CELL STRUCTURE AND FUNCTION 28: 195–204 (2003)


# c-*myc* Induces Autophagy in Rat 3Y1 Fibroblast Cells

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*ABSTRACT.* **The proto-oncogene c-*myc* is a multifunctional gene that regulates cell division, cell growth, and apoptosis. Here we report a new function of c-*myc*: induction of autophagy. Autophagy is a bulk degradation system for intracellular proteins. Autophagy proceeds with characteristic morphologies, which begins with the formation of a double-membrane structure called the autophagosome surrounding a portion of the cytoplasm, after which its outer membrane then fuses with the lysosomal membrane to become an autolysosome. Autophagosomes and autolysosomes are generally called autophagic vacuoles. When c-Myc protein was overexpressed in rat 3Y1 fibroblasts or when the chimeric protein c-MycER was activated by estrogen, the number of autophagic vacuoles in cells increased significantly. The formation of autophagic vacuoles induced by c-Myc was completely blocked by a specific inhibitor of autophagosome formation, 3-methyladenine. A c-Myc mutant lacking Myc Box II induced neither apoptosis nor oncogenic transformation, but still stimulated autophagy. An inhibitor of caspases suppressed apoptosis but not autophagy. These results suggest that the autophagy caused by c-*myc* is not due to the apoptosis or tumorigenesis induced by c-*myc*. Taken together, our results suggest that the induction of autophagy is a novel function of c-*myc*.**

**Key words:** autophagy/apoptosis/transformation/*myc*/mutant/rat fibroblast

When animal cells are exposed to stress, such as serum starvation, they initiate specific reactions including autophagy (Mortimore and Poso, 1987). Autophagy was observed in eukaryotes from yeast to mammals, and begins with the formation of a double-membrane structure called the autophagosome surrounding a portion of the cytoplasm (Blommaart *et al.*, 1997; Klionsky and Ohsumi, 1999). Its outer membrane then fuses with the lysosomal membrane, and it becomes an autolysosome. Autophagosomes and autolysosomes are generally called autophagic vacuoles (Seglen *et al.*, 1996). The contents of autolysosomes are subsequently digested by lysosomal enzymes. Therefore, autophagy had been suggested to be responsible for degradation of intracellular bulk proteins. Induction of autophagy had been also suggested to lead to the cell death characterized as type II programmed cell death in some cells (Kitanaka and Kuchino, 1999). Several genes involved in autophagy have been cloned (Klionsky and Emr, 2000; Ohsumi, 2001), and the basic machinery of autophagy has begun to be unraveled. However, the mechanism for induction of autophagy is still not clear.

In response to stress, animal cells also initiate another response, apoptosis, which ends in cell death due to an ordered cascade of enzymatic events (Fraser and Evan,

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Abbreviations: c-MycER, a chimeric protein consisting of c-Myc and the estrogen binding portion of an estrogen receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; 3MA, 3-methyladenine.

1996; Nagata, 1997). Apoptosis has been well studied, and several classes of regulatory proteins for apoptosis have been identified. Because both apoptosis and autophagy are responses to stress and have often been observed in cells simultaneously (Sandvig and Deurs, 1992; Anglade *et al.*, 1997; Xue *et al.*, 1999), autophagy may be induced by the same molecules responsible for the induction of apoptosis.

The *myc* family of proto-oncogenes consists mainly of three well-characterized members; c-*myc*, N-*myc*, and L-*myc*. While the three *myc* genes exhibit distinct patterns of expression with respect to cell types and developmental stages, they can substitute for each other in certain situations and appear to have basically the same biological activities (Henriksson and Lüscher, 1996; Malynn *et al.*, 2000). Deregulated expression of *myc* family genes, through gene amplification, viral promoter insertion, chromosomal translocation, or promoter mutation, has long been known to be associated with neoplastic diseases in a wide range of vertebrates including humans (Henriksson and Lüscher, 1996; Evan and Littlewood, 1998). Myc protein contains two conserved regions, Myc Box I and Myc Box II, which are highly conserved between c-Myc, N-Myc, and L-Myc as well as between species, suggesting that the two Myc Boxes are functionally important (Grandori *et al.*, 2000; Claassen and Hann, 1999).

Here we report that c-*myc* induces autophagy in rat fibroblast cells. Studies using a c-Myc mutation of the Myc Boxes and an inhibitor of apoptosis suggest that this newly discovered function is a result of neither the apoptosis nor tumorigenesis induced by c-*myc*.

## Materials and Methods

### Plasmids

The expression vector for c-*myc*, Pc-myc/CDM8, was described previously (Tsuneoka *et al.*, 1997). Vectors for expression of c-Myc (CMV-*myc*), c-Myc with the Myc Box I deleted (Δ46-63) (CMV-dMBI) and c-Myc with the Myc Box II deleted (Δ128-144) (CMV-dMBII) (Philipp *et al.*, 1994) were kind gifts from Dr. M. Eilers. The v-*ras* expression vector was p4E, a Ki-MSV provirus clone, in which v-Ki-*ras* was driven by the Ki-MSV LTR promoter (Tsuchida *et al.*, 1982).

### Cells and cell culture

Rat 3Y1 fibroblasts (Kimura *et al.*, 1975; Yamashita *et al.*, 1980) and their derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (10FBS-DMEM). ME-7 is a cell line that expresses c-MycER chimeric protein from 3Y1 cells (Tsuneoka *et al.*, 1997). The Myc activity in the chimeric protein is stimulated by adding estrogen to the medium. Although the estrogen derivative 4-hydroxy-tamoxifen (OHT) also activates MycER (Solomon *et al.*, 1995), OHT itself induced autophagy in rat 3Y1 fibroblasts (data not shown), as reported previously in human mammary carcinoma cell line MCF-7 cells (Bursch *et al.*, 2000). Therefore, we used estrogen instead of OHT to activate c-MycER protein in this study. To establish 3Y1 derivatives expressing c-Myc or various types of mutant c-Myc, 3Y1 cells were transfected with 10 μg of a vector for expressing c-*myc* or various types of mutant c-*myc* and 0.2 μg of pActHyg, which confers hygromycin resistance, and grown in the presence of 100 μg/ml of hygromycin. DNA transfection was performed by the calcium-phosphate procedure described previously (Chen and Okayama, 1988). Colonies were picked up and regrown to establish a cell line. The expression of c-Myc was detected by Western blotting. Cell extracts were separated on 4–20% polyacrylamide gel and transferred to a polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA). After treatment with rabbit anti-c-Myc antibody (Tsuneoka *et al.*, 1997), bands were detected using an enhanced chemiluminescence technique (Amersham Biosciences, Piscataway, NJ).

