BTG1, a member of a new family of antiproliferative genes

Jean-Pierre Rouault, Ruth Rimokh, Chantal Tessa, Glaucia Paranhos, Martine Ffrench, Laurent Duret, Monique Garoccio, Daniel Germain, Jacques Samarut and Jean-Pierre Magaud

Equipe de Cytologie Analytique et de Cytogénétique Moléculaire, Hôpital Edouard Herriot, 69437 Lyon, 1 BioMérieux, Centre de Recherche de Gerland, 69007 Lyon, 2 Laboratoire de Biométrie, Génétique et Biologie des Populations (CNRS, URA 243), Université Claude Bernard, 69622 Villeurbanne, and 3 Laboratoire de biologie Moléculaire et Cellulaire, CNRS UMR 49, Ecole Normale Supérieure, 69007 Lyon, France

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The BTG1 gene locus has been shown to be involved in a t(8;12)(q24;q22) chromosomal translocation in a case of B-cell chronic lymphocytic leukemia. We report here the cloning and sequencing of the human BTG1 cDNA and establish the genomic organization of this gene. The full-length cDNA isolated from a lymphoblastoid cell line contains an open reading frame of 171 amino acids. BTG1 is maximal in the G0/G1 phases of the cell cycle and is down-regulated when cells progress throughout G1. Furthermore, transfection experiments of NIH3T3 cells indicate that BTG1 negatively regulates cell proliferation. The BTG1 open reading frame is 60% homologous to PC3, an immediate early gene induced by nerve growth factor in rat PC12 cells. Sequence and Northern blot analyses indicate that BTG1 and PC3 are not cognate genes. We then postulate that these two genes are the first members of a new family of antiproliferative genes.

Key words: BTG1/PC3/cell proliferation/antiproliferative genes/cell cycle

Introduction

In normal tissue, homeostasis is maintained through negative and positive growth controls which affect the proliferation and differentiation cellular genetic program. An alteration of this subtle balance can result in developmental anomalies or in neoplasia. Proto-oncogenes, genes that promote cell division, were the first to be identified and more than 60 of them have been described so far (reviewed in Bishop, 1991). On the other hand, little is known about the genes that negatively regulate cell proliferation; these genes are nevertheless crucial to counteract the growth-inducing elements and are likely to have the same importance as proto-oncogenes in controlling cell division (reviewed in Marshall, 1991; Weinberg, 1991).

Different methodological approaches have been used to isolate genes with an antiproliferative activity and several overlapping families have been defined. Tumor suppressor genes have been identified on the basis of an association between neoplasia and the loss of function in both copies of the gene. The existence of 12 tumor suppressor genes is predicted on the basis of such an association but molecular clones are available only for five of them: the retinoblastoma gene (RB1) (Friend et al., 1986), p53 (reviewed in Levine et al., 1991), the Wilms’ tumor gene (WT1) (Call et al., 1990), the DCC gene deleted or mutated in colon carcinoma (Fearon et al., 1990) and the NF1 gene involved in neurofibromatosis I (Wallace et al., 1990). The genes whose loss of function kills the cell constitute a second category of genes whose absence might cause death during embryogenesis. The mammalian prohibitin gene, analogue of the development Cc gene in Drosophila, was the first member of this hypothetic class to be cloned (Nuell et al., 1991). A family of RAS-related transformation suppressor genes, the KREVIRAP genes, has also been demonstrated to be directly antiproliferative (Kitayama et al., 1989). The last group of antiproliferative genes consists of genes coding for extracellular growth inhibitory proteins. A few examples include transforming growth factor beta (Moses et al., 1990), JE gene (Rollins et al., 1988; Rollins and Sunday, 1991) and leukemia inhibiting factor (Stahl et al., 1990). Other genes, such as the members of the GAS family, whose expression increases as the cells become quiescent or senescent are also good candidates but there is no direct evidence of their antiproliferative activity in vitro or in vivo (Schneider et al., 1988; Manfioletti et al., 1990).

