Biochimie (1997) 79, 717–723
© Société française de biochimie et biologie moléculaire / Elsevier, Paris

Decreased expression of the high-mobility group protein T160 by antisense RNA impairs the growth of mouse fibroblasts

L Hertel[a], P Foresta[a], G Barbiero[b], GG Ying[a], G Bonelli[b], FM Baccino[b, d], S Landolfo[c, d], M Gariglio[a]*

[a]*Department of Medical Sciences, Medical School of Novara, Via Solaroli, 17, 28100, Novara;*
[b]*Department of Experimental Medicine and Oncology;*
[c]*Department of Public Health and Microbiology, Medical School of Torino;*
[d]*Center of Immunogenetics and Experimental Oncology, CNR, 10126 Torino, Italy*

(Received 9 October 1997; accepted 27 November 1997)

Summary — The T160 protein belongs to the HMG-1 box protein family and preferentially binds to non-B-DNA conformations with no sequence specificity. Its exact role has yet to be defined, though it seems to participate in processes involving DNA, such as replication, transcription and recombination. We have used an antisense RNA strategy to investigate its role in cell growth and proliferation. T160 expression is strongly suppressed by stable introduction of an antisense construct into NIH3T3 cells, and this decrease is accompanied by substantial changes in the growth properties of the stable transfectants. Impaired growth of $T160^-$ cells was mainly related to two mechanisms: i) decreased rates of cell proliferation at normal serum concentration; and ii) occurrence of cell death by apoptosis at low serum concentration, as demonstrated by both flow cytometry and microscopy. The finding that decreased T160 availability affects cell proliferation, provides further evidence of its involvement in a basic cell function, such as DNA replication.

T160 / HMG protein / antisense RNA / apoptosis

Introduction

The high mobility group protein HMG1/2 family has been divided into two subfamilies according to the number of HMG domains, their specificity of sequence recognition and their evolutionary relatedness [1, 2]. The first subfamily comprises the HMG1/2 proteins and is present in all cell tissues. It contains many HMG domains and preferentially binds to non-B-DNA conformations, such as B-Z junctions, stem-loops, cruciforms, four-way junctions, and cisplatin-modified DNA with no sequence specificity [3, 4]. The second subfamily includes the lymphoid enhancer-binding factors LEF-1 and TCF-1, has a single HMG-box domain, interacts with specific nucleotide sequences (A/TA/TCAAAG) and has a restricted cell-type distribution [5, 6].

It is already clear that the HMG-box sequence is an important, evolutionary conserved and versatile DNA-binding motif required for transcriptional regulation of key cellular pathways, such as the processes of DNA replication, nucleosome positioning and chromatin organization, though the mechanisms involved are uncertain [7, 8].

We have previously described the nuclear factors binding to the interferon-stimulated response element (ISRE) of the Ifi 200 genes [9, 10]. Besides the ISGF3 complex that appears to be primarily responsible for transcription activation by IFN, another protein belonging to the HMG-1/2 family seems capable of binding the ISRE [11]. Nucleotide sequencing has revealed that it is identical to the mouse HMG1-related DNA-binding protein T160 isolated with the V-(D)-J recombination signal probes [12]. This protein was specific for 12-bp spacer signals and failed to bind to a sequence with a base change in the third position of the heptamer. It was therefore inferred that the T160 protein could be specifically involved in the V-(D)-J recombination process that leads to immunoglobulin production by B lymphocytes and development of the antigen receptor by T lymphocytes. However, DNA binding by the T160 protein could only be detected with Southwestern blot analysis; the *in vitro* translated material could not be demonstrated to bind DNA according to electrophoretic mobility shift and DNA footprinting analysis; lastly, the T160 mRNA was expressed not only in lymphoid cells, but also in fibroblasts, which are very unlikely to undergo V-(D)-J recombination. The human homologue was isolated by screening a mature B cell cDNA library with a platinated (non-joining-signal) DNA probe and found to be ubiquitously expressed at the mRNA level [13]. The T160 yeast homologue, designated Pob3 on account of its capability to interact with the DNA polymerase 1, has been knocked out and the phenotype was

*Correspondence and reprints

lethal [14]. Although these studies shed some light on the binding characteristics of the T160 protein, they have not shown whether it is concerned in any cellular function, such as proliferation, differentiation, involvement in the IFN response or in virus replication.

To address this question we decided to suppress T160 expression by using antisense RNA technology, which has greatly facilitated the understanding of protein function in the context of its normal counterpart. In this study, we show that sustained down-regulation of endogenous T160 expression in NIH3T3 cells significantly alters their growth.

Materials and methods

Cells

The parental NIH3T3 cell line and its transfected clones were cultured in Dulbecco minimal essential medium (DMEM) supplemented with 10% donor calf serum (DS) (Gibco/BRL). G418-resistant cells were selected and maintained in the same medium containing G418 at 0.8 mg/mL (Gibco/BRL).

Construction of the antisense vector

The antisense expression vector pcDNA1asT160 was constructed by inserting the T160 cDNA into pcDNA1 expression vector (Invitrogen, San Diego, CA) in the opposite orientation to the cytomegalovirus (CMV) promoter, thereby allowing constitutive expression of antisense T160 mRNA. The T160 cDNA fragment, *Avr*II-BsU36I, 2250 bp in length, was ligated into the *Eco*RV site of pcDNA1 after Klenow filling.

