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ARTICLES

Suppression of AP-1 Constitutive Activity Interferes With Polyomavirus MT Antigen Transformation Ability

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Abstract Polyomavirus (Py) encodes a potent oncogene, the middle T antigen (MT), that induces cell transformation by binding to and activating several cytoplasmic proteins which take part in transduction of growth factors-induced mitogenic signal to the nucleus. We have previously reported that the AP-1 transcriptional complex is a target for MT during cell transformation although, its activation was not sufficient for establishment of the transformed phenotype. Here we show that expression of a dominant-negative cJun mutant in MT transformed cell lines inhibits its transformation ability, indicating that constitutive AP-1 activity is necessary for cell transformation mediated by MT. Evidences also suggest that proliferation of MT transformed cells in low serum concentrations and their ability to form colonies in agarose are controlled by distinct mechanisms. J. Cell. Biochem. 90: 253–266, 2003.

Key words: polyomavirus; middle T antigen; AP-1 activation; cell transformation

The early region of polyomavirus (Py) encodes three tumor antigens, namely, large T (LT), middle T (MT), and small T (ST). The LT antigen causes immortalization of primary cell lines [Schlegel and Benjamin, 1978; Rassoulzadegan et al., 1982; Cherington et al., 1986] by binding to the product of the Rb tumor suppressor gene (Retinoblastoma) [Freund et al., 1992]. It has been shown that interaction between LT and RB affects p53-dependent cell cycle arrest [Doherty and Freund, 1997]. More recently, it has been suggested that coexpression of Py MT and ST antigens can inhibit both p53-induced cell cycle arrest and apoptosis [Qian and Wiman, 2000]. The ST antigen is able to induce cell proliferation, in a manner dependent on its binding to protein phosphatase 2A [Mullane et al., 1998]. ST expression promotes both high saturation densities and changes in the cell cytoskeleton [Liang et al., 1984; Cherington et al., 1986]. Since MT alone can induce morphological transformation and alterations in the growth properties of established cell lines [Schlegel and Benjamin, 1978; Treisman et al., 1981; Cherington et al., 1986], it is believed that it plays a central role during Py transformation and tumorigenesis.

The MT phosphoprotein associates with the cell membrane and does not appear to display any enzymatic activity of its own. Instead, it exerts its effects in the host cell by interacting with, and altering the activities of essential growth regulatory proteins that have been implicated in normal cell growth control [Pallas et al., 1988; Armelin and Oliveira, 1996; Oliveira et al., 1999; Ichaso and Dilworth, 2001; Dilworth, 2002]. Examples of cellular proteins that bind to MT are: $pp60^{c\text{-}src}$, c-*yes*, and c-*fyn* kinases [Courtneidge and Smith, 1983; Kornbluth et al., 1986; Kaplan et al., 1987; Horak et al., 1989; Su et al., 1995; Dunant et al., 1996], phosphatidylinositol 3-kinase (PI3K) [Whitman et al., 1985], protein phosphatase 2A [Glenn and Eckhart, 1993; Campbell et al., 1995], Shc [Campbell et al., 1994; Dilworth et al., 1994; Blaikie et al., 1997], phospholipase Cγ (PLCγ) [Su et al., 1995], and 14-3-3 [Pallas et al., 1994; Culleré et al., 1998]. Association of MT antigen with these cellular proteins occurs, in general, through SH2 or PTB domains present in these proteins and MT

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phosphotyrosine residues. Generation of a large number of MT mutants [Carmichael et al., 1984; Morgan et al., 1988; Druker and Roberts, 1991; Druker et al., 1992] was important for identification of the MT domains that are responsible for binding to these cellular proteins and determination of their importance in MT-induced cell transformation [Druker et al., 1990; Glenn and Eckhart, 1993; Campbell et al., 1994, 1995; Oliveira et al., 1998].

MT overexpressing cell lines have been shown to display high constitutive mRNA levels of some of the immediate early genes induced by PDGF (platelet derived growth factor) as well as high constitutive AP-1 binding activity [Rameh and Armelin, 1991, 1992; Schöntal et al., 1992; Oliveira et al., 1998]. The AP-1 transcription factors (Jun–Jun homodimers or Jun–Fos heterodimers) bind to phorbol 12-*o*-tetradecanoate-13-acetate responsive elements present in the promoters of genes that are regulated by this agent. Increased AP-1 activity can result from increased synthesis of Jun and Fos proteins or from post-translational modifications [Angel and Karin, 1991; Karin, 1995]. Five phosphorylation sites have been identified in the Jun protein: two (Ser63 and Ser73) in the N-terminal region of the transactivation domain and three (Ser243, Ser249, and Thr231) in the DNA binding domain. Dephosphorylation of the sites present in the DNA binding domain and phosphorylation of those in the transactivation domain increase the ability of cJun to bind DNA [Binétruy et al., 1991; Pulverer et al., 1991; Minden et al., 1994].

Overexpression of MT in the NIH-3T3 cell line results in phosphorylation of the transactivation domain and dephosphorylation of the DNA-binding domain of cJun, leading to activation of its transcriptional activity, as determined by reporter assays [Srinivas et al., 1994]. We previously demonstrated the relationship between MT-mediated activation of several cellular proteins and the constitutive AP-1 activity displayed by MT-expressing cell line. Using different MT mutant overexpressing cell lines, we were able to identify the PI3K binding domain as being responsible for AP-1 activation [Oliveira et al., 1998]. This result points to an essential role for PI3K in the AP-1 activation by MT. However, constitutive AP-1 activity was not sufficient for cell transformation, since some MT mutants that are completely transformation-defective still display high AP-1 activity [Oliveira et al., 1998]. Analysis of the AP-1 complex components induced by MT revealed that cJun and JunB proteins, but not cFos or FosB proteins, are present in the AP-1 complex of MT overexpressing cell lines [Oliveira et al., 1998]. It remains to be determined whether other Fos (Fra1, Fra2) and Jun (JunD) proteins are important components of the AP-1 complex.

