Differential Expression of gas and gadd Genes at Distinct Growth Arrest Points during Adipocyte Development

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Abstract
The characterization of growth arrest-associated genes has revealed that cells actively suppress mitotic growth in response to extracellular signals. Mouse 3T3-L1 cells grow arrest at multiple distinct points during terminal differentiation to adipocytes. We examined the expression of growth arrest-specific (gas) and growth arrest- and DNA damage-inducible (gadd) genes as a function of 3T3-L1 growth arrest and adipocyte development. These growth arrest-associated genes are differentially expressed throughout adipocyte development. Some of the gas/gadd genes are preferentially expressed in a subset of growth arrest states. In contrast, gas1 and gas3 are expressed in serum-starved adipoblasts, contact-inhibited adipoblasts, and post-mitotic adipocytes. However, in post-mitotic adipocytes, gas1 and gas3 are induced in response to nutrient deprivation, not altered growth status. gas6 is an exception to the general concordance of mitotic growth arrest and gas/gadd expression in that gas6 is preferentially expressed during the clonal expansion of postconfluent adipoblasts. Combined, the expression patterns indicate that growth arrest-associated genes are regulated by numerous signal transduction pathways throughout a discrete developmental transition.

Introduction
Adipose conversion of cultured 3T3-L1 cells provides a model system for examining the relationship between differentiation and cellular growth control. In the standard differentiation protocol, adipoblasts are cultured to postconfluence, exposed to adipogenic hormones to initiate differentiation, and then maintained in differentiation media to promote the accumulation of cytoplasmic fat droplets (reviewed in Ref. 1). The addition of the adipogenic hormones stimulates the growth-arrested, postconfluent cells to reenter the mitotic cell cycle, which the cells exit again upon withdrawal of the hormones. Thus, a discrete period of mitotic division separates postconfluent adipoblasts from phenotypically differentiated, postmitotic adipocytes. To differentiate, 3T3-L1 cells require the transcription factor C/EBPα2 (2), which has been shown to trans-activate the promoters of several adipocyte-specific genes (3). Furthermore, forced expression of C/EBPα in logarithmically growing adipoblasts results in mitotic growth arrest independent of adipogenesis, whereas its premature expression after exposure to adipogenic hormones hastens differentiation (4). These observations lead us to speculate that C/EBPα may be a component of a signal transduction pathway that coordinates mitotic growth control and phenotypic differentiation.

To better understand the relationship between cellular growth control and adipocyte development, we have characterized the expression of growth arrest-specific (gas) and growth arrest- and DNA damage-inducible (gadd) genes during adipose conversion of 3T3-L1 cells. The gas genes were isolated by virtue of their preferential accumulation in serum-starved NIH-3T3 cells (5), whereas the gadd family was originally described as genes induced in response to DNA damage in Chinese hamster ovary cells (6). In general, within each family, the gas and gadd genes have been observed to be coordinately induced at growth arrest (5–8). We report here that the gas/gadd genes are differentially expressed throughout adipocyte development. Certain of these growth arrest-associated genes, such as gas5, are induced by serum starvation and contact inhibition. Others, including gas1, gas3, and gadd153, are expressed in serum-starved adipoblasts, contact-inhibited adipoblasts, and in postmitotic adipocytes. In differentiated cells, gas1 and gas3 appear to be induced in response to nutrient deprivation of the medium, an expression profile reported previously for gadd153/CHOP (9). gas6 mRNA is exceptional by virtue of its accumulation in growing cells. Specifically, gas6 accumulates in response to the addition of adipogenic hormones that stimulate the postconfluent cells to reenter the cell division cycle. The results indicate that growth arrest-associated genes are targets of multiple signaling pathways and play diverse roles in cellular physiology.