Transfections and DNA amplification (PCR)

DNA transfections in NIH3T3 cells were performed by the calcium phosphate precipitation technique modified according to Jordan *et al* [15]. To obtain stable transformants, 5 × 10⁵ NIH3T3 cells were cotransfected with pcDNA1asT160 (15 μg) and the plasmid pSTneoB (0.3 μg), containing the neomycin resistance gene under the control of the simian virus 40 early gene promoter [16]. Stable transfectants were selected by the addition of G418 (0.8 mg/mL). Resistant clones were isolated from the parental line by limiting dilution. Amplification of fragments from the T160 cDNA was performed with the Hybaid thermal cycler using cloned Taq polymerase (Dynazyme). The protocol was composed of 35 cycles at 92°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension of 10 min at 72°C. Oligonucleotides were selected to discriminate amplified products of the transfected cDNA from the endogenous T160 gene. The coding primer was positioned in the T7 promoter of pcDNA1 and the non-coding primer in the SP6 promoter. Thus, the predicted size of the amplified fragment from the transfected cDNA was 2300 bp.

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and disrupted in cold lysis buffer (125 mM Tris HCl, pH 6.8, 3% SDS, 10 mM DTT, 10% glycerol and protease inhibitors) and briefly sonicated. Insoluble material was removed by centrifugation. Proteins were electrophoresed through 8.5% SDS-PAGE, and electrotransferred to PVDF membrane (Amersham). Immunoblots were blocked with 5% non-fat milk for 2 h at room temperature and then incubated with affinity purified rabbit polyclonal antibody (diluted 1:3000) recognizing the N-terminus moiety. Goat anti-rabbit IgG-horseradish-peroxidase conjugate was used as second antibody at a dilution of 1:3000 and detected by ECL (Amersham). Monoclonal anti-actin antibodies (Boehringer) were used as internal control.

Cell growth

Cells were seeded at 10^4/cm^2 into a 24-well plate in DMEM containing 10% DS, and shifted after 24 h to DMEM-0.5% DS for 48 h. The medium was then renewed (time 0, t0), half of the cultures being placed in 10% DS and the other half in 0.5% DS. At the indicated times, cells from duplicate wells were trypsinized, combined with any floating cells, pelleted and counted. The trypan blue exclusion test was routinely performed. More than 300 cells for each time point and experimental condition were scored to evaluate the proportion of cells with a leaky plasma membrane. These were subtracted from total counts.

DNA distribution and physical parameters by flow cytometry

DNA staining was performed as previously described [17, 18]. Cells were harvested by trypsinization, washed with ice-cold PBS, and fixed in 70% ice-cold ethanol for at least 30 min. After centrifugation, cells were incubated at room temperature in the presence of DNase-free RNase (type 1-A) and propidium iodide (PI) at final concentrations of 0.4 and 0.18 mg/mL respectively. Fluorescence was measured with a FACScan flow cytometer (Becton & Dickinson, Mountain View, CA) equipped with a 488-nm argon laser. Two filters were used to collect the red fluorescence due to PI staining of the DNA, one transmitting at 585 nm with a bandwidth of 42 nm (FL2), the other transmitting above 620 nm (FL3). FL2 and FL3 were registered on a linear and a log scale respectively. Forward (FSC) and side (SSC) light scatter were measured as well. Approximately 10^4 cells were analyzed for each sample at a flow rate of about 200 cells/s. Debris was excluded from the analysis by raising the FSC and FL3 thresholds to experimentally selected values [19]. The percentages of cells in G_1, S, and G_2/M were determined by analysis with the CellFit computer program provided by Becton & Dickinson.

Cytochemical identification of apoptotic cells by in situ nick end-labeling

DNA breaks were detected as described by Fehsel *et al* [20] with a few modifications. NIH3T3 cells grown on cover glass were washed with PBS, fixed for 20 min at room temperature in 3.7% formaldehyde, washed in Tris-EDTA buffer, pH 8 (10 mM Tris-HCl, pH 8, 1 mM EDTA) then equilibrated with nick translation buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 7.5 mM DTT, pH 7.5). Nick end-labeling was performed by incubating samples for 1 h at room temperature with digoxigenin-DNA labeling mixture (Boehringer) and *E coli* DNA polymerase I (0.5 U/sample) (Biolabs) in the same buffer. The reaction was stopped in enriched Tris-EDTA buffer pH 8 (10 mM Tris-HCl, pH 8, 5 mM EDTA). After permeabilization with Triton X-100, digoxigenin-labeled DNA was revealed by an enzyme-linked immunoassay. Coverslips were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody, followed by an enzyme-catalyzed color reaction

employing 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate, Sigma) and nitroblue tetrazolium salt (NTB, Sigma), which produces insoluble blue precipitates. To exclude non-specific staining, negative controls were performed in which either DNA polymerase or anti-digoxigenin antibody were omitted. Coverslips mounted with Mowiol (Calbiochem) were observed with a Dialux 20 microscope (Leitz).

Results

Stable expression of T160 antisense RNA in NIH 3T3 cells decreases HMG T160 expression

Since transient expression of antisense RNA did not reduce the steady-state level of T160 (data not shown), probably because of its long half-life, we decided to generate stable transfectants in NIH 3T3 cells by using the pcDNA1 eukaryotic expression vector carrying the antisense T160 cDNA. The resulting construct pcDNA1asT160, which expresses T160 antisense RNA, was cotransfected with pSTneoB into NIH 3T3 cells. Empty pcDNA1 together with pSTneoB was transfected into NIH 3T3 cells as control. Geneticin resistant clones were isolated by the limiting dilution assay, and the presence of the antisense HMG T160 construct was monitored by PCR amplification with genomic DNA. The choice of the two primers allowed us to discriminate between amplification of the transgene and that of the endogenous T160 gene (see *Materials and methods*). To determine if NIH 3T3 cells transfected with antisense T160 construct had an altered level of T160 protein, Western blot analysis was performed on total cell extracts from 10 independent Neo^R cell clones transfected with pcDNA1asT160. Although total suppression of T160 was never achieved, we selected three clones (T160$^-$.1, T160$^-$.2, T160$^-$.3) which had a substantially lower level of T160 protein compared to the Neo^R clones Neo$^+$1 and Neo$^+$2 (fig 1, upper panel). Densitometric analysis of the labeled bands indicated that the magnitude of the reduction was about 60%. Actin immunodetection, used as internal control, showed that the steady-state level of this protein was not affected (fig 1, lower panel).