Here we describe the expression of a dominant-negative cJun mutant in the MT transformed cell line MTWT to analyze the role of constitutive AP-1 activity for cell transformation induced by this tumor antigen. The results show that constitutive AP-1 activity is necessary for cell transformation induced by Py MT antigen. In addition, we also show that proliferation of MT transformed cells in low serum concentrations and their ability to form colonies in agarose are controlled by distinct mechanisms.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Culture Conditions

Balb-3T3 cells (clone A31) were obtained from Dr. C. Stiles' laboratory (Dana Farber Cancer Institute, Boston, MA). The MTWT cell line was generated in Dr. T. Roberts' laboratory (Dana Farber Cancer Institute) by infection of Balb-3T3 cells (clone A31) with wild type MT cDNA using retroviral vectors [Cherington et al., 1986] and has been described elsewhere [Carmichael et al., 1984; Morgan et al., 1988; Druker and Roberts, 1991; Druker et al., 1992]. A control cell line (PLJ) was generated in the same laboratory, by infection of A31 cells with a retroviral vector expressing only the G418 resistance marker. The pCMV-67 vector, containing the dominant-negative cJun mutant (TAM67) cDNA, was constructed in Dr. M.J. Birrer's laboratory (National Cancer Institute) [Brown et al., 1993, 1994]. The p343X vector, derived from the pY3 vector [Blochlinger and Diggelmann, 1984] confers resistance to hygromicin-B upon transfection. MTJ clones were derived by co-transfection of the MTWT cell line with the pCMV-67 vector and the p343X vector. MTC clones were derived by co-transfection of the MTWT cell line with the empty pCMV vector and the p343X vector. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 25 mg/L ampicillin, and 100 mg/L streptomycin.

Plasmids used in the gene reporter assays were: (a) pSEAP-AP-1 vector (BD Biosciences Clontech, Palo Alto, CA), that contains a specific DNA binding sequence, corresponding to the AP-1 responsive element, localized upstream relative to the TATA-like promoter region from the basal promoter of the herpes simplex virus thymidine kinase (HSV-TK) linked to the *SEAP* (a secreted form of the human placental alkaline phosphatase) gene; (b) pGL3-promoter vector (Promega Corp., Madison, WI), that contains the SV40 promoter located immediately upstream relative to the luciferase reporter gene.

Growth Curves

Cells (5.0×10^4 cells) were plated onto 35 mm plates in 10% FCS–DMEM. In the following day, plates were washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2PO_4) and the medium was changed to 0.5 or 10% FCS–DMEM. Duplicate plates were collected by trypsinization at the indicated periods of times, fixed in 3.7% formaldehyde, and counted in a CELM CC-530 electronic cell counter (São Paulo, Brasil).

Growth in Agarose Suspension

Cells were plated at low cell density (250 cells/cm^2) onto solid (0.6%) agarose in 10% FCS–DMEM and rapidly overlayed with the soft (0.3%) agarose in 10% FCS–DMEM. Upon jellification, liquid medium was carefully placed over the soft agarose layer and was replaced, by aspiration, every 3 days. Colonies were observed under a phase-contrast in the Nikon Diaphot microscope after 2 weeks.

MT-Associated Kinase Assay

Confluent cultures were lysed by incubation with NP40 lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM EDTA, 1 mM PMSF, 0.03 U/ml aprotinin, and 1 mM sodium vanadate) for 15 min at 4°C. Cell lysates were normalized for MT expression and incubated with a polyclonal anti-MT antiserum for 2 h at 0°C. Protein A Agarose (Gibco-BRL, Grand Island, MD) was added to the lysates for 1 h at 4°C, under agitation. Upon washing twice with PBS, twice with 0.1 M LiCl, and twice with kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MnCl_2$), immunoprecipitates were incubated with kinase buffer containing 10 μCi [γ ^{32}P] ATP (3,000 mCi/mmol, Amersham Life Science, Buckinghamshire, NA, UK) for 20 min, at room temperature and fractionated by 10% SDS–PAGE.

Transfection and Gene Reporter Assays

Twenty-four hours before transfection, cells were plated onto 12 well plates (3.0×10^4 cells/well). Plasmid DNAs, purified using the QIAfilter plasmid midi kit (Qiagen Inc., Valencia, CA) were used for transfection with cationic lipid reagents. To detect the AP-1 element promoter activity, cells were co-transfected with 1 μg of the pSEAP-AP-1 plasmid (Clontech) and 0.25 μg of the pGL3-promoter plasmid (Promega) using LipofectAMINE PLUS™ Reagent (Life Technologies) in serum-free medium. The pGL3-promoter plasmid, that carries the luciferase gene driven by a constitutive SV40 promoter, was used as an internal control to normalize for transfection efficiency. After incubation for 3–6 h at 37°C in an atmosphere containing 5% CO_2, cells were incubated in DMEM supplemented with 10% calf serum for 48 h. Supernatant samples were collected to measure SEAP activity. Cells were harvested using passive lysis buffer (Promega) and the lysates were utilized to measure the luciferase activity. SEAP activity was analyzed using the Great EscAPe™ Chemiluminescent Detection Kit (Clontech). Luciferase activity was analyzed using the Luciferase Reporter Assay System (Promega). Light intensity emitted by the samples was measured during 10 s using a EG&G Berthold, Microplate Luminometer LB 96 V.

Statistical Analysis

Data were statistically evaluated using analysis of variance and Student's *t*-test. A P value of <0.05 was considered significant.