### Enumeration of autophagic vacuoles by electron microscope

Cells were collected after treatment with trypsin and EDTA at 37°C for 5 min and centrifugation at 1000×g at 4°C. The cells were then fixed in 2.5% glutaraldehyde in 0.1 M Na-phosphate buffer, pH 7.4, at 4°C overnight and washed with the buffer three times. The pellet of cells was post-fixed in 1% $OsO_4$ in 0.1 M cacodylate buffer, pH 7.4, for 1 hour, washed with distilled water, dehydrated with a graded ethanol series, and embedded in epoxy resin. Ultrathin sections were doubly stained with uranyl acetate and lead citrate. Autophagosomes (double membrane structures sequestering intact cytoplasm) and autolysosomes (single membrane structures containing partially degraded cytoplasm) were identified from their morphology using a Hitachi H-7000 electron microscope. The numbers of autophagic vacuoles (autophagosomes+autolysosomes)/cell profile (30 cells) were counted. Tests of significance (t-test) were performed to confirm increment of autophagic vacuoles by Myc in some cases.

### Assay for cell transformation

The ability of DNAs to induce oncogenic transformation was measured as follows: 3Y1 cells in the exponentially growing state were re-plated on culture dishes ($5\times10^4$ cells/21 $cm^2$). The next day, cells were transfected with 2 μg of v-Ki-*ras* expression vector and 2 μg of vector expressing various types of mutant c-*myc* by the calcium-phosphate procedure. One day after transfection, cells were re-plated on cell-culturing dishes (55 $cm^2$) and cultured for 2 weeks. The cells were then washed once with phosphate-buffered saline (PBS), fixed with methanol for one hour, and stained with Giemsa's solution for a few hours. After rinsing cells with water, stained foci that indicate transformed cells were counted by eye.

### Other materials

3-methyladenine (3MA) was purchased from Sigma-Aldrich, St. Louis, MO. Estrogen (1,3,5[10]-estratriene-3, 17β-diol) was pur-

chased from Sigma, and dissolved in ethanol. A peptide inhibitor of caspases, zVAD-fms, was purchased from Calbiochem, La Jolla, CA, and dissolved in DMSO. Giemsa's solution was purchased from Merck, Darmstadt, Germany, and diluted with PBS finally to 5% (volume/volume) before use.

## *Results*

### *Overexpression of c-***myc** *stimulates autophagy*

Rat fibroblast 3Y1 is a cloned cell line that had been established from a Fisher 344 rat embryo according to the transfer schedule for murine 3T3 cells, and retains *in vivo* characteristics, including regulated growth and a diploid karyotype (Kimura *et al.*, 1975; Yamashita *et al.*, 1980). To permit conditional activation of c-Myc in 3Y1 cells by addition of estrogen, we isolated an ME-7 cell line that expresses c-MycER (Tsuneoka *et al.*, 1997). c-MycER is a chimeric protein consisting of c-Myc and the estrogen-binding portion of an estrogen receptor. c-MycER anchors to the cytoskeletal components in the absence of estrogen. When estrogen binds to the chimeric protein, it becomes free to function as c-Myc (Eilers *et al.*, 1989).

Deprivation of serum is an accepted condition for the initiation of autophagy in mammalian cells (Mortimore and Poso, 1987). When ME-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (10FBS-DMEM), where they grow well, autophagic morphology was hardly detected (Fig. 1A). When ME-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 0.5% fetal bovine serum (0.5FBS-DMEM) for 2.5 hours, we detected double- and single-membrane vacuolar structures containing visible cytoplasmic contents (Fig. 1B), which is a hallmark of autophagosomes and autolysosomes, while the nucleus was well preserved. These results confirm that the cell line used here is capable of formation of autophagic vacuoles.

When ME-7 cells were maintained in 10FBS-DMEM in the presence of estrogen to activate c-MycER, autophagic vacuoles formed (Fig. 1C). The quantitative studies suggest that Myc activation quadrupled the number of autophagic vacuoles (Fig. 2A). When ME-7 cells were cultured in 0.5FBS-DMEM in the presence of estrogen for 2.5 hours, the number of autophagic vacuoles was increased by 60% compared to that in ME-7 cells maintained in 0.5FBS-DMEM without estrogen (Fig. 1D and Fig. 2A). Thus, the induction of autophagic vacuole formation by Myc was clearly observed in 10FBS-DMEM when c-Myc was overexpressed. Estrogen had no effect on the number of autophagic vacuoles in parent 3Y1 cells (Fig. 2A, control cells).

3-Methyladenine (3MA) is a specific inhibitor of autophagosome formation (Seglen and Gordon, 1982). When ME-7 cells were cultured with 3MA, autophagic vacuole formation induced by Myc activation and/or serum deprivation was almost completely inhibited (Fig. 2B), indicating that the vacuoles observed here are autophagic vacuoles.

There are differences in number of autophagic vacuoles between Fig. 2A and B even in the same experimental conditions. For example, the number in ME-7 cells without estrogen in 0.5% serum is about 7 in the experiment A, whereas it is about 3.5 in the experiment B. Deprivation of serum induced autophagy, suggesting that serum factor(s) are involved in induction of autophagy. Since different lots or ages of serum may contain different amounts of serum factors, the distinct series of experiments can result in different number of autophagic vacuoles. Although we observed different number of autophagic vacuoles in Fig. 2A and B, there are similar induction rates by Myc activation, that is, in the both experiments ME-7 cells with Myc activation contained about 4 and 1.5 times as many autophagic vacuoles as cells without Myc activation did in 10FBS-DMEM and 0.5FBS-DMEM, respectively.

To confirm that c-Myc stimulates autophagy, we introduced the c-*myc* expression vector into 3Y1 cells and isolated two cell lines with constitutive expression of c-Myc (Myc-1 and Myc-2 cells). The expression of c-Myc was confirmed by Western blotting in both Myc-1 and Myc-2 cells (Fig. 3A), and the number of autophagic vacuoles was counted in these cells. As shown in Fig. 3B, although Myc-1 and Myc-2 cells in 0.5FBS-DMEM showed similar levels of autophagic vacuoles to those of control cells, Myc-1 and Myc-2 cells in 10FBS-DMEM had approximately 2.5 times the number of autophagic vacuoles that control cells did. These results show that c-*myc* stimulates autophagy even in 10FBS-DMEM cell-growing conditions.