Expression of T160 antisense RNA increases serum dependency

Preliminary evidence suggested that T160 could be involved in DNA replication and hence in cell proliferation [21]. To investigate whether antisense T160 RNA modulates cell growth, the T160$^-$ clones and the matching Neo$^+$ clones were seeded at equal densities and grown for 24 h in DMEM containing 10% DS, then shifted to medium containing 0.5% DS for 48 h to synchronize cells in G0/G1. The medium was then renewed (t0) and a set of cultures was maintained for 5 days in DMEM supplemented with 0.5% DS, while another set was shifted back to 10% DS (fig 2).

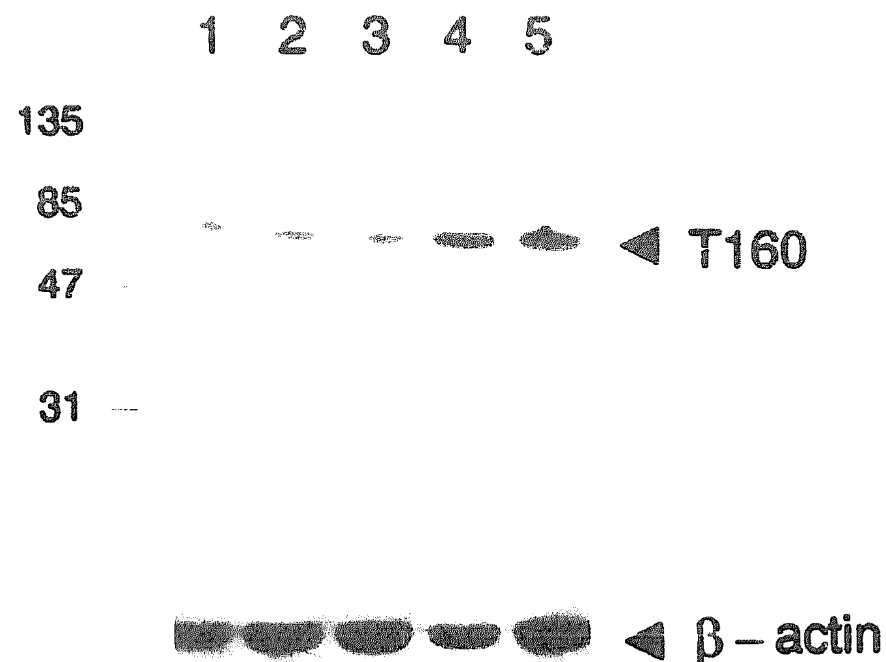

Fig 1. Western blot analysis of cell extracts from both Neo$^+$ and T160$^-$ cells. Western blot analysis of cell extracts from T160$^-$ or Neo$^+$ clones. Fifty μg of total cell protein extracts were separated by gel electrophoresis and blotted onto nitrocellulose. The presence of T160 was detected with a rabbit polyclonal antibody recognizing its N-terminus. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody and visualized with an ECL kit (Amersham). Immunodetection of actin using a monoclonal antibody (Boehringer) was performed as internal control. Lanes 1–3: T160$^-$.1, T160$^-$.2 and T160$^-$.3; lanes 4, 5: Neo$^+$.1 and Neo$^+$.2, respectively. Numbers on the left are sizes of molecular mass markers (kDa).

Since the density of the T160$^-$ cultures at t0 was significantly lower (about half) than that of the Neo$^+$ clones (see section below), cell densities were normalized at t0 and data expressed as relative cell number for an easier comparison. The rate of growth of the Neo$^+$ clones in 10% DS (fig 2A) did not significantly differ from that for non-transfected control NIH 3T3 cultures (data not shown) and a plateau state was soon achieved. In comparison, growth was somewhat stunted for T160$^-$ clones (fig 2B), which increased in number at definitely reduced rates (in spite of the lower initial density), and by day 5 reached lower densities (less than half), and showed no plateau phase. An even sharper difference between Neo+ and T160$^-$ clones was observed in 0.5% DS, while the former showed some growth during day 1, then maintained the same density up to day 5, the latter steadily decreased in number throughout the observation period. This finding clearly indicated that, under conditions of serum deprivation, cell death was occurring in the T160$^-$ clones, and prompted a more detailed analysis of this phenomenon.

