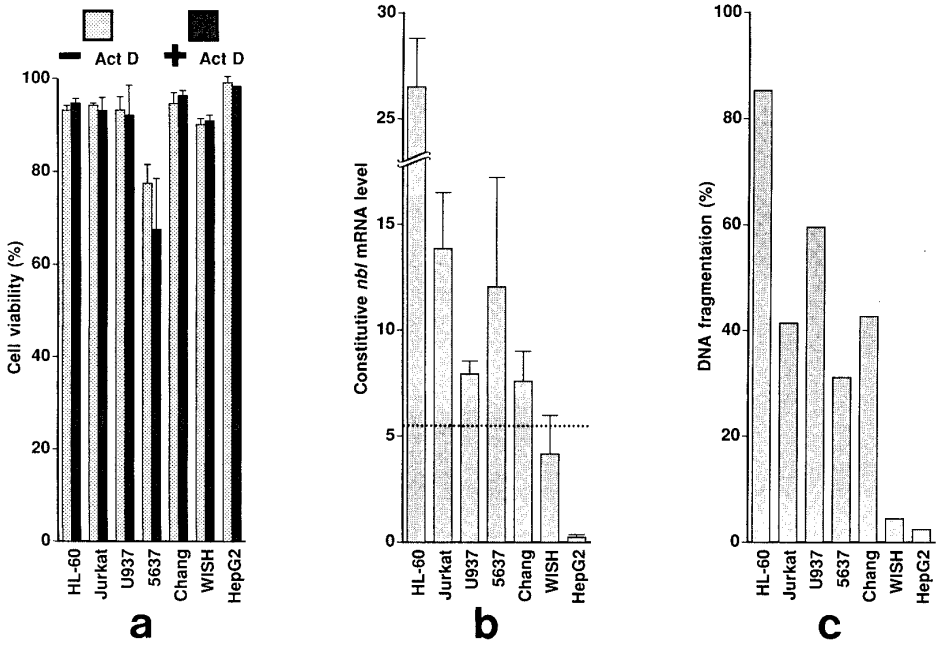

FIG. 5. (a) Viability of cultures incubated for 6 h in the absence (−) and presence (+) of Act D (1 μg/ml). Results are representative of two independent experiments. **(b)** The constitutive *nbl* mRNA level in each human cell line was quantified by Phosphor-Imager analysis and expressed relative to the *nbl* mRNA level in human placenta. This value was corrected for RNA content using 18S ribosomal RNA hybridization signals and is expressed as the mean±SD of results obtained from 3 or 4 independent experiments. **(c)** The extent of DNA fragmentation, induced at 6 h after addition of Act D (1 μg/ml), was quantified as previously described (2) and is expressed as an increment above the low basal level of DNA fragmentation detected in untreated cells. Internucleosomal DNA cleavage appears to be induced by Act D when *nbl* is constitutively expressed above the ''threshold'' level indicated by the dotted line shown in (b).

not shown). Susceptibility to Act D was also not restricted to cells of haemopoietic origin nor to suspension cell cultures.

Normal murine tissues possess low levels of *nbl* mRNA (2). In this study, the *nbl* mRNA level in normal human placenta was used as a reference to compare constitutive levels of *nbl* mRNA in the various cell lines. In general, cells in which internucleosomal DNA cleavage was induced by Act D were found to possess relatively high constitutive levels of *nbl* mRNA (Figs. 5b,c). In contrast, cells with relatively low constitutive levels of *nbl* mRNA, such as WISH and HepG2, exhibited no detectable ''DNA ladders'' (Figs. 4,5b). Although the correlation between constitutive *nbl* mRNA levels and the degree of ''DNA ladder'' formation is not strictly proportional, it suggests that the constitutive expression of *nbl* above a certain ''threshold'' may be associated with the susceptibility of a cell to undergo apoptosis involving internucleosomal DNA cleavage in response to Act D. Quantitation of *nbl* mRNA levels in the various cell lines indicates that this ''threshold'' is approximately 5- to 6-fold the *nbl* mRNA level in normal human placenta (Fig. 5b). ''DNA ladder'' formation was also induced by Act D in EL4.23 cells (Fig. 4), and the constitutive *nbl* mRNA level in this mouse cell line was found to be approximately 12-fold the level in normal mouse liver.