### *Induction of autophagy by c-***myc** *does not require Myc box II*

There are two conserved regions in *myc* family genes, Myc Box I and Myc Box II. 3Y1 cells were transfected with plasmids that express wild type c-Myc, mutant c-Myc lacking Myc Box I (MycdMBI), or mutant c-Myc lacking Myc Box II (MycdMBII) to isolate cell lines Myc-3, dMBI-1, or dMBII-1, respectively. Comparable levels of c-Myc expression were confirmed by Western blotting (Fig. 4A, upper panel). In 10FBS-DMEM, cells expressing wild type c-Myc (Myc-3) contained more autophagic vacuoles than control cells, which is consistent with the results described above. dMBI-1 cells and dMBII-1 cells cultured in 10FBS-DMEM also contained 3 times as many autophagic vacuoles as control cells did (Fig. 4A, middle panel). A mutant Myc lacking Myc Box II was reported to lack many activities of Myc including oncogenic transformation and apoptosis (Evan *et al.*, 1992). Therefore, to confirm further that the mutant Myc lacking Myc Box II induces autophagy, we isolated another cell line (dMBII-2 cells) that expresses MycdMBII (Fig. 4B). In 10FBS-DMEM, dMBII-2 cells also showed 3 times the number of autophagic vacuoles that control cells did (Fig. 4B). The results suggest that the stimulation of

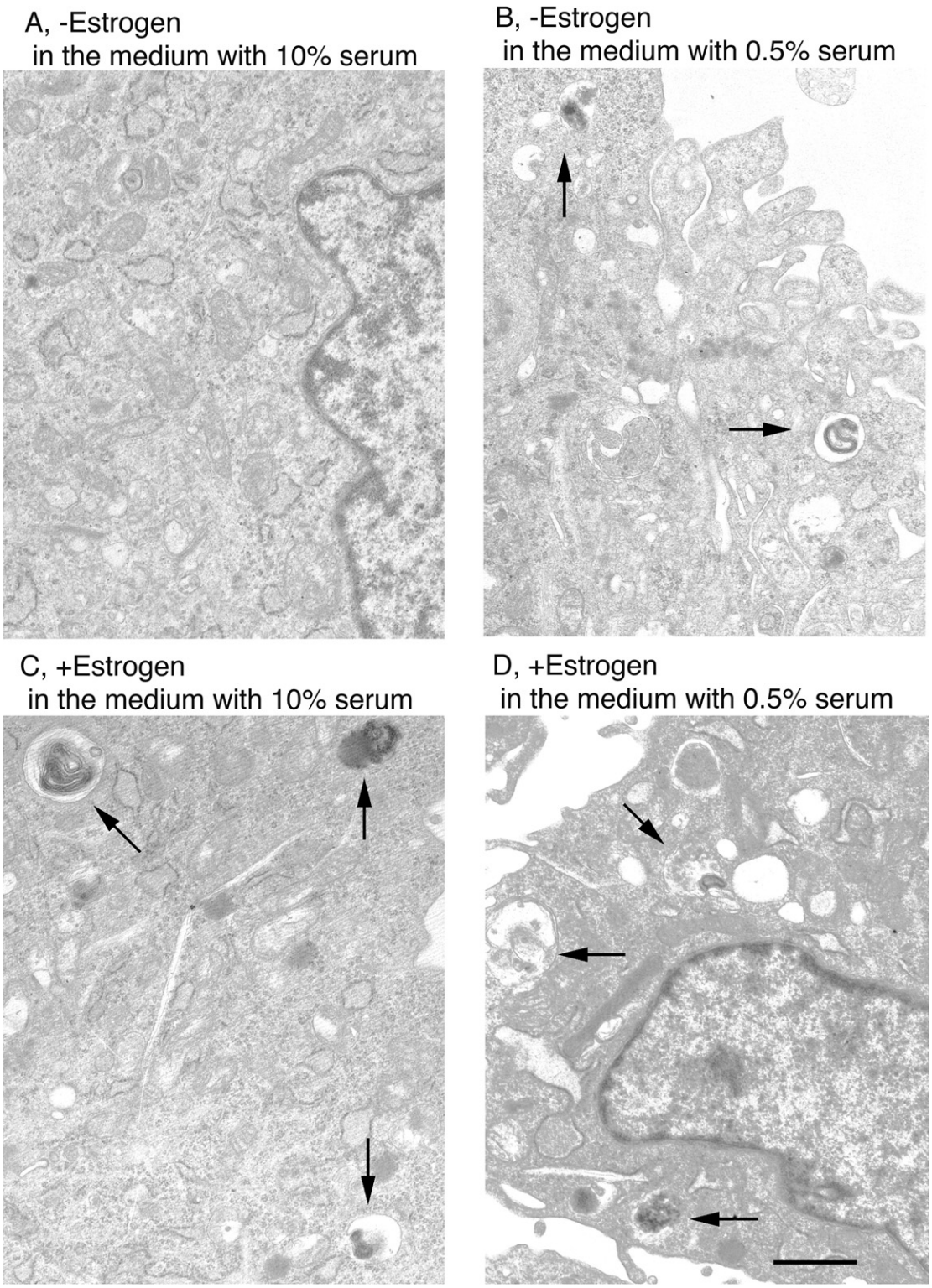


**Fig. 1.** Induction of autophagy by activation of c-MycER in ME-7 cells. ME-7 is a cell line expressing the MycER chimeric protein whose Myc is activated by estrogen. ME-7 cells were pre-cultured in the presence of 10% serum without (A and B) or with (C and D) estrogen for 3 days and further cultured for 2.5 hours in the presence of 10% (A and C) or 0.5% (B and D) serum with the same conditions for c-MycER activation as those in the pre-culture. Cells were collected, fixed and observed using a Hitachi H-7000 electron microscope. The bar indicates 1 μm. Arrows indicate autophagic vacuoles.