RESULTS

Dominant-Negative cJun Mutant Expression Leads to Morphological Alterations in the MTWT Cell Line

It has been reported that cell transformation induced by Py MT antigen leads to constitutive AP-1 activity [Rameh and Armelin, 1992; Glenn and Eckhart, 1993]. However, we previously described that AP-1 activation is not sufficient for cell transformation induced by MT, suggesting that additional events are necessary [Oliveira et al., 1998]. We have also described that Jun proteins (cJun and JunB) are present

in the constitutive AP-1 complex induced by MT [Oliveira et al., 1998]. Therefore, we set out to directly address the importance of the AP-1 complex in this system through overexpression of a dominant-negative cJun mutant (TAM67) [Brown et al., 1993, 1994] in a MT transformed cell line (MTWT).

The TAM67 mutant lacks the transactivation domain but retains the ability to form dimers with cellular Jun and Fos proteins (shown in Fig. 1). The TAM67 cDNA was cloned into the pCMV vector and named pCMV-67 [Brown et al., 1994].

The MTWT cell line was transfected with the pCMV-67 vector or with the empty pCMV vector, generating, respectively, the MTJ and MTC clones, as shown in Table I.

The MTWT cell line displays a disarrayed, transformed morphology with cells loosely attaching to the substrate and growing to high cellular densities, when compared with the PLJ cell line. Transfection of the MTWT cell line with the pCMV-67 vector generated several (MTJs) clones displaying morphological alterations. Cells became flat and started to grow in monolayer, resembling the PLJ cell line. Transfection of the MTWT cell line with the empty pCMV vector generated MTC clones that display the disarrayed, transformed morphology. The morphology of MTJ and MTC transfectant clones is shown in Figure 2.

MT Antigen Activity is not Altered by TAM67 Mutant Expression

To analyze if MT antigen is expressed and active in all cellular clones, we performed a kinase assay with the MTC and MTJ clones using the PLJ and MTWT cell lines as controls. No differences were found between the MTC and MTJ clones in the MT expression and ability to bind and phosphorylate specific cellular proteins, such as p85 subunit of PI3K (Fig. 3), suggesting that TAM67 is acting downstream of MT in the signal transduction pathway.

MTJ Clones Display low AP-1 Activity

Expression of the dominant-negative cJun mutant in the MTJ clones was analyzed by Northern-blot assays. A transcript corresponding to the expected size of TAM67 was detected in all MTJ clones but not in the MTC clones (data not shown).

To examine the effects of the TAM67 protein in the constitutive AP-1 activity, transient transfection studies were conducted with reporter constructs (pSEAP-AP-1 plasmid-Clontech and pGL3-Promoter plasmid-Promega) in MTJ clones. The pGL3-promoter was used as an internal control to normalize for transfection efficiency. SEAP activity was used as a measure of the AP-1 activity displayed by MTJ and MTC clones in comparison with PLJ and MTWT cell lines (Fig. 4). The MTWT cell line displays high AP-1 activity when compared with the PLJ cell line. This result is in agreement with the AP-1 DNA binding activity displayed by these cells [Oliveira et al., 1998]. Expression of TAM67 in MTJ clones led to about 20–60% inhibition of the AP-1 activity, when compared to the MTWT cell line. On the other hand, MTC clones display high AP-1 activity, with levels comparable to those displayed by the MTWT cell line. Statistical analysis shows that the results obtained for these two groups of clones (MTJs and MTCs) are significantly different, with $P < 0.05$, thus confirming the inhibition of constitutive AP-1 activity in MTJ clones.

AP-1 Activity is Necessary for the Cells Ability to Grow in Agarose Suspension

Normal cell growth in vitro is dependent on the addition of serum to the culture medium. Cell proliferation was analyzed through growth curves carried out in medium containing high (10%) and low (0.5%) serum (FCS) concentration. Figure 5 shows MTJ and MTC clones growth curves in comparison to those of the PLJ and MTWT cell lines. As expected, the PLJ cell line was incapable of growing in medium containing low serum concentration. On the other hand, MT expression (in the MTWT cell line) confers the ability to grow in low serum concentration, a hallmark of transformed cell lines.

The MTC clones were able to grow in medium containing low serum concentration, displaying growth curves similar to that of the MTWT cell line. On the other hand, the MTJ clones displayed a lower ability to grow under these conditions. Nevertheless, it is important to note that all MTJ and MTC clones analyzed were capable of growing in medium containing low serum concentration, showing a distinctly different behavior, when compared to the control PLJ cell line. However, the degree of inhibition of AP1 activity in the MTJ clones appears to directly correlate with their ability to grow in medium containing low serum concentration.