Serum deprivation affects survival of T160$^-$ cells

Neo$^+$ and T160$^-$ clones seeded at equal densities were grown for 48 h in DMEM-10% DS, then shifted to 0.5% DS

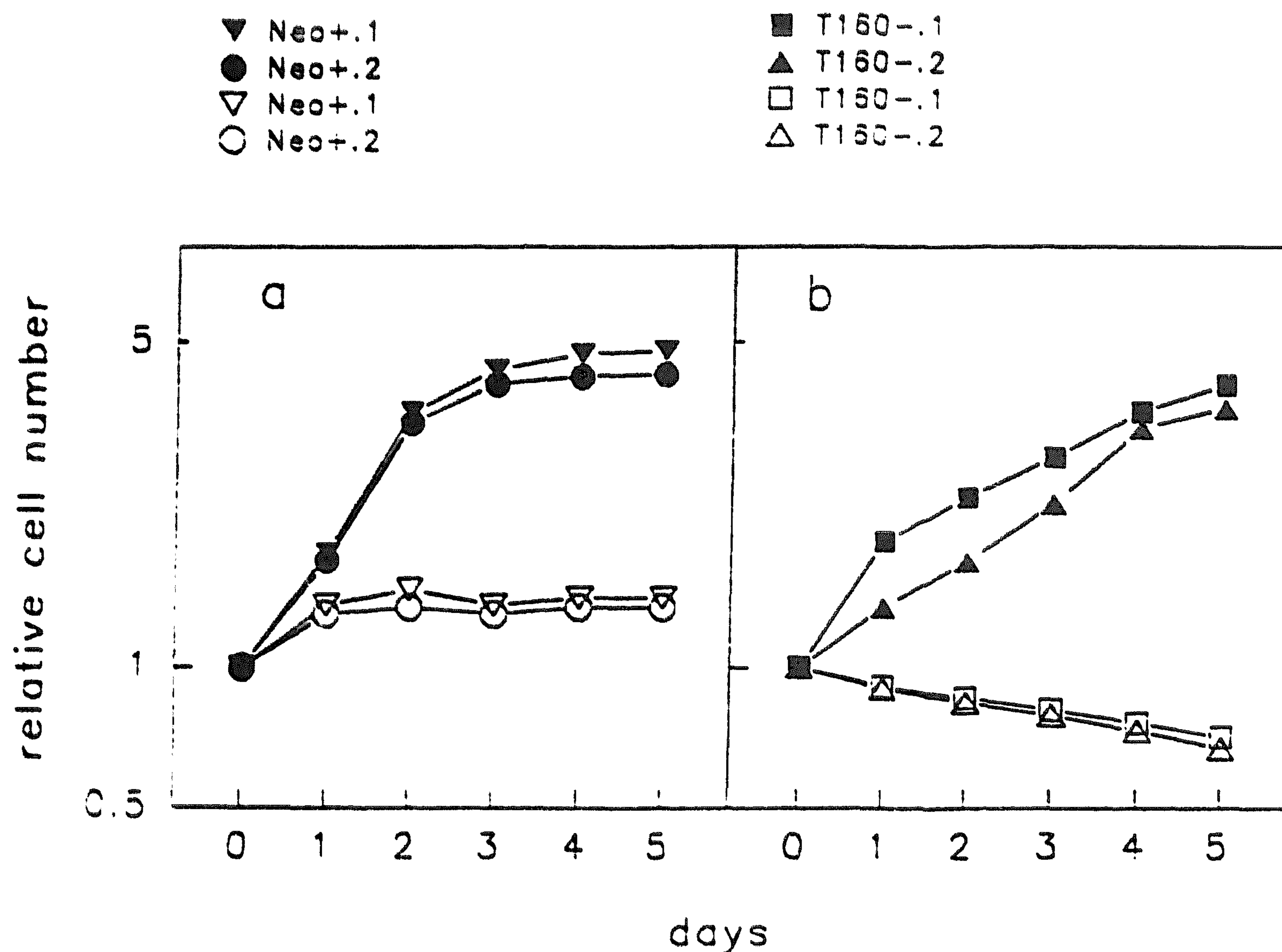

Fig 2. Growth of Neo$^+$ and T160$^-$ cells. Neo$^+$.1, Neo$^+$.2 (**A**) and T160$^-$.1, T160$^-$.2 (**B**) clone growth curves. Neo$^+$ and T160$^-$ cells, seeded at equal densities, were maintained in DMEM supplemented with 10% DS for 24 h, then shifted to 0.5% DS for another 48 h. At time 0 (end of serum deprivation) the medium was changed, a set of cultures being shifted again to 10% DS (full symbols) and another set being maintained in low serum (open symbols). Each point represents the average of duplicate cultures (cell counts in triplicate). Data, normalized at t0, refer to a representative experiment.

for up to 24 h. The histograms of figure 3 illustrate their DNA distribution profiles before and after serum restriction. After 48 h in 10% DS, their cell cycle distributions were much the same. After 24 h in 0.5% DS, however, while virtually all of the Neo$^+$ cells had accumulated in G0/G1 (not differently from other 3T3 cells) [17], the proportion of T160$^-$ cells in this phase was 65% and a significant fraction was still in S and G2/M. Furthermore, a subpopulation characterized by both reduced size (FSC, not shown) and subdiploid DNA fluorescence, which are features typical of cells with a fully developed apoptotic phenotype, emerged in the T160$^-$ clones only [22–24].

Figure 4 provides further information on the time-course of this cell death process in T160$^-$ clones, as evaluated by monitoring the reduction of DNA fluorescence to subdiploid levels combined with shifts of the SSC, which showed an early increase and a subsequent marked drop [17, 24]. Apoptotic death was already detectable after 3 h and particularly prominent after 6 h of serum deprivation.

We next examined the effect of serum deprivation microscopically on preparations either stained with DAPI to detect chromatin condensation (not shown) or processed for *in situ* 3′-OH-nick end-labeling to detect DNA fragmentation. A direct correlation was found between the decrease of T160 expression and the number of cells showing DNA fragmentation (fig 5).