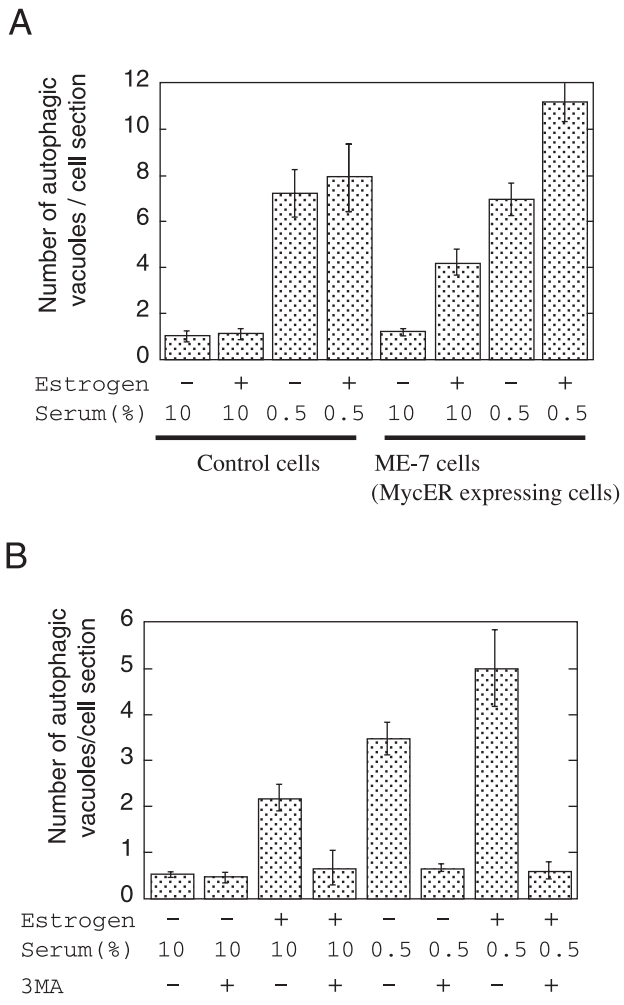


**Fig. 2.** Quantitation of vacuole formation in 3Y1 (control) and ME-7 cells. (A) Induction of autophagy by activation of c-MycER. ME-7 cells expressing c-MycER and control 3Y1 cells carrying the empty vector were cultured as described in Fig. 1. Cells were fixed, and autophagic vacuoles were counted under an electron microscope. Bars indicate standard deviations of 3 experiments. (B) Suppression of Myc-induced autophagic vacuole formation by 3-methyladenine (3MA), a specific inhibitor of autophagy. ME-7 cells expressing c-MycER were pre-cultured in the presence of 10% serum, with or without activation of MycER by estrogen, and further cultured in the presence of 10% or 0.5% serum, with or without 20 mM 3MA, for 2.5 hours with the same conditions for MycER activation as those of the pre-culture. Cells were fixed, and autophagic vacuoles were counted under an electron microscope. Bars indicate standard deviations of 3 experiments.

autophagy by Myc does not require Myc Box II.

It is well known that Myc induces apoptosis (Evan and Littlewood, 1998). When Myc-3 cells overexpressing c-Myc were cultured in 0.5FBS-DMEM for 15.5 hours, cells became detached from the culture dish and began floating (Fig. 4A, lower panel). DNA laddering, a hallmark of apoptosis (Fraser and Evan, 1996; Nagata, 1997), was detected in floating Myc-3 cells (data not shown), suggesting that apoptosis was induced in at least some of the floating cells and that the number of detached cells could be used as an indicator of the number of apoptotic cells. dMBI-1 cells were detached from the dish at a level comparable to that of wild type c-Myc (Myc-3) in 0.5FBS-DMEM. However, dMBII-1 cells did not detach from the dish in 0.5FBS-DMEM. These results suggest that the Myc Box II, but not the Myc Box I, region is required for induction of apoptosis in serum-deprived conditions, which is consistent with previous reports (Evan *et al.*, 1992; Prendergast, 1999). Since the Myc Box II region is not necessary for induction of autophagy, the region of c-Myc necessary to induce autophagy does not completely overlap with the region necessary to induce apoptosis.

We also measured the ability of these Myc mutants together with activated *ras* to transform cells. As shown in Table I, transfection with a mammalian expression vector containing the wild type c-*myc* or the mutant c-*myc* lacking Myc Box I transformed 3Y1 cells efficiently. However, the mutant Myc lacking Myc Box II did not transform 3Y1 cells, which is consistent with reports by others (Evan *et al.*, 1992). These results suggest that the region of c-Myc necessary to stimulate autophagy does not completely overlap with the region of c-Myc necessary to transform cells.

### *Induction of autophagy is not due to apoptosis induced by c-myc*

Next we investigated whether an inhibitor of caspases, the enzymes necessary for the execution of apoptosis, affects autophagy. When Myc-1 cells with overexpressed c-Myc were cultured in 0.5FBS-DMEM for 15.5 hours, cells became detached from the culture dish and began floating in the medium (Fig. 5A), suggesting that c-Myc induced apoptosis. When Myc-1 cells in 0.5FBS-DMEM were incubated with a caspase inhibitor, zVAD, which inhibits activities of all kinds of caspases, the number of floating cells dramatically decreased. Therefore, the inhibition of caspase activities suppressed apoptosis induced by Myc in Myc-1 cells. The result is consistent with previous reports (Kagaya *et al.*, 1997; Juin *et al.*, 1999). Overexpression of c-Myc elevated

**Table I.** TRANSFORMATION OF RAT 3Y1 CELLS BY *myc* AND *myc* MUTANTS WITH ACTIVATED *ras*.

| | Number of foci[a] | |
|---|---|---|
| c-*myc* plasmids | Exp. 1 | Exp. 2 |
| empty vector | 1 | 3 |
| wild type c-Myc | 42 | 38 |
| c-Myc lacking Myc Box I | 17 | 16 |
| c-Myc lacking Myc Box II | 4 | 2 |

[a] 3Y1 cells were transfected with c-*myc* or its deletion mutants' expression vector plus v-Ki-*ras* expression vector. After being cultured for 2 weeks, foci of transformed cells were counted.