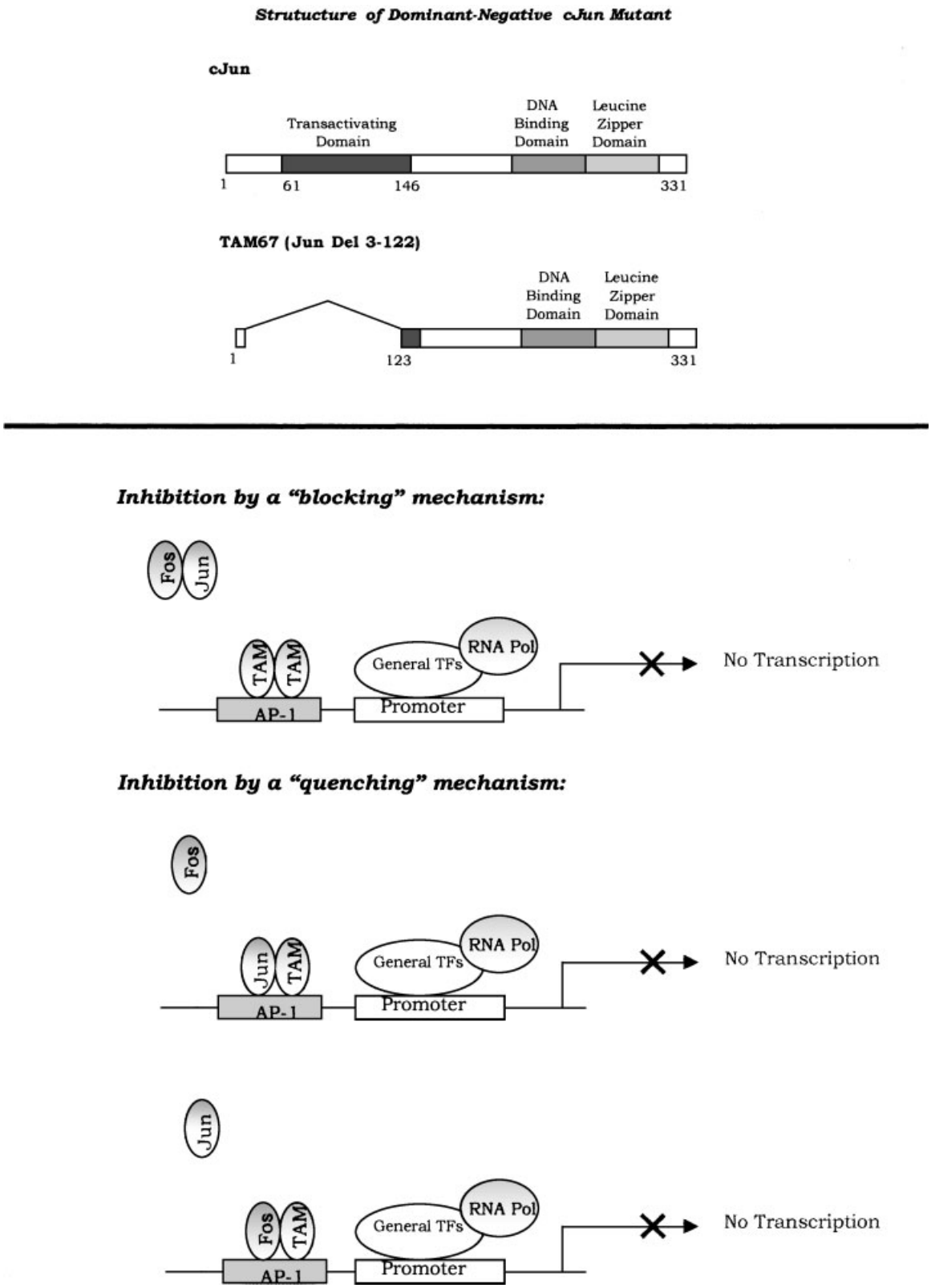

Fig. 1. Hypothetical mechanisms through which the TAM67 dominant-negative inhibitor may function. Jun and Fos represent any member of the Jun and Fos family of transcription factors which could interact with TAM67, while TF represents proteins of the general transcription apparatus (adapted from Brown et al., 1994).

TABLE I. Description of Cell Lines

Cell line	Description
A31	Parental cell line (Balb/c 3T3)
MTWT	Parental cell line infected with the PLJ-MT retroviral vector containing the middle T cDNA
PLJ	Parental cell line infected with the empty PLJ retroviral vector
MTJ	MTWT cell clone transfected with the pCMV-67 dominant-negative vector
MTC	MTWT cell clone transfected with the empty pCMV vector

Thus, both MTJ4 and MTJ16 clones display the highest (~60%) inhibition of AP1 activity (Fig. 4) and the lowest ability to grow in medium containing low serum concentration (Fig. 5).

High density-dependent growth inhibition is characteristic of normal cells. Table II shows the saturation densities and the doubling times obtained for MTJ and MTC clones, in comparison with the PLJ and MTWT cell lines, in medium containing high (10%) and low (0.5%) serum concentration.

Statistical analysis of saturation density and the doubling time data obtained for MTJ and MTC clones in medium containing high serum concentration, showed that the results are not significantly different. Using medium containing low serum concentration, it was possible to analyze the involvement of the AP-1 activity in the proliferation ability induced by Py MT antigen. Under this condition, the MTJ clones displayed similar doubling time when compared with MTC clones, but showed significantly lower saturation densities ($P < 0.05$), suggesting the involvement of the AP-1 transcriptional complex in MT-transformed cell proliferation.

It is important to note that none of the MTJ clones displayed growth characteristics similar to the normal control (PLJ) cell line, keeping some features of transformed cell lines, like, for instance, the ability to grow in low serum medium, reaching high saturation density in medium containing high serum concentration. These results suggest that yet unidentified factors could be involved in this process.

A common feature of transformed cells is the ability to grow in semi-solid substrates. This ability can be assessed by plating cells in agarose suspension, avoiding adherence to the substrate. Figure 6 shows the ability of MTJ and MTC clones to form colonies in agarose suspension, in comparison to PLJ and MTWT cell lines, after 2 weeks of culture. We observed that MTWT cells and MTC clones were able to grow in agarose suspension, forming several large colonies. The MTJ clones displayed greatly reduced ability to grow in agarose suspension, forming a small number of colonies that contained a small number of cells. Thus, the MTJ clones, displayed a dramatic reduction in their ability to grow in semi solid substrate, when compared to MTC clones and MTWT cell line, pointing to the involvement of the AP-1 transcriptional complex in the ability of MT-transformed cell lines to form colonies in semi-solid medium.

DISCUSSION

We have previously described that cell transformation mediated by Py MT antigen leads to constitutive AP-1 activity [Rameh and Armelin, 1992; Oliveira et al., 1998], which is dependent on MT-binding to PI3K [Oliveira et al., 1998]. However, activation of this transcriptional complex is not sufficient to convert cells to the malignant phenotype, suggesting the existence of other yet unidentified molecular events in the process [Oliveira et al., 1998].

The requirement for AP-1 activation in MT-mediated cell transformation was analyzed, in the present study, through inhibition of AP-1 activity in an MT transformed cell line (MTWT), using a dominant-negative cJun mutant.