Results
Expression of gas and gadd Genes in 3T3-L1 Cells. To begin characterizing growth arrest states in 3T3-L1 cells, we determined whether gas/gadd mRNAs are elevated in response to serum deprivation of logarithmically growing adipoblasts. 3T3-L1 cells are typically propagated in 10% calf serum (10). We examined the expression of several gas and gadd mRNAs after switching cells to 0.2% serum. Fig. 1 shows the results of a typical Northern blot analysis. Within 24 h incubation in 0.2% serum, gas1, gas3, and gadd153 transcripts are noticeably induced. gas1 is undetectable in logarithmically growing cells and increases throughout the 5-day period examined. gas3 mRNA levels are also elevated in serum-starved cells. However, unlike gas1, gas3 mRNA is detectable in the logarithmically growing cells.

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4 The abbreviation used is: C/EBPα, CCAAT/enhancer binding protein α.
and the level plateaus after 24 h (Fig. 2). The gadd53 homologue CHOP also exhibits elevated levels in response to serum deprivation, but the induced levels are lower than the maximal levels seen at other growth arrest points in 3T3-L1 cells (below). When 24-h serum-deprived cells are switched to 10% serum, mRNA levels for all three transcripts examined decline to preinduction levels within 12 h (Fig. 1). The accumulation of the gas1 and gas3 mRNAs in serum-starved NIH 3T3 cells. Specifically, gas1 and gas3 messages are induced in response to serum deprivation and decline when growth-inhibited cells are stimulated to reenter the cell division cycle (5). Two other mRNAs, gas5 and gas6, are also induced in serum-deprived adipoblasts comparable to their induction in serum-deprived fibroblasts (data not shown). Specifically, gas5 induction is similar to gas1, whereas gas6 exhibits the least induction among the gas genes in serum-deprived cells (5). gadd mRNAs have been shown to be induced in Chinese hamster ovary (CHO) cells in response to several growth arrest signals, including serum deprivation (7). Our results demonstrate that the gadd53 homologue CHOP is induced during 3T3-L1 growth arrest in response to serum deprivation (Fig. 1). Two other gadd transcripts, gadd34 and gadd45, were not detected (data not shown).

We next examined the expression of gas/gadd mRNAs as a function of adipose conversion of 3T3-L1 cells. This developmental transition includes several separate periods of mitotic growth arrest (1). In the standard differentiation protocol, logarithmically growing 3T3-L1 adipoblasts are grown to a postconfluent monolayer, resulting in growth arrest due to contact inhibition. The cells are then treated with differentiation inducers, which results in an increase in cell number during a period of clonal expansion (1). The differentiation inducers are subsequently removed, and the cell number again plateaus and remains constant throughout phenotypic differentiation, the accumulation of cytoplasmic fat droplets. We collected RNA from 3T3-L1 cells throughout this developmental transition and analyzed gas and gadd expression by Northern blots. The results of that analysis are shown in Fig. 2A. All of the growth arrest genes examined, except gadd153, are detectable in logarithmically growing, subconfluent adipoblasts. gas1 accumulates to maximal levels in the postconfluent, day-0 cells, declines during the period of clonal expansion, is induced again on day 3, and is expressed at low levels during phenotypic differentiation (days 4–8). gas3 accumulates on days 0 and 3, similar to gas1, but gas3 is also expressed at maximal levels on alternate days in the phenotypically differentiated cells (days 4–8). gas5 exhibits yet another expression pattern that it accumulates to maximal levels on day 0 only. Finally, consistent with analyses published previously of gadd53 expression (9), we find that CHOP accumulates to maximal levels on alternate days in phenotypically differentiated cells. However, we also detect CHOP expression in 3T3-L1 cells that are growth arrested in response to contact inhibition (Fig. 2A, day 0). gas6 is a notable exception to the general observation that the gas and gadd genes are expressed at growth arrest points during adipocyte development. Specifically, gas6 is maximally expressed on days 1 and 2, coincident with the exposure of cells to differentiation inducers and the period of clonal expansion.