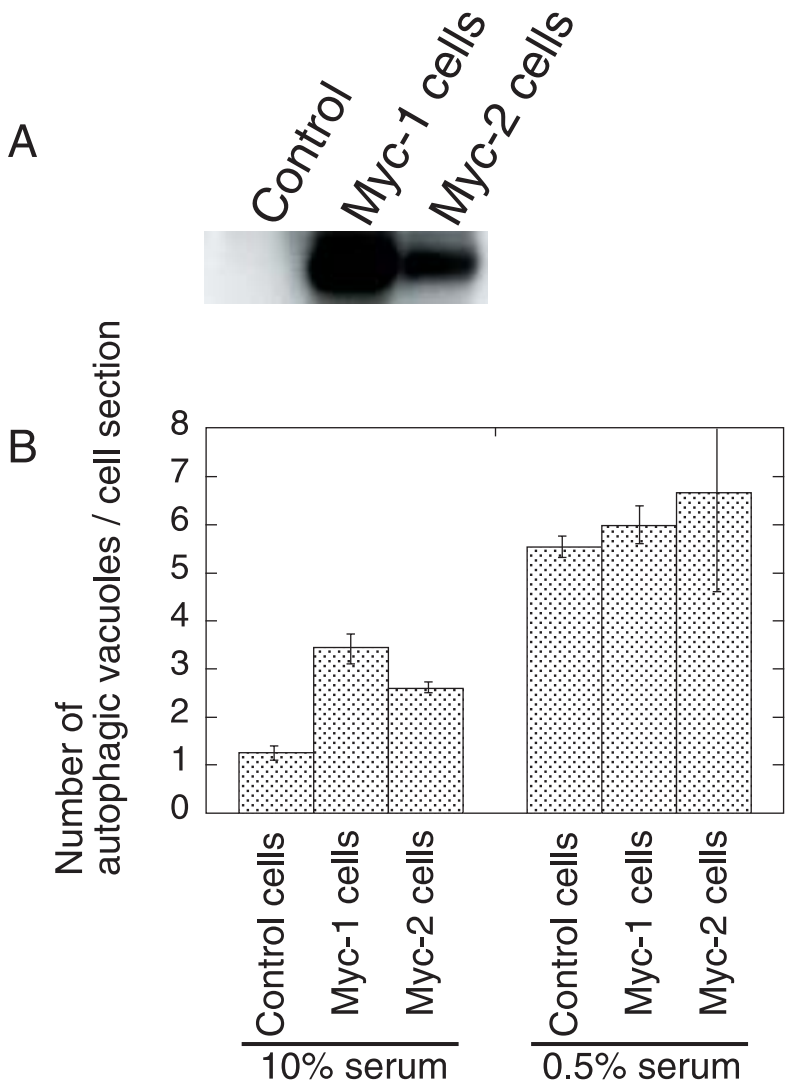


**Fig. 3.** Autophagic vacuole formation in cells overexpressing c-*myc*. (A) Overexpression of c-Myc in Myc-1 and Myc-2 cells. 3Y1 cell clones stably expressing c-*myc* (Myc-1 and Myc-2) were established. Expression of c-Myc protein was detected by Western blotting using anti-c-Myc antibody. Cells carrying the empty vector were used as control cells. (B) Increase in autophagic vacuole formation in Myc-1 and Myc-2 cells in 10FBS-DMEM. Cells were cultured in the presence of 10% or 0.5% serum for 2.5 hours, fixed, and autophagic vacuoles were counted using an electron microscope. Bars indicate standard deviations of 3 experiments. In 10FBS-DMEM numbers of autophagic vacuoles for Myc-1 and Myc-2 were significantly higher than that for control cells ($P<0.05$).

the number of floating cells also in 10FBS-DMEM, although the level of induction of apoptosis was lower (Fig. 5A). This elevation was also suppressed by zVAD, suggesting that Myc induces apoptosis even in the presence of 10% serum in Myc-1 cells. These results suggest that zVAD effectively suppresses Myc-induced apoptosis under these experimental conditions.

On the contrary, treatment with zVAD did not affect the number of autophagic vacuoles in Myc-1 cells in the presence of 10% serum (Fig. 5B). These results suggest that induction of autophagy by c-Myc does not require caspase activities or apoptotic events induced by caspases. Treatment with zVAD also did not affect the number of autophagic vacuoles induced by serum starvation (0.5% serum) in control cells. When Myc-1 cells were cultured for a long time (15.5 hours) in 0.5FBS-DMEM, apoptosis was strongly induced. Since the cytoplasmic structures were highly degenerated under these conditions, it was difficult to distinguish the autophagic vacuoles from the other cytoplasmic structures and count autophagic vacuoles.