Blocking of the constitutive AP-1 activity in the MTWT cells, by overexpression of the dominant-negative TAM-67 cJun mutant, led to morphological changes, without interfering with MT antigen's activity. MTJ clones isolated formed perfect monolayers in solid substrate and displayed a flat morphology, resembling the control PLJ cell line.

Downregulation of c-Jun activity (by using the TAM67 dominant-negative mutant) leads to cell growth inhibition in other systems, such as the NIH3T3 cell line transformed by different oncogenes [Rapp et al., 1994]. However this effect was not observed when we transfected the ST1 cell line (derived from C6 glioma cell line) with the TAM67 mutant (data not shown), indicating that TAM67-mediated regulation of cell growth is not a general effect.

MTJ clones displayed a reduction in the ability to grow in solid substrate in medium containing low serum concentration. The saturation densities reached by these cells were significantly lower ($P < 0.05$) when compared to control MTC clones. However, statistical

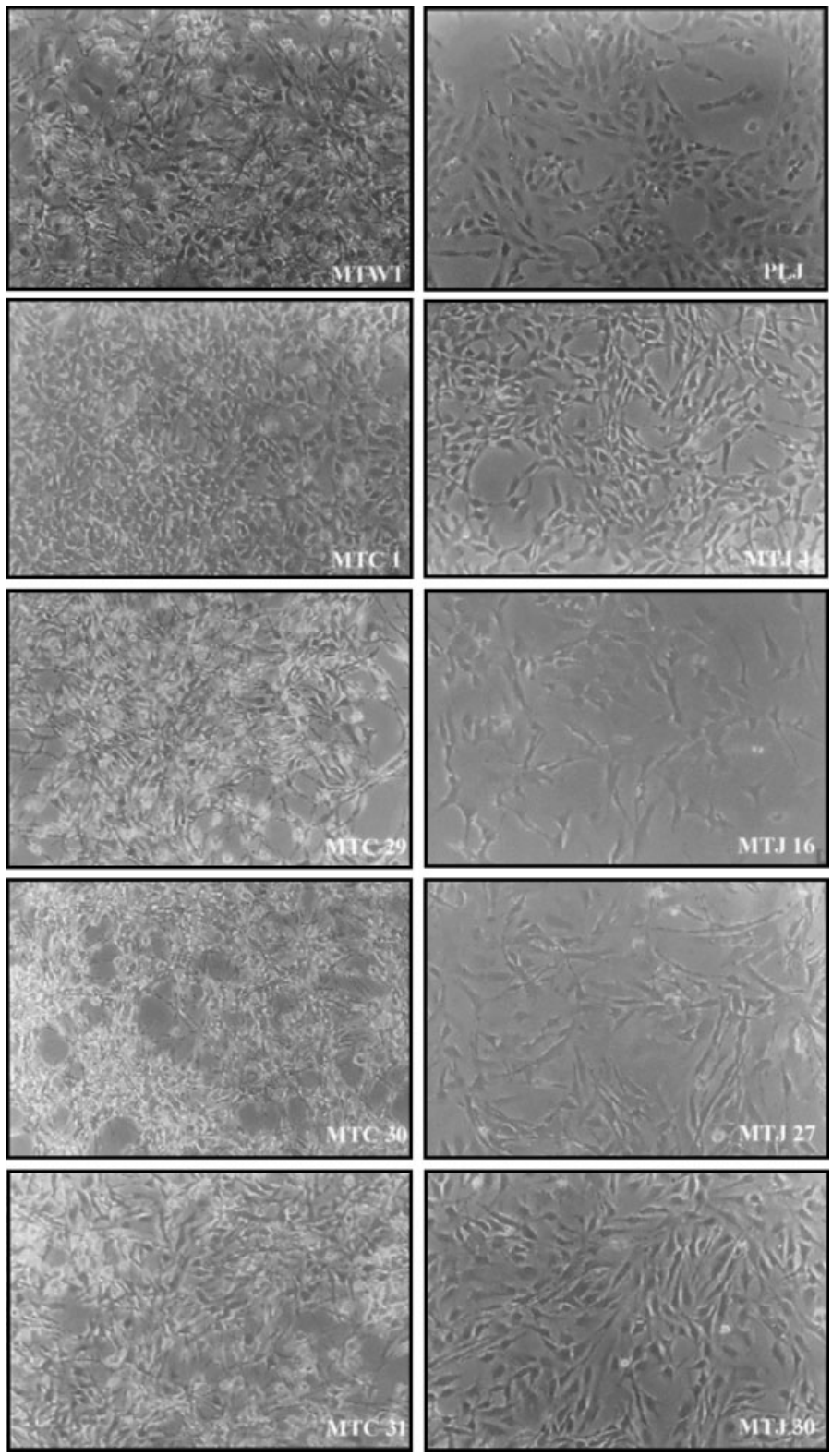

Fig. 2. Cell morphology. Transfection of the MTWT cell line with the dominant-negative cJun cDNA (TAM67) leads to changes in morphology. Cells become flat and grow to form a monolayer. However, transfection of the MTWT cell line with the pCMV vector does not cause changes in morphology, i.e., cells continue to display a disarrayed, transformed morphology. Phase-contrast photomicrographs were taken under a Nikon Diaphot microscope (100×).