DISCUSSION

In this study, apoptosis involving internucleosomal DNA cleavage was induced by Act D in various cell lines which constitutively expressed *nbl* at high levels above a ''threshold''.

In contrast, internucleosomal DNA cleavage was not induced by Act D in cell lines which constitutively expressed *nbl* at levels below the ''threshold''. This ''threshold'' was approximately 5- to 6-fold the *nbl* mRNA level present in normal human placenta. *nbl* mRNA levels in apoptotic mouse thymus are transiently elevated by a similar order of magnitude, relative to levels in other normal murine tissues (2). Enhancement of *nbl* expression to levels above the ''threshold'' may therefore be associated with apoptotic pathways which involve ''DNA ladder'' formation and which can be blocked or induced by Act D.

Enhanced *nbl* expression could be regarded as ''priming'' a cell for apoptosis. Thymocytes require an induction of *nbl* enhancement. However, cells such as HL-60, Jurkat, U937, 5637 and Chang could be thought of as having already undergone early ''priming'' steps involving at least *nbl* enhancement, and have maintained such enhanced levels constitutively. Martin (1993) has proposed a common pathway in which apoptosis can be triggered at different stages depending on the stimulus (12). Under this model, early signaling events are gene expression-dependent, whereas later events, such as internucleosomal DNA cleavage, are gene expression-independent. It should be noted that transiently enhanced *nbl* levels abruptly fall prior to maximal ''DNA ladder'' formation in apoptotic mouse thymus (2). The high constitutive *nbl* levels in cells such as HL-60 also fall following Act D addition, and treatment of HL-60 cells with *nbl* antisense sequences appears to induce typical apoptotic morphology and DNA fragmentation (Naora *et al.*, manuscript in preparation). This suggests that suppression of enhanced *nbl* expression may be involved in the apoptotic process. WISH and HepG2 cells may need to undergo the early ''priming'' step involving *nbl* enhancement, as appears to be the case in untreated thymocytes. The effect of enhancing and subsequently suppressing *nbl* expression in such cells is currently being investigated. In view of evidence that apoptosis in certain systems can occur in the absence of internucleosomal DNA cleavage (13), the ''smeared'' DNA patterns seen in WISH and HepG2 cells after prolonged Act D treatment are being investigated, for example, by detecting high molecular weight DNA fragments and single-stranded DNA breaks.

The precise biological action of the *nbl* gene product is as yet unclear. The predicted amino acid sequence of the original *nbl* cDNA clone appears identical, except for one residue, to the human *fte-1* gene product, whose rat homologue has been implicated as an effector of v-*fos* transformation (14). The human *fte-1* gene product is apparently identical to the human ribosomal protein S3a (15). *fte-1* expression is enhanced in v-*fos* transformed fibroblasts, and expression of many ribosomal proteins is enhanced in various tumours (14,16). In this and other work (2), *nbl* expression has been found to be generally higher in tumour tissues and tumour cell lines than in normal tissues. The *nbl* gene product may have multi-functional roles in apoptosis, transformation and normal cell growth depending on the nature of the signal which the cell receives. Many proteins, including several ribosomal proteins, appear to have multi-functional activities. Of particular interest is ribosomal protein S3, which seems structurally and functionally associated with S3a, and which possesses endonuclease activity (17,18).

ACKNOWLEDGMENTS

The authors wish to thank the following researchers at the John Curtin School of Medical Research, Canberra, Australia: Dr. A. J. Hapel for providing U937, 5637 and WISH cells; Dr. T. Suzuki for Chang and HepG2 cells; Prof. I. G. Young for EL4.23 cells; Dr. R. Baker for the ubiquitin probe. Authors also wish to thank Dr. N. J. Deacon (Macfarlane Burnet Centre for Medical Research, Melbourne, Australia) for providing Jurkat cells and Prof. B. Maden (University of Liverpool, Liverpool, U.K.) for plasmid pX1r14F. Human placenta tissue was provided by Woden Valley Hospital (Canberra). This work was supported by a grant from Otsuka Pharmaceutical Co. Ltd. (Japan).

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