Molecular cloning of the breakpoint of a t(8;12) chromosomal translocation enabled us to isolate a coding sequence mapping on chromosome 12 band q22. This sequence detects a 1.8 kb transcript in virtually all tissues tested except in the brain and the muscle where the signal is barely detectable. The putative gene corresponding to this sequence (termed BTG1 for B-cell translocation gene 1) was shown to be highly conserved in evolution and a similar 1.8 kb transcript can be detected in murine and chicken tissue by using a human BTG1 DNA probe (Rimokh et al., 1991).

This report describes the cloning and analysis of human BTG1 cDNA with a complete open reading frame (ORF) and shows that this new gene is able to negatively regulate NIH3T3 cell proliferation when over- or inappropriately expressed. We also establish the genomic organization of the human BTG1 gene. Comparison of the cDNA sequence with those in the GenBank and in the EMBL bank revealed a very strong homology of the BTG1 protein to the product of the rat PC3 gene (Bradbury et al., 1991) whose expression is induced by nerve growth factor in neuronal PC12 cell line. Since BTG1 is different from the human cognate of this rodent gene, it is likely that these two genes are members of a new gene family.
Fig. 1. (A) Restriction map of the BTGI locus on chromosome 12q22. Exons are indicated by boxes, horizontal lines under the map show the location of the RIA and R7 probes. CEN, centromere; TEL, telomere; B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI; R, EcoRV. Bg* polymorphic site detected by the R7 probe (Rouault et al., 1991). (B) Nucleotide sequence of BTGI 5' flanking region and exons with predicted amino acid sequence. Candidate TATA sequence is underlined. Number +1 corresponds to the 5' extremity of the longest cDNA cloned. The promoter region contains two SPI (---), one PEA3 (-----), one CRE (-----) and one AP1-like (-----) potential binding sites. Bold type AATAAA indicates the polyadenylation site.
Results

Cloning and sequencing of the human BTG1 cDNA
Polyadenylated RNA was used as the template to construct a λZap II cDNA library which was screened with the human RIA probe (Figure 1A). Twelve positive clones were isolated and their partial sequences showed that they all coded for the same protein. Three overlapping clones representing the entire cDNA were sequenced by generating progressive deletions and by dideoxy sequencing procedure. The sequence exhibits an ORF coding for a peptide of 171 amino acids, predicted molecular weight 19 207, and a 961 nucleotide long 3' unregioned region. The latter is rich in A/U nucleotides (65%) and contains one AUUUA motif (nucleotides 908–912), all elements that have been implicated in the control of mRNA instability (Shaw and Kamen, 1986; Bonnieu et al., 1990). The first three ATG codons of the cDNA (nucleotides 10, 32 and 76) are followed by numerous stop codons. The longest ORF contains two possible ATG initiation codons (nucleotides 303 and 333); of the two, the second has the better initiation sequence consensus: ACCATGA (Kozak, 1986; Cavener and Ray, 1991). There is an in-frame terminator codon (TAA) 180 bases upstream of the first ATG. A consensus polyadenylation site AATAGG (nucleotides 1745–1750) is found 929 bases 3' from the stop codon (Birnstiel et al., 1985). A poly(A) tail was found on the cDNA 26 bases downstream of this polyadenylation signal.

Comparison of the human cDNA and genomic DNA sequences revealed that the BTG1 gene contains two exons of respectively 450 and 1326 bp separated by a 0.9 kb intron with typical splice donor and splice accceptor sites at the intron—intron junctions (Mount et al., 1982). The sequenced promoter region contains, in addition to a consensus TATA box, several other consensus: a potential PEA3 binding site (AXXGGAA) (Karim et al., 1990); an API consensus (TGCGTCA) (Sassone-Corsi et al., 1988) and a cAMP responsive element (TGACGTA) (Montminy et al., 1986) both identical to those found in the fos promoter; and two SP1 binding sites GGCGGC (Dyban et al., 1986) (see Figure 1B for localization).

The predicted amino acid sequence of the largest open reading frame was determined from the nucleotide sequence. Computerized analysis of the amino acids sequence (PROSITE program) showed that the btg1 protein contains a potential N-glycosylation site on Asn136, potential phosphorylation sites by protein kinase C on Ser33, 112 and 148, by casein kinase II on Ser43, 90 and 148 and by p34cdc2 M-phase kinase on Ser159. The hydropathic profile of btg1 obtained by the Kyte and Doolittle program (Kyte and Doolittle, 1982) showed a unique region of hydrophobicity at the N terminus (amino acids 8–27) of the protein. As there does not exist any potential cleavage site, it seems unlikely that the hydrophobic domain corresponds to a signal peptide and that the btg1 protein might be secreted. Finally, the btg1 protein is rich in PEST residues, which could be a characteristic of unstable proteins (Rogers et al., 1986).