Discussion

The experiments described in this paper specifically address the potential role in cell growth of T160, a member of the HMG-1 protein family, by examining the ability of an antisense construct to impair the expression of its gene. Here we show that T160 expression can be significantly suppressed by stable introduction of an antisense construct into NIH3T3 cells, and that this decrease is accompanied by substantial changes in the growth properties of the transfec-

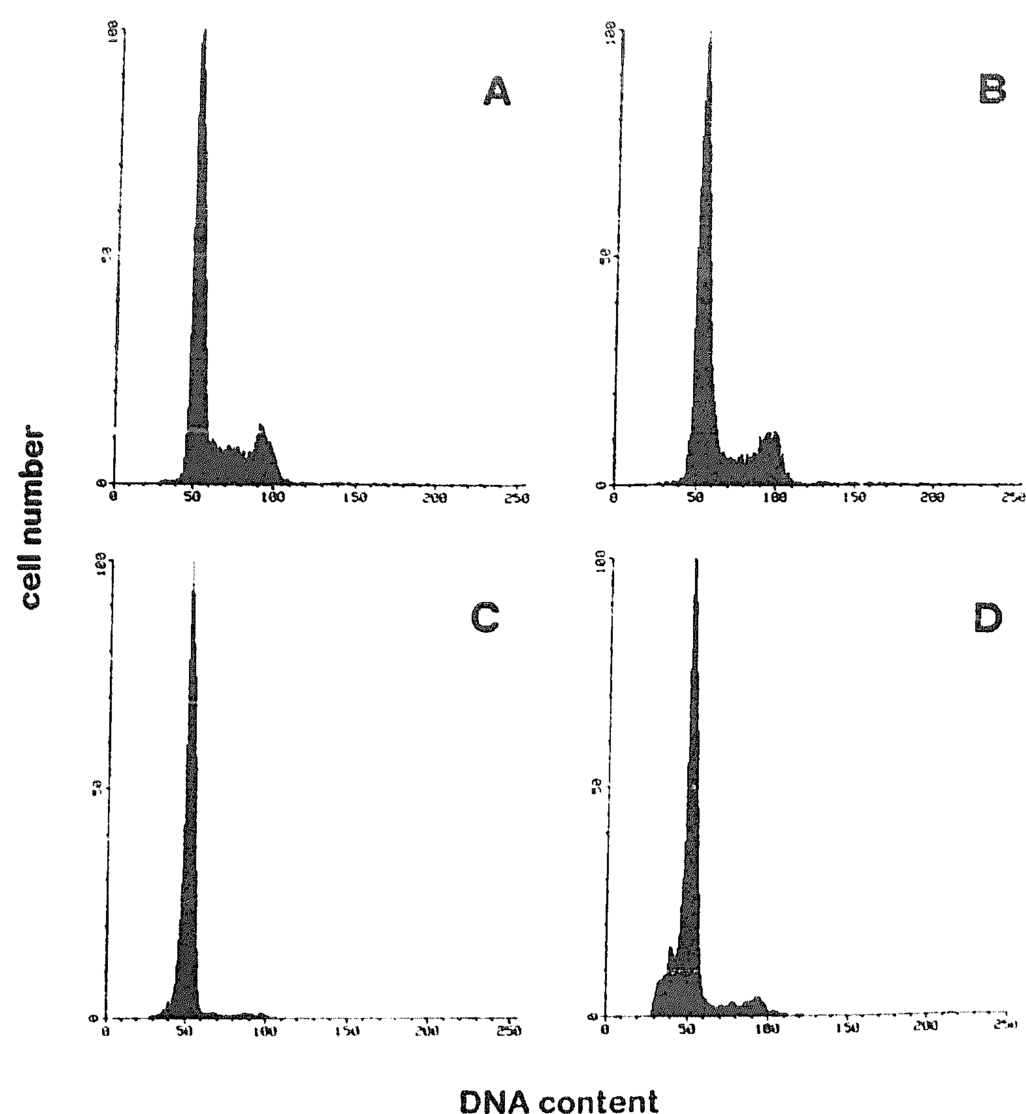

Fig 3. Effect of serum deprivation on cell cycle phase distribution in both Neo$^+$ and T160$^-$ cells. Neo$^+$.1 (**A, C**) and T160$^-$.1 cells (**B, D**), before (**A, B**) and after (**C, D**) 24-h serum deprivation. The DNA distribution patterns in **A** and **B** are typical of exponentially growing cells. After 24 h of serum deprivation, in Neo$^+$ cultures (**C**) virtually all (95%) cells had accumulated in the G0/G1 phase; in T160$^-$ cultures (**D**) only 65% of cells were in G0/G1, and a significant fraction was still distributed in S or G2/M, and a sub-G1 area corresponding to apoptotic cells was evident.