## *Discussion*

Activation of c-MycER increased the number of autophagic

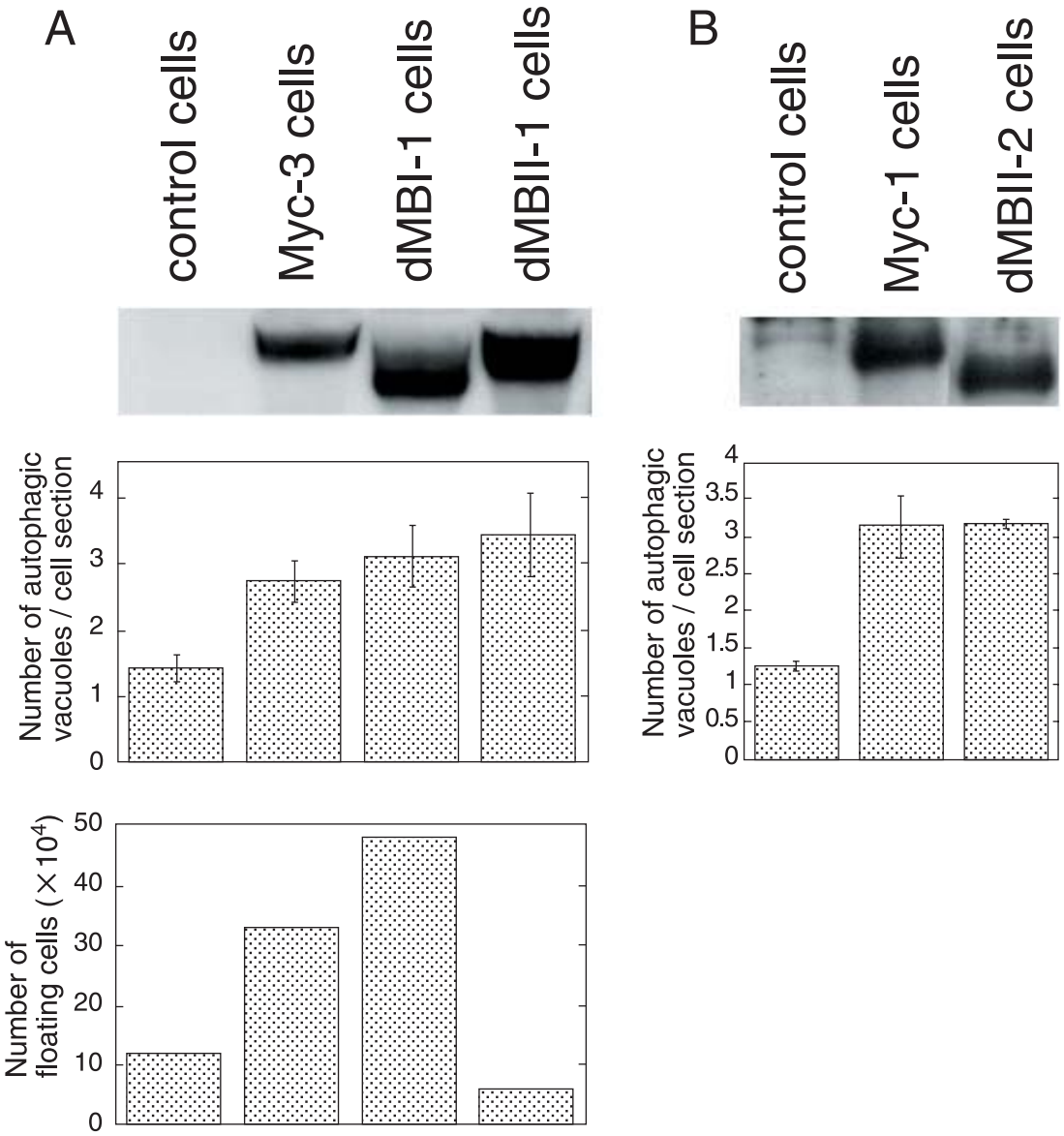


**Fig. 4.** Myc Box I and Box II are not required to stimulate autophagy. (A) Effects of Myc Boxes on autophagy and apoptosis. 3Y1 cells were transfected with c-*myc* or a mutant c-*myc* expression vector, and cell clones stably expressing Myc were selected. Myc-3 cells express wild type c-Myc protein, dMBI-1 cells express c-Myc protein lacking Myc Box I, and dMBII-1 cells express c-Myc protein lacking Myc Box II. Cells carrying the empty vector were used as control cells. c-Myc proteins were detected by Western blotting using anti-c-Myc antibody (upper panel). Myc-3, dMBI-1, dMBII-1, and control cells were cultured in fresh 10FBS-DMEM for 2.5 hours to examine autophagic vacuole formation. Cells were fixed and the number of autophagic vacuoles was counted using an electron microscope (middle panel). Bars indicate standard deviations of 3 experiments. Numbers of autophagic vacuoles for Myc-3, dMBI-1 and dMBII-1 were significantly higher than that for control cells ($P<0.05$). Cells were also cultured in 0.5FBS-DMEM for 15.5 hours to examine cell death. Apoptotic cells were estimated by counting floating cells. A representative result is shown (lower panel). (B) Myc Box II not required for autophagy. The second cell line expressing c-Myc protein lacking Myc Box II was selected (dMBII-2 cells). Expressions of c-Myc (upper panel) and autophagic vacuoles (lower panel) were detected as in Fig. 4A. The cell line expressing wild type c-Myc (Myc-1) described in Fig. 3 and control cells (carrying the empty vector) were also investigated. Bars indicate standard deviations of 3 experiments. Numbers of autophagic vacuoles for Myc-1 and dMBII-2 were significantly higher than that for control cells ($P<0.05$).

vacuoles in the cell-proliferating medium (10FBS-DMEM). Cells overexpressing c-Myc contained more autophagic vacuoles than control cells. 3-Methyladenine (3MA), which is a specific inhibitor of autophagy, suppressed autophagic vacuole formation induced by c-Myc. These results indicate that overexpression of c-Myc stimulates autophagy in 10FBS-DMEM, and suggest that c-Myc can induce autophagy without any stressful condition.

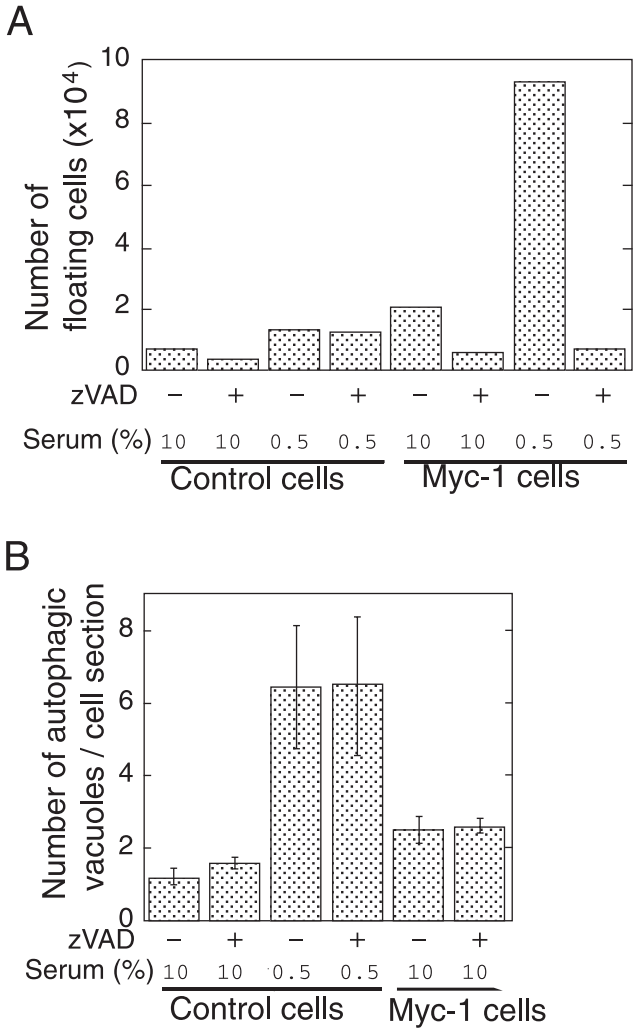


**Fig. 5.** Autophagy stimulated by Myc is not a result of apoptosis induced by c-Myc. (A) Inhibition of apoptosis by the caspase inhibitor zVAD in Myc-1 cells. Cells expressing c-Myc (Myc-1) or cells carrying the empty vector were cultured in the presence of 10% or 0.5% serum, with or without 150 μM zVAD, for 15.5 hours. Apoptotic cells were detected by counting floating cells. (B) autophagic vacuole formation not inhibited by zVAD in Myc-1 cells. Cells were cultured in the conditions described in Fig. 5A. Cells attached to culture dishes were fixed, and autophagic vacuoles were counted using electron microscope. Bars indicate standard deviations of 3 experiments.

c-Myc is a multifunctional protein that participates in the control of apoptosis and tumorigenesis (Evan *et al.*, 1992; Evan and Littlewood, 1998; Hunter, 1997) and of intracellular proteolytic pathways (Gavioli *et al.*, 2001). Treatment of cells with a caspase inhibitor that inhibits all kinds of caspases suppressed the apoptosis but not the autophagy induced by c-Myc. The c-Myc mutant with Myc box II deleted did not induce apoptosis, but it still induced autophagy. Therefore, autophagy does not result from apoptosis induced by c-Myc. The c-Myc mutant with Myc Box II deleted did not induce oncogenic transformation, but still induced autophagy, indicating that autophagy is not a result of transformation induced by c-Myc.