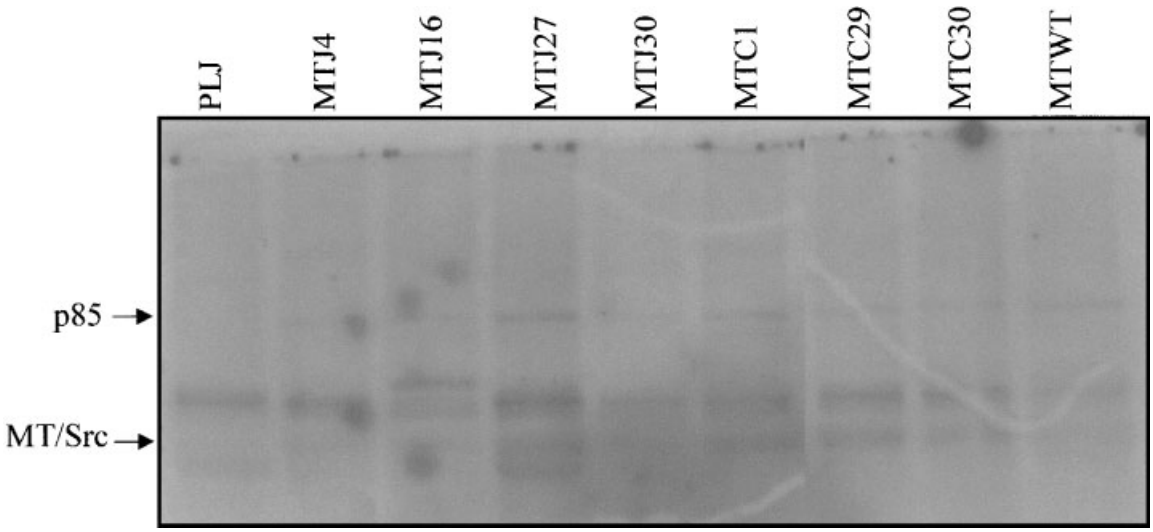

Fig. 3. MT-associated kinase assay. Cellular lysates obtained from PLJ, MTWT, MTJ, and MTC clones were collected and subjected to immunoprecipitation assays using specific polyclonal anti-MT. Kinase assays were performed using ^{32}P-γ-ATP and phosphorylated proteins were fractionated by SDS–PAGE electrophoresis. No difference was found between the MTC and MTJ clones in the MT-ability to bind and phosphorylate specific cellular proteins, such as p85 subunit of PI3K.

analysis of the saturation density and doubling time of MTJ clones in medium containing high serum concentration showed that the expression of the TAM67 mutant did not completely block their ability to proliferate in solid substrate and to grow to high cellular density. These results suggest not only the involvement of the AP-1 transcriptional complex in cell proliferation of MT-transformed cell line but also that other yet unidentified important factors could be involved in this process. Nevertheless, we cannot discard the possibility that residual AP-1 activity of the MTJ clones (in comparison with PLJ cell line) could be responsible for some of the transforming functions that still remain in these cellular clones.

It has been reported that cJun is required for progression through the G1 phase of fibroblasts cell cycle, by mechanisms that involve transcriptional control of the cyclin D1 gene, through AP-1 sites present in these gene promoter [Wisdom et al., 1999]. Involvement of the

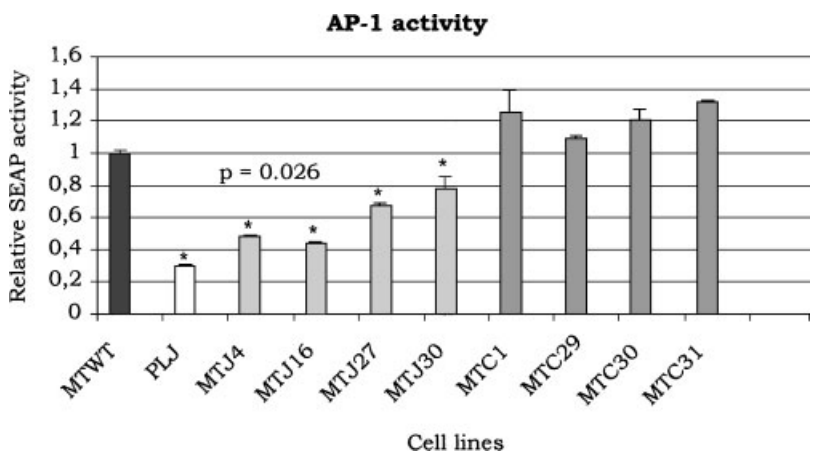

Fig. 4. Detection of AP-1 activity in the MTJ and MTC clones. The SEAP reporter construct was co-transfected together with the pGL3-SV40 reference plasmid, into MTWT, PLJ, MTC, and MTJ clones, and the cell lysates were subjected to the SEAP assays. Student's *t* test was performed between the two groups of clones (MTJ and MTC) in comparison with the parental cells (PLJ and MTWT), and *P* value are presented. Bars represent means ± SD from three independent experiments.