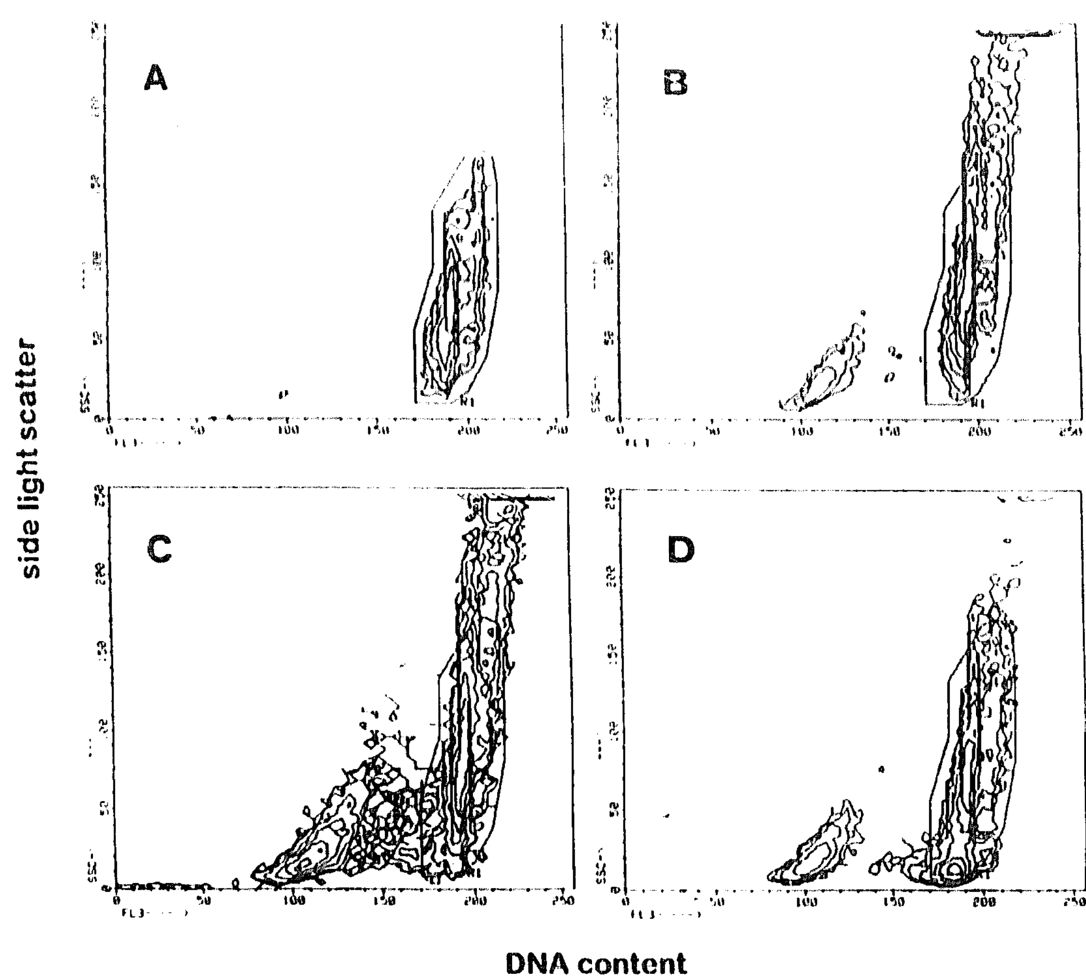

Fig 4. Flow cytometric evaluation of T160$^-$ cell death elicited by serum deprivation. Contour plots of side light scatter (SSC) *vs* DNA fluorescence. **A.** Exponentially growing cells in complete medium. **B–D.** Cells after 3, 6 and 24 h serum deprivation. R1 corresponds to cells with normal SSC and DNA fluorescence. Upon serum deprivation, a marked, transient increase of SSC was already detectable at 3 h, while an apoptotic subpopulation characterized by decreased SSC and DNA fluorescence was particularly prominent at 6 h.