Comparison of the cDNA sequence with those in the EMBL bank and in the GenBank (September, 1991) revealed homology to the putative protein encoded by the PC3 rat gene (Bradbury et al., 1991). The btg1 and the pc3 proteins are 59% identical in amino acid sequence (Figure 2), the degree of homology being much higher (75%) if we take into account the conservative residues. The amino acids 1–10 are not conserved, suggesting that the second ATG in-frame at position 11 is likely to be the initiation codon. More interesting is the fact that the btg1 protein differs from the pc3 protein in having 10 extra amino acids (amino acids 135–144), this insertion occurring at a position that does not correspond to any splice junction in the human and rodent gene (Figure 2). At the nucleotide level, the BTG1 coding sequence is ~60% identical to the coding sequences of the PC3 gene.

Northern blot analysis of BTG1 and PC3 transcripts in murine tissues
In murine tissues, the human BTG1 RIA probe (encompassing nucleotides 530–931 on the cDNA sequence) detects, even under high stringency conditions, a transcript similar in size (1.8 kb) to the human BTG1 mRNA (Figure 3). The 55% identity between the human RIA probe and the corresponding PC3 counterpart cannot account for this result; there must then exist, in rodent tissues, another sequence more homologous to BTG1 than to PC3. In the murine tissues analyzed, the RIA probe also detects, in addition to the normal 1.8 kb BTG1 transcript, a 2.6 kb weak signal similar in size to the normal rodent PC3 transcript (Bradbury et al., 1991). Hybridization of the same blot with a PC3 probe showed that this 2.6 kb transcript corresponded to the normal PC3 transcript (Figure 3), thus confirming that BTG1 and PC3 albeit related, are not cognate genes.

**Fig. 2.** Comparison of the amino acid sequences of the predicted btg1 and pc3 proteins. The amino acid sequences are aligned for optimal homology.

* Identical residue. ** conservative substitution.
The BTG1 gene is strongly expressed in quiescent cells and down-regulated as the cells enter the growth cycle

The idea that BTG1 expression could be related to certain phases of the cell cycle came from the observation that BTG1 expression was found to be significantly higher in quiescent fibroblasts than in proliferating cells (data not shown). The two systems that we have utilized are phytohemagglutinin (PHA) activated peripheral blood lymphocytes (PBL) and serum stimulation of NIH3T3 cells arrested by serum starvation.

To assess the relative abundance of the BTG1 transcript during the cell cycle, Northern blot analysis of BTG1 expression was carried out at various times after stimulation of PBL with PHA. Ethidium bromide staining of the gel before blotting is shown as a control of the amount of RNA loaded in each lane, and the percentage of cells entering in S phase was calculated by flow cytometric analysis (Figure 4B). The highest level of BTG1 transcripts was observed in quiescent unstimulated PBL; after PHA stimulation BTG1 RNA decreased significantly and reached a minimum after 36–48 h when the cells enter the S phase of the cell cycle (Figure 4A). Sequential hybridizations of the same blots with BTG1 and MYC probes revealed that the levels of the transcripts of these two genes vary in the opposite direction during the cell cycle (Figure 4A).

We confirmed that BTG1 expression varies across the cell cycle by assessing BTG1 RNA level of NIH3T3 cells synchronized by serum starvation. After 72 h of serum starvation, the expression of BTG1 in NIH3T3 cells is barely detectable; when the cells were refed with 20% of fresh serum, BTG1 RNA increased steadily, reaching a peak after 2 h. After the peak, BTG1 RNA level decreased as the cells progressed through the G1 phase and reached a minimum at the entry of the S phase (Figure 5A and B). The fact that BTG1 is highly expressed in quiescent cells after density-dependent growth inhibition (Figure 5A) confirms that BTG1 expression is serum dependent in quiescent NIH3T3 cells and thus that BTG1 expression is not required for the quiescence of serum-depleted cells.