A number of genes, which include ornithine decarboxylase (Bello-Fernandez *et al.*, 1993), cdc25A (Galaktionov *et al.*, 1996), RCC1 (Tsuneoka *et al.*, 1997), cyclin D2 (Bouchard *et al.*, 1999), Id2 (Lasorella *et al.*, 2000), and *mina53* (Tsuneoka *et al.*, 2002), have been identified to be directly transactivated by c-Myc (Dang, 1999). Myc also represses expression of particular genes, including gas1, C/EBPα and cyclin D1 (Grandori *et al.*, 2000; Claassen and Hann, 1999; Lüscher, 2001). These Myc-controlling genes appear to be involved in Myc functions, and the two Myc Boxes have been shown to be important for both the activities of transactivation and repression (Grandori *et al.*, 2000; Claassen and Hann, 1999). In this study, it was found that Myc mutants with either Myc Box I or Myc Box II deleted stimulate autophagy, suggesting that control of gene expression may not be involved in induction of autophagy. However, since it was also reported that a Myc mutant lacking Myc Box I and a Myc mutant lacking Myc Box II can transactivate or repress the expression of some Myc target genes (Li *et al.*, 1994; Grandori *et al.*, 2000), it is still possible that either the transactivation or repression of gene expression by Myc is involved in the control of autophagy.

The process of autophagy has been reported to be defective in hepatoma cells (Kisen *et al.*, 1993; Canuto *et al.*, 1993). Decreased expression of the protein beclin 1, which is involved in induction of autophagy, may contribute to the development or progression of breast and other human malignancies (Liang *et al.*, 1999). These observations suggest that autophagy functions against tumorigenesis. Induction of autophagy leads to the cell death characterized as type II programmed cell death in some cells (Kitanaka and Kuchino, 1999), suggesting that autophagy suppresses tumorigenesis by killing cells. Another possibility is controlling cell mass. Differences in the rate of protein synthesis between tumor cells and their normal counterparts are rather small (Canuto *et al.*, 1993), and it is suggested that cell growth in cancer development is mainly induced by inhibition of protein degradation (Blommaart *et al.*, 1997). Since autophagy had been suggested to be responsible for the degradation of intracellular bulk protein, autophagy may suppress tumorigenesis by decreasing cell mass. In any case, as *myc* is a causal gene for tumorigenesis, induction of autophagy by *myc* may sound paradoxical. But there is a similar case: induction of apoptosis by *myc* as described below.

Myc controls cell division and cell growth (Evan *et al.*, 1992; Hunter, 1997; Evan and Littlewood, 1998). These activities of Myc appear to be involved in tumorigenesis by *myc*. However, overexpression of *myc* alone does not usually lead to tumorigenesis (Land *et al.*, 1983; Tsuneoka and Mekada, 2000; Pelengaris *et al.*, 2002), which may be due to another activity of Myc, apoptosis (Pelengaris *et al.*, 2002). These results suggest that apoptosis functions as a

safeguard to suppress tumorigenesis by *myc* (Pelengaris *et al.*, 2002). Here we reported that *myc* induces autophagy in rat fibroblast cells. This new function does not result from apoptosis or tumorigenesis and may function against tumorigenesis. Thus, *myc* itself may suppress oncogenic transformation by two distinct activities: apoptosis and autophagy.

Recently autophagy induced by another oncogene, the activated *ras*, has been reported (Chi *et al.*, 1999). The mutation of *ras* proto-oncogenes is frequently involved in tumorigenesis, but is very rare in some tumor types such as glioblastomas and gastric cancers. In these cells, oncogenic *ras* induces cell death, accompanied by cellular degeneration due to autophagy. These results suggest that induction of autophagy by the oncogenic *ras* appears to function as a safeguard for cell transformation. Accordingly, induction of autophagy in response to activation of proto-oncogenes may not be an unusual mechanism to prevent neoplasia.

*Acknowledgments.* We thank Dr. M. Eilers for providing vectors for expression of c-Myc (CMV-*myc*), c-Myc with deleted Myc Box I (Δ46-63) (CMV-dMBI), and c-Myc with deleted Myc Box II (Δ128-144) (CMV-dMBII). This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, from the Novartis Foundation (Japan) for the Promotion of Science, and from the Ishibashi Foundation (Japan) for the Promotion of Science.

***References***

Anglade, P., Vyas, S., Javoy-Agid, F., Herrero, M.T., Michel, P.P., Marquez, J., Mouatt-Prigent, A., Ruberg, M., Hirsch, E.C., and Agid, Y. 1997. Apoptosis and autophagy in nigral neurons of patient with Parkinson's disease. *Histol. Histopathol.*, **12**: 25–31.

Bello-Fernandez, C., Packham, G., and Cleveland, J.L. 1993. The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA*, **90**: 7804–7808.

Blommaart, E.F.C., Luiken, J.J.F.P., and Meijer, A.J. 1997. Autophagic proteolysis: control and specificity. *Histochemical J.*, **29**: 365–385.

Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. 1999. Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. *EMBO J.*, **18**: 5321–5333.

Bursch, W., Hochegger, K., Torok, L., Marian, B., Ellinger, A., and Hermann, R. 2000. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. *J. Cell Sci.*, **113**: 1189–1198.

Canuto, R., Tessitore, L., Muzio, G., Autelli, R., and Baccino, F. 1993. Tissue protein turnover during liver carcinogenesis. *Carcinogenesis*, **14**: 2581–2587.

Chen, C.A. and Okayama, H. 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques*, **6**: 632–638.

Chi, S., Kitanaka, C., Noguchi, K., Mochizuki, T., Nagashima, Y., Shirouzu, M., Fujita, H., Yoshida, M., Chen, W., Asai, A., Himeno, M., Yokoyama, S., and Kuchino, Y. 1999. Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. *Oncogene*, **18**: 2281–2290.

Claassen, G. and Hann, S. 1999. Myc-mediated transformation: the repression connection. *Oncogene*, **18**: 2925–2933.

Dang, C.V. 1999. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell. Biol.*, **19**: 1–11.

Eilers, M., Picard, D., Yamamoto, K.R., and Bishop, J.M. 1989. Chimaeras of Myc oncoprotein and steroid receptor cause hormone-dependent transformation of cells. *Nature*, **340**: 66–68.

Evan, G. and Littlewood, T. 1998. A matter of life and cell death. *Science*, **281**: 1317–1322.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z., and Hancock, D.C. 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell*, **69**: 119–128.

Fraser, A. and Evan, G. 1996. A license to kill. *Cell*, **85**: 781–784.