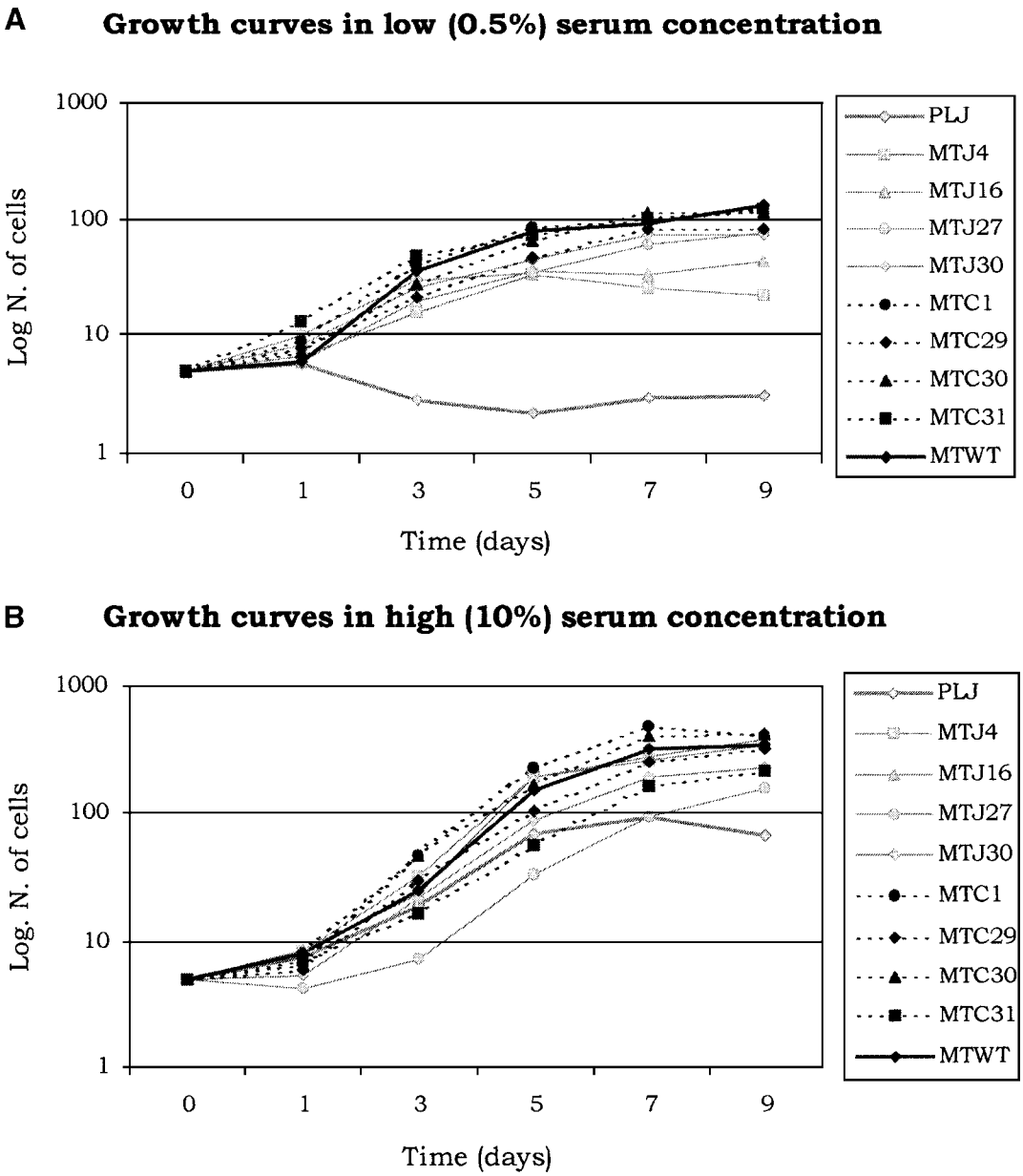

Fig. 5. Growth curves. Expression of the dominant-negative cJun mutant (TAM67) in MTJ clones does not block their ability to grow in monolayer, in low or high serum concentration medium. Cells (5.0×10^4) were plated in 35 mm dishes. Triplicates were collected every 2 days and counted using an electronic counter (CELM). **A**: Growth curve using low (0.5%) serum concentration DMEM medium. **B**: Growth curve using high (10%) serum concentration DMEM medium.

AP-1 complex in the transcriptional activation of the cyclin D1 gene has already been described in other systems [Watanabe et al., 1996; Albanese et al., 1999], establishing a molecular link between growth factor signaling and cell cycle regulators.

In addition, we showed that MTJ clones display a reduced ability to grow in semi-solid medium, forming a few, small sized colonies in agarose suspension, when compared to the MTC clones and to the fully transformed MTWT cell line. It is important to point out that the ability to grow in semi-solid substrate, a hallmark of transformed cells, is the best correlate with tumorigenic potential.

This observation suggests that the AP-1 transcriptional complex could be responsible for modulating the expression of different

TABLE II. Characteristics of the MTJs and MTCs Clones in Low (0.5%) and High (10%) Serum Containing Media

	Doubling time (h)[a]		Saturation density[b] (number of cells $\times 10^5$/cm^2)	
Cell lines	0.5% FCS	10% FCS	0.5% FCS	10% FCS
PLJ	—	24	0.034	0.7
MTJ4	52.1	16	0.24	3.4
MTJ16	29.3	12	0.49	3.8
MTJ27	32.0	21	0.83	1.6
MTJ30	30.6	24	0.80	2.4
MTC1	21.4	20	1.3	4.1
MTC29	35.2	27	0.9	3.3
MTC30	25.0	26	1.3	4.3
MTC31	25.5	29	1.4	2.2
MTWT	16.0	16	1.5	3.5

[a]Measured between the first and the third days.
[b]Average number of cells per cm^2.

extracellular matrix components, contributing to the remodeling of the extracellular matrix during neoplastic progression. Analysis of the 5′-flanking region of some genes such as fibronectin [Matsubara et al., 2000; Tamura et al., 2000], SPARC [Vial et al., 2000], cytokeratin 19 [Choi et al., 2000], matrix metalloproteinase-13 [Solis-Herrugo et al., 1999], Ecm1 [Smits et al., 1999] has revealed the presence of AP-1 binding sites in the promoter regions of these genes, suggesting a possible role of the AP-1 transcriptional complex as an important regulator of their expression.

Our results demonstrate the need for constitutive AP-1 activity during Py MT-cell transformation, since blocking of the constitutive AP-1 activity, by a dominant-negative cJun mutant, promoted selection for cellular clones that display a flat morphology, grow as monolayers, and are unable to grow in agarose suspension. They also suggest that during cell transformation induced by the Py MT antigen, distinct mechanisms control on one hand, cells ability to grow in solid substrate in the presence of low serum concentration and, on the other hand, the ability to form colonies in agarose suspension.