Forced expression of exogenous BTG1 in NIH3T3 cells is evidence for its antiproliferative activity

Since the BTG1 gene was strongly expressed in quiescent cells and appeared to be down-regulated as the cells enter the growth cycle, we hypothesized that BTG1 might be involved in the control of the early phases of the cell cycle. To test this possibility we performed transfection experiments of NIH3T3 cells with the pIRV-9 expression vector. In this vector the BTG1 gene is under the control of the β-actin promoter and the 3' AU-rich untranslated region of the gene is deleted. In this configuration, expression of BTG1 is thought to be independent of its normal physiological mechanisms of regulation (Figure 6B). After transfection, 21 G418 resistant clones were isolated and Southern blot analysis of the integration pattern permitted us to select and to extend six independent clones. Northern blot analysis using a U3 proviral probe specific to the LTR, showed that the BTG1 gene was expressed from the expression vector in all six clones (Figure 6C, data shown for the three clones that were further characterized). Growth kinetics of the clones were analyzed and compared with those of control NIH3T3 cells. The most striking characteristics of these clones was that the cells grew to a much lower density at confluence than control cells transfected with the pIRV vector alone (3- to 5-fold range) (Figure 6A). At confluence, the pIRV-9 transfected cells were characterized by a pseudo-

![Fig. 3. Expression of BTG1 and PC3 in rodent tissues (S, spleen; T, thymus). Ten µg of RNA were loaded in each lane and sequentially hybridized to the human RIA and to the rodent PC3 probes under high stringency conditions. The scale is in kb.](image)

![Fig. 4. BTG1 expression after PHA stimulation of peripheral blood mononuclear cells. (A) Total RNA was extracted and processed for Northern blot analysis at the indicated times as described in Materials and methods. The ethidium bromide staining is shown at the top; for the most part, lanes contain comparable quantities of RNA (10 µg). The Northern blot was successively hybridized to BTG1 and MYC as illustrated. (B) Analysis of DNA synthesis level (%S: percentage of cells in S phase as determined by flow cytometric analysis).](image)
epithelial aspect contrasting with the normal morphology of the control cells (Figure 6A). Diagrams of growth curves of clones 3T3-9.16, -9.12 and -9.21 showed that overexpression or inappropriate expression of the BTG1 cDNA resulted in an increase of the doubling time of the analyzed clones with a plateau reached earlier than in the control cells (Figure 6A). At the plateau, the cells could be replated normally, showing that they were not dead. It must be pointed out that the six independently selected clones contained only one copy of the transfected gene and that the pIRV-9 vector yielded roughly half the number of resistant colonies as did the control vector. These two data constitute additional pieces of evidence in favour of the antiproliferative role of BTG1 and suggest that the effects observed on the cell kinetics are due to the inappropriate expression of BTG1 during the cell cycle rather than to its overexpression which is likely to kill the cells. To confirm that BTG1 suppresses the outgrowth of neomycin-transfected cells, we performed another set of transfection experiments using another BTG1 expression vector (pX71-9). In this vector, BTG1 is under the control of the herpes simplex virus thymidine kinase promoter. The dose–response curve showed that inclusion of the pX71-9 vector in the transfection mixture markedly reduced the number of neomycin-resistant foci by a factor of 3 to 4, thus confirming that overexpression of BTG1 alters cell growth (Figure 7).

**Discussion**

In this study we have identified a new gene, termed BTG1, which is able to negatively regulate cell proliferation. The BTG1 gene encodes a peptide of 171 amino acid residues homologous to the putative protein encoded by the rat PC3 gene (Bradbury et al., 1991).

Northern blot analysis of the BTG1 and PC3 transcripts in rodent tissues, comparison of their sequences (with in particular the presence of a 10 amino acids insertion in the btg1 protein) indicate that, albeit related, these two genes are not cognate genes but are likely to constitute the first members of a new gene family. An additional argument comes from the observation that their tissue distribution is slightly different since unlike PC3, BTG1 is expressed in liver and kidney (Rimohk et al., 1991).