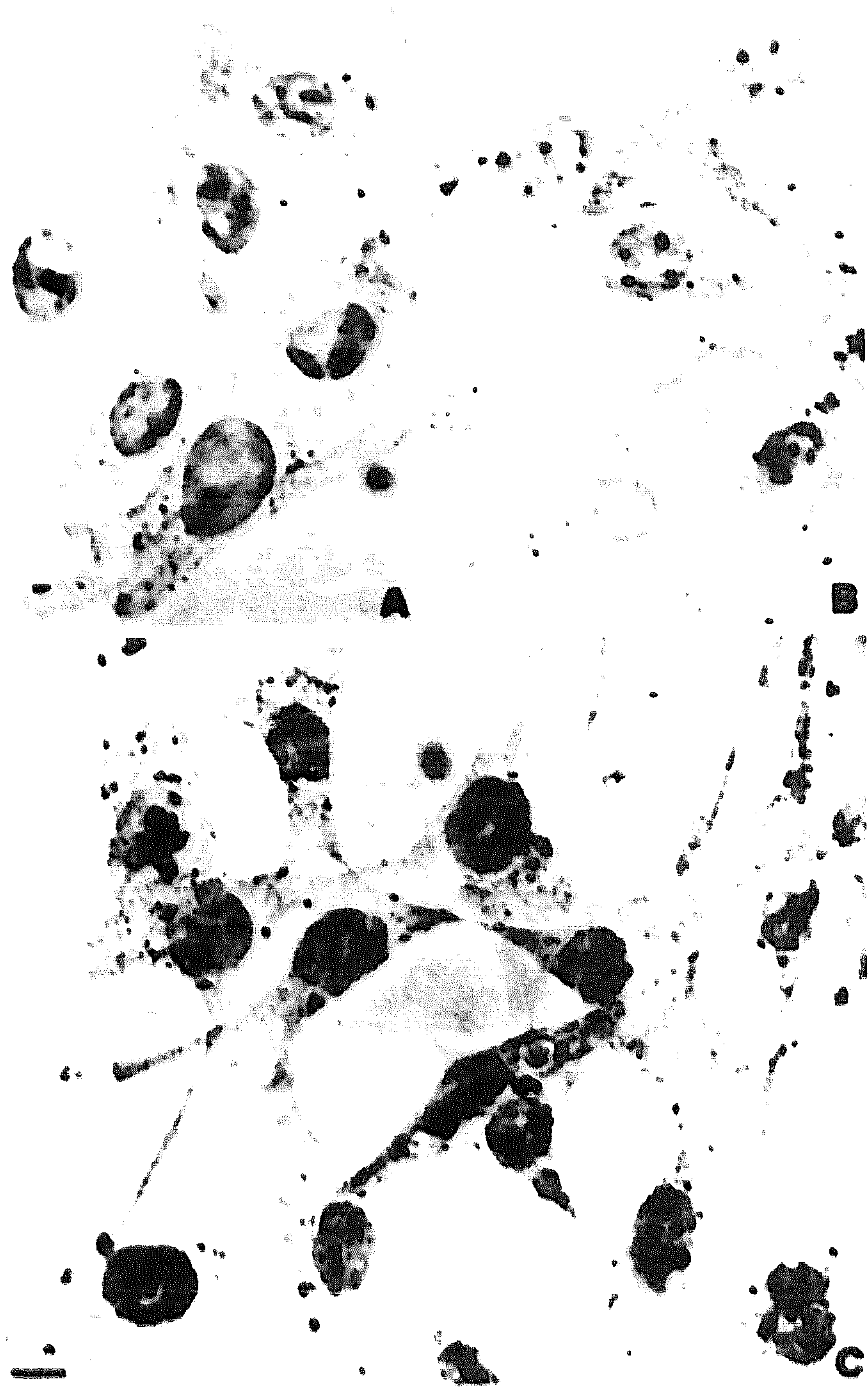

Fig 5. Light microscopic detection of DNA strand breaks by *in situ* nick end-labeling. Cells were grown on glass coverslips and serum-deprived for 6 h: untransfected controls (A) and Neo⁺ cultures (B) show rare labeled cells, while many strongly positive nuclei are evident in T160⁻ cultures (C) (scale bar, 10 μm).

tants. Impaired growth of T160⁻ cells was mainly related to two mechanisms: i) decreased rates of cell proliferation; and ii) occurrence of cell death. At 10% serum concentration, which is adequate to support optimal growth of control cells, suppression of T160 protein expression resulted in a significant reduction of the growth rate in the monolayers, whereas a substantial net loss was observed at 0.5%. This negative growth contrasted with the ability of the Neo⁺ clones to maintain a steady growth state or even slightly increase in number in 0.5% serum. Therefore, a restriction in T160 expression impaired cell proliferation at serum concentration, adequate to support at least the survival of control cells. Further support to these conclusions comes from the finding that transient transfection of T160⁻ cells with a vector expressing sense T160 under the control of a strong viral promoter restored the proliferation rate to a level comparable to that obtained with Neo⁺ cells (data not shown). This appears to be the first report of an involvement of T160 in cell proliferation and death.

The decreased growth rate of T160⁻ cells at a high serum concentration did not seem to be associated with their accumulation in any specific phase of the cycle, since their distribution did not differ from that of control cells. At low serum concentration, death of T160⁻ cells occurred by the apoptotic mode, as demonstrated by both flow cytometric and microscopic analysis, including *in situ* nick end-labeling of DNA strand breaks. In cells with a low T160 level, apoptosis was particularly evident after 6 h of serum deprivation, though it also occurred subsequently. Interestingly, when the clones were exposed to low serum for 24 h virtually all the Neo⁺ cells accumulated in the G0/G1 phase, as expected for cells strictly dependent on exogenous factors for their engagement in the proliferative cycle, though an appreciable proportion of the T160⁻ cells were still found in the S or G2/M phases, pointing to some loosening in their proliferative controls.

The exact role of T160 is unknown. We have recently observed that it is tightly associated with chromatin and undergoes post-translational modifications during certain phases of the cell cycle (LH and MG, unpublished results). It is therefore not surprising that its scarcity affects cell proliferation. Although not further investigated in the present work, the possibility that a common basic process links suppression of T160 expression in NIH 3T3 cells to both impaired cell proliferation at high serum and enhanced cell death at low serum is compatible with the present data. Both phenomena, for instance, could reflect an increased requirement of T160⁻ cells for serum factors needed for both progression through the cycle and survival (*eg* see the seminal paper by Evan *et al* [25]). On the other hand, by impairing the optimal functioning of the cell replicative machinery, the limited availability of T160 protein plus a serum shortage, could disrupt the normal coordination of the cell cycle to the point of triggering apoptosis. These two possibilities are not necessarily alternative. The observation of apoptosis in low serum is probably not irrelevant, since it is now recognized that this option may be exercised not only at cycle checkpoints, if genome damage cannot be conveniently repaired, but also at phase transitions, whenever incoherent signaling does not permit further progression through the cycle [26].

T160 belongs to the HMG-1 box protein family and preferentially binds to non-B-DNA conformations, such as B-Z junctions, stem loops, cruciforms, four-way junctions, and cisplatin-modified DNA with no sequence specificity [11]. Its major target, therefore, appears to be DNA. Its exact role has yet to be defined, though it seems to participate in processes involving structured DNA, such as replication, transcription and recombination. In addition, we have recently

demonstrated that T160⁻ clones that express low levels of T160 protein do not support replication of a DNA virus such as the murine cytomegalovirus [21]. As yet, it is unclear what functional role is played by the recognition of irregular DNA structures by HMG domain proteins. However, this property might be important in various biological processes that use irregular DNA structures as substrates or generate them as intermediates, such as DNA replication, DNA repair and recombination [4, 5, 7]. Interference with T160 expression greatly disturbs cell growth and this can lead to programmed cell death. Furthermore, the broad evolutionary conservation and *in vivo* expression pattern of T160 solely in actively proliferating tissues indicate that it may perform a critical function, very likely related to DNA replication [27]. Targeted disruption of the yeast homologue consistently results in a lethal phenotype, suggesting that the T160 protein plays an essential role for normal cell cycle progression [14].