The AP-1 transcriptional factor plays an important role in the origin of malignancy in several cellular systems. Activation of Erk1 and/or Erk2 promotes the transactivation of AP-1, which is required for neoplastic transformation of Balb/C JB6 cells [Watts et al., 1998]. Members of the Jun family, specially cJun, have also been described as targets for cell transformation. Recently, the involvement of Jun family members, specially cJun and JunB, has been described in human colorectal adenocarcinomas, since their expression levels were significantly increased in this tumoral tissue [Wang et al., 2000]. Involvement of the AP-1 transcriptional complex and of cJun proteins in progression of mouse skin carcinogenesis has also been suggested, since phosphorylated cJun and JNK kinase activity could be associated with tumor progression and highly malignant carcinoma [Zoumpourlis et al., 2000]. In addition, blocking of AP-1 activity with the TAM67 dominant-negative mutant, inhibits breast cancer growth both in vitro and in vivo by suppressing multiple signal transduction pathways [Liu et al., 2002]. Study of the mechanisms underlying AP-1 activation, as well as identification of AP-1 cellular targets could provide important clues for the molecular basis of cell growth control and tumorigenesis.

Recent studies suggest association of oncogenic viruses, including Py, with various types of human cancer [Testa et al., 1998; Krynska et al., 1999]. Expression of JCV human neurotropic Py T-antigen was demonstrated in various human brain tumors [Del Valle et al., 2001a]. Associations between JC virus T antigen and p53 and Rb tumor suppressor genes were observed in human medulloblastomas, suggesting that the JC virus might be involved in the formation and progression of this kind of tumor [Del Valle et al., 2001b]. The results presented here, showing requirement for AP-1 transcriptional complex activation for Py MT antigen-mediated cell transformation, suggest that suppression of constitutive AP-1 activity, by a dominant-negative c-*jun* construct, could be used as a therapeutic strategy in some human cancers.

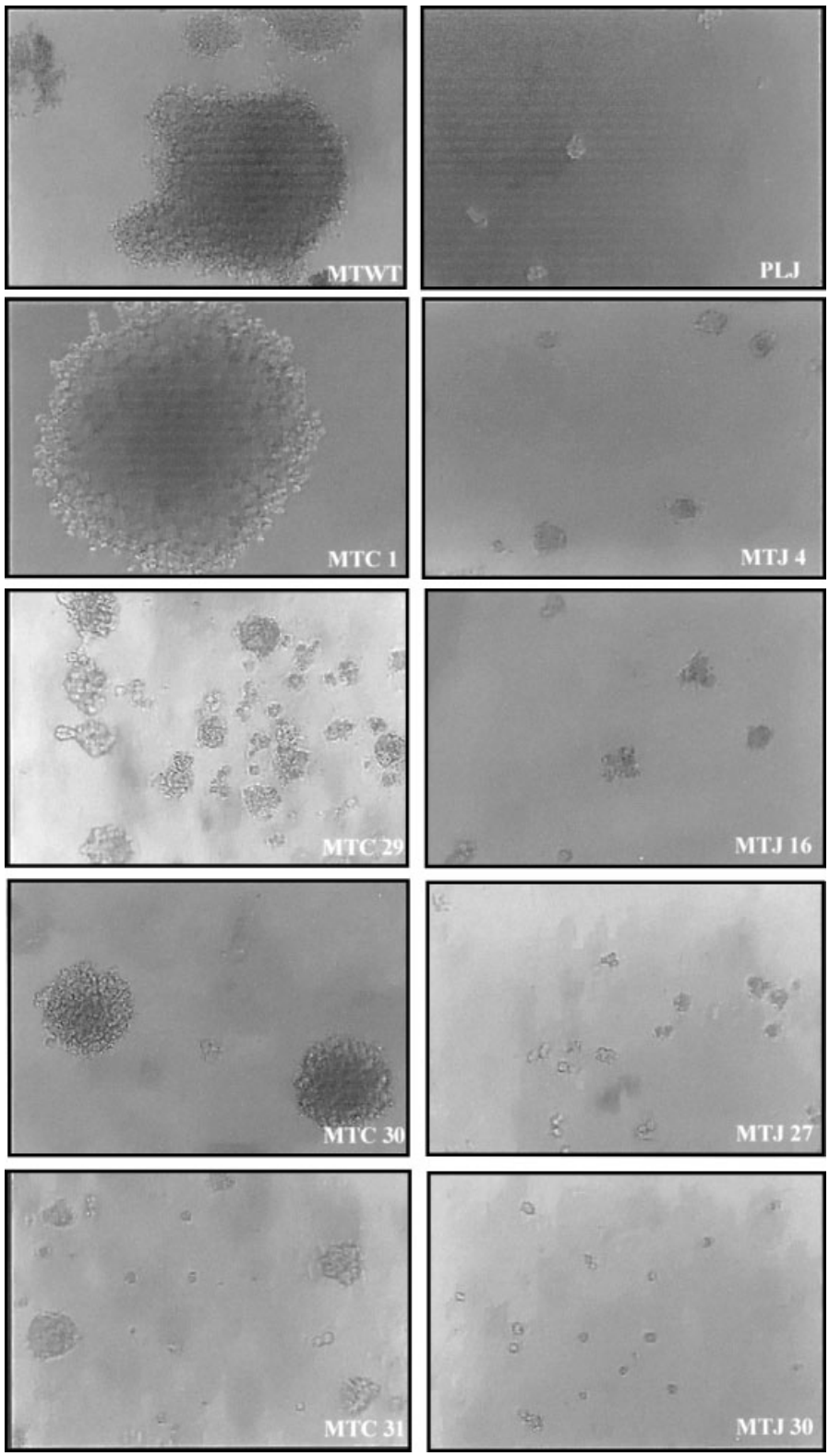

Fig. 6. Growth in agarose suspension. Cells (10^3) were plated onto semi-solid 0.6% agarose–DMEM–10% FCS and overlaid with 0.3% agarose–DMEM–10% FCS. Transfection of the MTWT cell line with the empty pCMV vector had no effect in the cells ability to form colonies in agarose suspension. On the other hand, transfection of the MTWT cell line with the dominant-negative cJun cDNA (TAM67) dramatically decreases the cells ability to form colonies in agarose suspension. Phase-contrast photomicrographs were taken under a Nikon Diaphot microscope (100×).

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