Galaktionov, K., Chen, X., and Beach, D. 1996. Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature*, **382**: 511–517.

Gavioli, R., Frisan, T., Vertuani, S., Bornkamm, G.W., and Masucci, M.G. 2001. c-*myc* overexpression activates alternative pathways for intracellular proteolysis in lymphoma cells. *Nature Cell Biol.*, **3**: 283–288.

Grandori, C., Cowley, S.M., James, L.P., and Eisenman, R.N. 2000. The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu. Rev. Cell Dev. Biol.*, **16**: 653–699.

Gronostajski, R. and Pardee, A. 1984. Protein degradation in 3T3 cells and tumorigenic transformed 3T3 cells. *J. Cell. Physiol.*, **119**: 127–132.

Henriksson, M. and Lüscher, B. 1996. Proteins and the Myc network: essential regulators of cell growth and differentiation. *Adv. Cancer Res.*, **68**: 109–182.

Hunter, T. 1997. Oncoprotein networks. *Cell*, **88**: 333–346.

Juin, P., Hueber, A.-O., Littlewood, T., and Evan, G. 1999. c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release. *Genes Dev.*, **13**: 1367–1381.

Kagaya, S., Kitanaga, C., Noguchi, K., Mochizuki, T., Sugiyama, A., Asai, A., Yasuhara, N., Eguchi, Y., Tsujimoto, Y., and Kuchino, Y. 1997. A functional role for death proteases in s-Myc- and c-Myc-mediated apoptosis. *Mol. Cell. Biol.*, **17**: 6736–6745.

Kimura, G., Itagaki, A., and Summers, J. 1975. Rat cell line 3Y1 and its virogenic polyoma- and SV40-transformed derivatives. *Int. J. Cancer*, **15**: 694–706.

Kisen, G., Tessitore, L., Costelli, P., Gordon, P., Schwarze, P., Baccino, F., and Seglen, P. 1993. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. *Carcinogenesis*, **14**: 2501–2505.

Kitanaka, C. and Kuchino, Y. 1999. Caspase-independent programmed cell death with necrotic morphology. *Cell Death Differ.*, **6**: 508–515.

Klionsky, D.J. and Emr, S.D. 2000. Autophagy as a regulated pathway of cellular degradation. *Science*, **290**: 1717–1721.

Klionsky, D.J. and Ohsumi, Y. 1999. Vacuolar import of proteins and organelles from the cytoplasm. *Annu. Rev. Cell Div. Biol.*, **15**: 1–32.

Land, H., Parada, L.F., and Weinberg, R.A. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature*, **304**: 596–602.

Lasorella, A., Noseda, M., Beyna, M., and Iavarone, A. 2000. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoprotein. *Nature*, **407**: 592–598.

Li, L., Nerlov, C., Prendergast, G., MacGregor, D., and Ziff, E. 1994. c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J.*, **13**: 4070–4079.

Liang, X., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. 1999. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*, **402**: 672–676.

Lüscher, B. 2001. Function and regulation of the transcription factors of the Myc/Max/Mad network. *Gene*, **277**: 1–14.

Malynn, B.A., Alboran, I.M., O'Hagan, R.C., Bronson, R., Davidson, L., DePinho, R.A., and Alt, F.W. 2000. N-*myc* can functionally replace c-*myc* in murine development, cellular growth, and differentiation. *Genes Dev.*, **14**: 1390–1399.

Mortimore, G.E. and Poso, A.R. 1987. Intracellular protein catabolism and its control during nutrient deprivation and supply. *Annu. Rev. Nutr.*, **7**: 539–564.

Nagata, S. 1997. Apoptosis by death factor. *Cell*, **88**: 355–365.

Ohsumi, Y. 2001. Molecular dissection of autophagy: two ubiquitin-like systems. *Nat. Rev. Mol. Cell. Biol.*, **2**: 211–216.

Pelengaris, S., Khan, M., and Evan, G.I. 2002. Suppression of Myc-induced apoptosis in β-cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. *Cell*, **109**: 321–334.

Philipp, A., Schneider, A., Vasrik, I., Finke, K., Xiong, Y., Beach, D., Alitalo, K., and Eilers, M. 1994. Repression of cyclin D1: a novel function of Myc. *Mol. Cell. Biol.*, **14**: 4032–4043.

Prendergast, G. 1999. Mechanisms of apoptosis by c-Myc. *Oncogene*, **18**: 2967–2987.

Sandvig, K. and Deurs, B.V. 1992. Toxin-induced cell lysis: protection by 3-methyladenine and cycloheximide. *Exp. Cell Res.*, **200**: 253–262.

Seglen, P.O., Berg, T.O., Blankson, H., Frengsrud, M., Holen, I., and Stromhaug, P.E. 1996. Structural aspects of autophagy. *Adv. Exp. Med. Biol.*, **389**: 103–111.

Seglen, P.O. and Gordon, P.B. 1982. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc. Natl. Acad. Sci. USA*, **79**: 1889–1892.

Solomon, D., Philipp, A., Land, H., and Eilers, M. 1995. Expression of cyclin D1 mRNA is not upregulated by Myc in rat fibroblasts. *Oncogene*, **11**: 1893–1897.

Tsuchida, N., Ryder, T., and Ohtsubo, E. 1982. Nucleotide sequence of the oncogene encoding the p21 transforming protein of Kirsten murine sarcoma virus. *Science*, **217**: 937–939.

Tsuneoka, M., Koda, Y., Soejima M., Teye, K., and Kimura, H. 2002. A novel Myc target gene, *mina53*, that is involved in cell proliferation. *J. Biol. Chem.*, **277**: 35450–35459.

Tsuneoka, M. and Mekada, E. 2000. Ras/MEK signaling suppresses Myc-dependent apoptosis in cells transformed by c-*myc* and activated *ras*. *Oncogene*, **19**: 115–123.

Tsuneoka, M., Nakano, F., Ohgusu, H., and Mekada, E. 1997. c-*myc* activates RCC1 gene expression through E-box elements. *Oncogene*, **14**: 2301–2311.

Xue, L., Fletcher, G.C., and Tolkovsky, A.M. 1999. Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. *Mol. Cell Neurosci.*, **14**: 180–198.

Yamashita, T., Yamamoto, K., Shiroki, K., Kimura, G., Matsui, K., Osato, T., Shimijo, H., and Fujinaga, K. 1980. Chromosomal alterations of rat cell lines transformed by human adenovirus type-12 virion, whole DNA and left-end DNA fragments. *Int. J. Cancer*, **26**: 435–442.

(*Received for publication, May 9, 2003 and accepted, June 6, 2003*)