Acknowledgments

We are grateful to Ludovica Verdun di Cantogno for the *in situ* nick end-labeling shown in figure 5. This study was supported by grants from the Italian National Research Council (CNR) (PF 'Biotecnologie') to MG (PF 'ACRO') and 'MURST' to FMB, and from the Associazione Italiana per la Ricerca sul Cancro (AIRC) to SL and FMB Gi Ba was supported by a fellowship first from Fondazione Gigi Ghirotti and then from the CNR (PF 'Invecchiamento').

References

1 Grosschedl R, Giese K, Pagel J (1994) HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Tig* 10, 94–100
2 Landsman D, Bustin M (1993) A signature for the HMG-1 box DNA-binding proteins. *BioEssays* 15, 539–546
3 Johns EW (ed) (1982) *The HMG Chromosomal Proteins*. Academic Press, London
4 Bianchi ME (1995) The HMG-box domain. *In: DNA-protein structural interactions* (Lilley DMJ, ed) Oxford University Press, Oxford, UK, 177–200
5 Baxevanis AD, Landsmann D (1995) The HMG-1 box protein family: classification and functional relationship. *Nucleic Acids Res* 23, 1604–1613
6 Bustin M, Lehn DA, Landsman D (1990) Structural features of the HMG chromosomal proteins and their genes. *Biochim Biophys Acta* 1049, 231–243
7 Crothers DM (1993) Architectural elements in nucleoprotein complexes. *Curr Biol* 3, 675–676
8 Lilley DM (1992) HMG has DNA wrapped up. *Nature* 357, 282–283
9 Gariglio M, Gaboli M, Mana C, Ying GG, Gribaudo G, Cavallo R, Landolfo S (1994) Characterization of nuclear factors involved in 202 gene induction by Interferon-a in murine leukemia cells. *Eur J Biochem* 221, 731–739
10 Gariglio M, Foresta P, Ying GG, Gaboli M, Lembo D, Landolfo S (1996) Host genotype controls the ability of the ISGF3 complex to activate transcription of IFN-inducible genes. *J Cell Biochem* 60, 83–94
11 Gariglio M, Ying GG, Hertel L, Gaboli M, Clerc RG, Landolfo S (1997) The high-mobility group protein T160 binds to both linear and cruciform DNA and mediates DNA bending as determined by ring closure. *Exp Cell Res* 236, 472–481
12 Shirakata M, Hüppi K, Usuda S, Okazaki K, Sakano H (1991) HMG1-related DNA-binding protein isolated with V-(D)-J recombination signal probes. *Mol Cell Biol* 11, 4528–4536
13 Bruhn SL, Pil PM, Essigmann JM, Housman DE, Lippard SJ (1992) Isolation and characterization of human cDNA clones encoding a high mobility group protein that recognize structural distortions to DNA caused by binding of the anticancer agent cisplatin. *Proc Natl Acad Sci USA* 89, 2307–2311
14 Wittmeyer J, Formosa T (1997) The *Saccharomyces cerevisiae* DNA polymerase a catalytic subunit interacts with Cdc68/Spt16 and with Pob3, a protein similar to an HMG1-like protein. *Mol Cell Biol* 17, 4178–4190
15 Jordan M, Schallhorn A, Wurm FM (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res* 24, 596–601
16 Katoh K, Takahashi Y, Hayashi S, Kondoh H (1987) Improved mammalian vectors for high expression of G418 resistance. *Cell Struct Funct* 12, 575–580
17 Barbiero G, Duranti F, Bonelli G, Amenta JS, Baccino FM (1995) Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. *Exp Cell Res* 217, 410–418
18 Barbiero G, Munaron L, Antoniotti S, Baccino FM, Bonelli G, Lovisolo D (1995) Role of mitogen-induced calcium influx in the control of the cell cycle in Balb-c 3T3 fibroblasts. *Cell Calcium* 18, 542–556
19 Bianciotto V, Barbiero G, Bonfante P (1995) Analysis of the cell cycle in an arbuscular mycorrhizal fungus by flow cytometry and bromodeoxyuridine labelling. *Protoplasma* 188, 161–169
20 Fehsel K, Kolb-Bachofen V, Kolb HJ (1991) Analysis of TNF alpha-induced DNA strand breaks at the single cell level. *Am J Pathol* 139, 251–254
21 Gariglio M, Foresta P, Sacchi C, Lembo M, Hertel L, Landolfo S (1997) Suppression of high mobility group protein T160 expression impairs mouse cytomegalovirus replication. *J Gen Virol* 78, 665–670
22 Telford WG, King LE, Fraker PJ (1991) Evaluation of glucocorticoid-induced DNA fragmentation in mouse thymocytes by flowcytometry. *Cell Prolif* 24, 447–459
23 Canuto RA, Muzio G, Maggiora M, Autelli R, Barbiero G, Costelli P, Bonelli G, Baccino FM (1997) Rapid and extensive lethal action of clofibrate on hepatoma cells *in vitro*. *Cell Death Differ* 3, 224–232
24 Bonelli G, Sacchi MC, Barbiero G, Duranti F, Goglio G, Verdun di Cantogno L, Amenta JS, Piacentini M, Tacchetti C, Baccino FM (1996) Apoptosis of L929 cells by etoposide: a quantitative and kinetic approach. *Exp Cell Res* 228, 292–305
25 Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69, 119–128
26 Elledge SJ (1996) Cell cycle checkpoints: preventing an identity crisis. *Science* 274, 1664–1672
27 Bruhn SL, Housman DE, Lippard SJ (1993) Isolation and characterization of cDNA clones encoding the *Drosophila* homolog of the HMG-box SSRP family that recognize specific DNA structures. *Nucleic Acids Res* 21, 1643–1646