Analysis of the amino acid sequence of the putative btg1 protein failed to provide insights into its function and subcellular localization. The existence of a hydrophobic N-terminal domain suggests however, that this protein might be anchored into a cell membrane. The production of a specific antibody will enable us to address this question.

Assessment of BTG1 RNA level during the growth cycle showed that this gene is preferentially expressed in quiescent cells and during the early subphases of G1 in a serum-dependent manner; it is then down-regulated to reach a minimum level as the cells enter the S phase. These results suggest the existence of a functional link between BTG1 and the cell cycle process. Analysis of the nucleotide sequence of the BTG1 gene argues also in favour of this hypothesis. Many of the genes involved in the control of the cell cycle are characterized by the instability of their transcripts and of the proteins for which they code. Instability of the BTG1 transcript, predicted from the presence of a long 3' AU-rich untranslated region was confirmed by the assessment of its half-life which is ~1 h (data not shown). The abundance of PEST residues in the primary structure of the btg1 protein argues also in favour of a rapid turnover of the BTG1 product (Rogers et al., 1986). The existence of a potential p34cdc2 kinase phosphorylation site in the btg1 protein may have the same significance since one of the most important biochemical events leading to cell division is the phosphorylation of specific substrates by the p34cdc2 kinase (Moreno and Nurse, 1990).

In the present report, we also showed by transfection experiments that inappropriate and overexpression of the BTG1 gene negatively regulates NIH3T3 cell proliferation. Considering all these experimental data, it can be postulated that BTG1 expression constitutes a marker of quiescent cells (in G0-G1 phases of the cell cycle) in the presence of serum; its down-regulation in response to optimal doses of mitogenic factors or after suppression of cell contact inhibition allows the cells to progress further through the cell cycle. We have previously reported (Rimohk et al., 1991) that BTG1 is preferentially expressed in tissues (lymphoid tissue, liver, placenta) containing non-dividing cells likely to re-enter the cell cycle upon different stimuli. Conversely, the expression of BTG1 is barely detectable in fully differentiated tissues such as brain and muscle. The expression of BTG1 appears then to be associated with the G0-G1 transition only in cells which have kept the ability to respond to various mitogenic signals.

The finding that PC3 is an immediate early gene induced by nerve growth factor (NGF) in PC12 cells (Bradbury et al., 1991), a cell line derived from a tumor of the adrenal medulla, is in agreement with the observation that BTG1 negatively regulates cell proliferation. Effectively, it has been shown that addition of NGF to exponentially growing cells results in their accumulation in G1 phase before they differentiate (Rudkin et al., 1989). The members of the PC3/BTG1 family could thus act as growth inhibitors in different physiological circumstances that remain to be analyzed.
Fig. 6. Consequences of a forced expression of exogenous BTG1 in NIH3T3 cells. (A) Diagrams of growth curves of three clones of pIRV-9 transfected NIH3T3 cells (3T3-9.16, 9.20, 9.21), of normal NIH3T3 cells (3T3) and of NIH3T3 cells transfected with pIRV alone (3T3-pIRV). Morphology of BTG1 transfected NIH3T3 cells (3T3-9.20 clone, lower photo) and of pIRV-transfected NIH3T3 cells (upper photo). In both cases confluent cells (day 8) were examined by phase contrast microscopy, scale bar indicating 100 μM. (B) Schematic diagram of the pIRV expression vectors. NEO, neomycin resistance gene; βACT, rat β-actin promoter. (C) Northern blot analysis of exogenous BTG1 expression in transfected clones. Equal amounts (10 μg) of total RNA were loaded in each lane, the filter was then hybridized with a U3 proviral probe. N, NIH3T3 cells; P, pIRV transfected NIH3T3 cells; 16, 20 and 21, pIRV-9 transfected clones.

Although the mechanism of action of btg1 protein is presently unknown, the results presented here raise the possibility that this protein might be involved in the transduction of growth inhibitory signals such as those responsible for cell contact inhibition. A similar mechanism is proposed to explain the consequence of overexpression
of the gene coding for the 1,2-cyclic phosphate 2-phospho-
hydrolase (cyclic hydrolase), an enzyme involved in the
metabolism of inositol cyclic phosphate (reviewed in Majerus
et al., 1990). Cells which express high levels of cyclic hydrolase
grow to lower density at confluence than cells expressing
low levels of activity. Furthermore, cyclic hydrolase levels are also lower during active cell growth than when
cells are confluent (Majerus et al., 1990). Altern-
atively, we cannot rule out the possibility that BTGI might
antagonize a protein involved in the transduction of a positive
growth signal.

The possibility that BTGI might constitute a new tumor
suppressor gene arises from the observation that band q22
of chromosome 12 is often involved in non-random structural
abnormalities of chromosome 12. In particular, band 12q22
which contains the BTGI locus, is deleted in > 40% of the
testicular or extragonadal non-seminomatous germ cell
tumors (Samaniego et al., 1990). In the tumors carrying this
karyotypic alteration, the study of BTGI expression and the
search for mutation and/or deletion of the second allele
should bring some data elements. The t(8;12) translocation
which allowed us to clone the BTGI gene neither changed
the BTGI structure nor led to the creation of a fusion
transcript since, on chromosome 12, the break was localized
at 10 kb upstream of the first exon of BTGI; furthermore,
BTGI expression appeared to be 'normal' in this leukemia
(Rimokh et al., 1991), and we did not find any mutation in
the coding sequence of the translocated allele (data not
shown). We cannot however, rule out the possibility that
the t(8;12) translocation might have separated the BTGI
coding sequence from important cis-activating regions
located far upstream and that the 'normal' RNA level
observed in this leukemia might be due to the non-
translocated (mutated?) allele. This hypothesis, if it is proved
correct, would also argue in favour of BTGI being a tumor
suppressor gene. On the other hand, BTGI could be
functionally related to the prohibitin gene (Nuell et al.,
1991); it remains to be analyzed whether homozygous
deletion of BTGI kills the cells or alters embryonic
development.

Materials and methods

Cell lines and cell culture conditions
NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium
(DMEM) containing 10% newborn calf serum (NCS), 0.03% L-glutamine,
100 μg/ml of penicillin G and 100 μg/ml of streptomycin sulfate (growth
medium). For serum starvation, the medium was changed to 0.5% NCS
when cells were subconfluent, and the cells were then left in the same medium
for 72 h. For induction of DNA synthesis, fresh medium containing 20%
NCS was added to the arrested cells. The cells were then harvested at various
times for RNA isolation.

Peripheral blood lymphocytes (PBL) were obtained from the blood of
normal volunteers by centrifugation on Triosil Ficoll. Mitogen-stimulated
lymphocytes were obtained by incubating PBL at a concentration of
2 x 10^6 cells/ml, in the presence of phytohemagglutinin (PHA, Welcome) diluted
1:200 in RPMI 1640 medium supplemented with 20% fetal calf serum
plus 0.03% L-glutamine, 100 μg/ml of penicillin G and 100 μg/ml of
streptomycin sulfate. BTGI RNA level was then assessed at various times
after PHA stimulation.

Flow cytometric analysis of cell cycle kinetics
The flow cytometry technique was carried out on PBL and NIH3T3 cells
as previously described (Firench et al., 1985). Briefly, propidium iodide
was used to stain DNA in ethanol-fixed cells after RNase treatment. The
stained cells were analyzed in a Cytofluorograf H50 (Ortho Instruments,
Westwood, MA). The percentage of cells in the cell cycle phases was
calculated from the DNA histogram according to model 1 of Baisch’s
method. Flow cytometric studies and Northern blot analysis of the BTGI
RNA level were performed on the same samples.

DNA and RNA isolation and analysis
High molecular weight DNA was extracted from fresh cells or frozen material
following standard procedure (Maniatis et al., 1982). After digestion with
appropriate restriction endonucleases as recommended by the suppliers
(Boehringer), DNA fragments were electrophoresed on 0.6–0.8% agarose
gels and transferred onto nylon filters (Southern, 1975).

Total cellular RNA was isolated from cultured cells and from frozen
samples by the acid guanidium thiocyanate—phenol—chloroform method
(Chomczynski and Sacchi, 1987). For Northern blot analysis, 10 μg of total
RNA were size fractionated in formaldehyde–1.2% agarose gels and
transferred onto nylon filters (Thomas, 1981).

DNA probes and hybridization procedures
RIA probe is a human genomic probe which extends from nucleotides 530
to 931 on the sequence shown in Figure 1B and contains almost all of the
coding sequence of BTGI second exon (Figure 1A). A human genomic probe
for MYC third exon was kindly provided by D.Stehelin (Lille, France), probe
GAPDH was a gift of J.M.Blanchard (Montpellier, France). A 264 bp PCR-
amplified DNA segment encompassing nucleotides 208–472 of the murine
PC3 gene sequence (Fletcher,B.S., Lim,R.W., Varum,B.C., Kubujo,D.A.,
Kuski,R.K. and Herschman,H.R., unpublished results; EMBL, bank access
number M64292) was used as PCR probe. Probe α-32P-labeling,
prehybridization, hybridization and washing conditions were carried out as
previously described (Rimokh et al., 1989).

Preparation and analysis of DNA and cDNA libraries
Construction of genomic DNA library in EMBL3 λ phage and isolation
of recombinant clones containing the BTGI gene have been previously
reported (Rimokh et al., 1991).

Poly(A)+ RNA extracted from the EBV+ lymphoblastoid cell line UD53
was used to construct a human cDNA library (Clontech custom library
service) in the λ Zap II vector (Stratagene).

Sequencing
Overlapping deletions of BTGI cDNA cloned into Bluescript. SK(−)
(Stratagene) were obtained by the unidirectional exonuclease III digestion
method (Erase-a-base system, Promega). Deletion clones were sized on
agarose gels, and the inserts of the selected clones were sequenced using
the double-stranded DNA sequencing technique (dideoxy chain termination
procedure) with Sequenase II as described by the manufacturer (USB).

Plasmids
The BTGI expression vector was constructed by subcloning the 0.9 kb
BglII–BglII cDNA segment (nucleotides 22–930, Figure 1B) into the
BamHI site of the retroviral pRV-Neo-Act vector (Figure 5C) which confers
G418 resistance to the host cells (description of the vector in Bedington et al.,
1989). In the resulting plasmid, termed pRV-9, the BTGI gene is
under the control of the β-actin promoter and the polyadenylation signal
is provided by the 3’ LTR of the provirus (Figure 5C). Another BTGI
expression vector was constructed by subcloning the 0.9 kb BglII–BglII
cDNA segments into the BglII site of the pX1T-9 vector (Stratagene). In
the resulting plasmid, termed pX1T-9, the BTGI gene is under the control of
the herpes simplex virus thymidine kinase promoter.

DNA transfection experiments
NIH3T3 cells (1 x 10^6) were plated 1 day prior to transfection on 60 mm
Petri dishes. Transfections were carried out using the calcium phosphate

Fig. 7. Effect of BTGI expression plasmid pXT1-9 on the outgrowth
of neomycin-resistant NIH3T3 cells.
method (Cellispet transfection kit, Pharmacia) with 5 µg of pRTV-9 DNA, pRTV DNA without insert being used as control. Cells were exposed to the precipitate for 16–20 h at 37°C in a CO2 incubator. After two washings with DMEM, the cells were treated with 1.5 ml of 15% glycercol in HEPES buffer, pH 7.5 for 3 min, rinsed with DMEM and refed with 3 ml of growth medium. The following day the cells were trypsinized and replated in 100 mm Petri dishes with 10 ml of growth medium containing 500 µg/ml of G418 (Geneticin, Gibco-BRL). The medium was changed every 2 days thereafter. Colonies of resistant cells were observed 10–14 days after transfection, and independent clones were isolated by dilution cloning. Before transfection, the pRTV vectors were linearized by BglII digestion in order to favour expression of the exogenous BGFI gene. To study growth kinetics of the different clones, 105 cells of each clone were plated on 30 mm Petri dishes in growth medium; the growing viable cells were then counted every day thereafter by using an automatic cell counter (from day 1 to day 8). In another set of experiments, NIH3T3 cells were transfected, following the same procedure as described above, with increasing amounts of pXT1-9 DNA to establish a dose–response curve. For each transfection, the total amount of DNA used (5 µg) was kept constant by mixing pXT1 and pXT1-9 DNA.

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