We monitored adipocyte differentiation by the appearance of fat droplets (data not shown) and the accumulation of the 422 mRNA that encodes an intracellular fatty acid-binding protein (11). The pattern of 422 expression (Fig. 2A) is similar to that observed previously (11). To monitor equivalent loading of RNA samples, we have hybridized the same blots to detect two different mRNAs for normalization. The pAL15 cDNA encodes a mitochondrial ribosomal protein subunit whose mRNA abundance is constant from days 0–8 (12). However, we reproducibly find pAL15 mRNA to be less abundant in semiconfluent and confluent cells (e.g., Fig. 2A). We have also probed the blots to detect a ubiquitously expressed tubulin isoform mRNA (13). Tubulin mRNA levels decline throughout phenotypic differentiation but are constant at earlier time points (14). The expression profiles of gas1, gadd6, gas3, and gadd153 are shown graphically for days 0–8 in Fig. 2B. mRNA abundance is expressed as a percentage of the maximum expression of each species after normalization to pAL15. The top graph contrasts the profiles of gas1 and gas6 during clonal expansion (days 1 and 2), while the bottom graph reveals the coordinate fluctuation of gas3 and gadd53 levels during days 5–8.

The mRNA levels of gas1, gas3, and gas5 decline during clonal expansion (Fig. 2, CE). This reduction in gas mRNA levels is coincident with the exposure to differentiation inducers and the accompanying increase in cell number (1). In contrast to the decline in gas1, gas3, and gas5 mRNA on days 1 and 2, gas6 mRNA levels rise dramatically at the same time (Fig. 2). To more precisely correlate gas/gadd expression and mitotic growth arrest points during adipogenesis, we analyzed 3T3-L1 cells for DNA content

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5. P. Cornelius and D. Lane, personal communication.
Fig. 2. Expression profiles of gas and gadd transcripts during adipose conversion of 3T3-L1 cells. A, total RNA was harvested from 3T3-L1 cells throughout the conversion from adipoblasts to adipocytes using a standard protocol (10). Beginning with a semiconfluent (sc) population of adipoblasts, the cells were grown to confluence (c), incubated 48 h postconfluence (day 0), exposed to differentiation inducers for 48 h (days 1 and 2), and refed differentiation media every 48 h thereafter (days 3–8). The periods of growth, including logarithmic growth (LG) and clonal expansion (CE) are indicated. Ten µg of total RNA was analyzed at each step by Northern blot analysis as in Fig. 1. Membranes were successively probed, stripped, and reprobed with cDNAs of gas1, gas3, gas5, gas6, or CHOP (gadd153) and 422 (encoding a fatty acid-binding protein), tubulin, and pAL15 (a ribosomal subunit encoding mRNA). Each blot was analyzed for tubulin and pAL15 expression to normalize the expression profiles. B, the relative abundance of each transcript was determined by quantification of the band intensity (ImageQuant software, Molecular Dynamics); subsequent normalization to the pAL15 signal was obtained for the same sample on the same membrane. The sample expressing a given mRNA at the highest level on days 0–8 was set at 100%, and all other samples are reported relative to that amount.

throughout the developmental transition. Fig. 3 shows the result of a flow cytometric analysis of cells harvested at various time points throughout adipocyte conversion of 3T3-L1 cells. The resulting area graph reveals that the adipoblast are an asynchronous population at semiconfluence that growth arrest in G_, as they progress from confluence to postconfluence (day 0). The cells reenter the mitotic division cycle in response to the addition of differentiation inducers, as evidenced by the decline in the percentage of cells in G_, Twenty-four h after the withdrawal of adipogenic hormones (day 3), the distribution is similar to that of the contact-inhibited cells (day 0) and remains so throughout phenotypic differentiation. Combined with the results presented in Fig. 2, the flow cytometric analysis reveals that the induction of gas1, gas3, and gadd153 coincide with exits from the mitotic division cycle, both in response to contact inhibition and the removal of differentiation inducers.

Regulation of gas and gadd Genes in Post-Mitotic Adipocytes. We noticed that gas3 accumulates to maximal levels on alternate days in post-mitotic, phenotypically differentiated 3T3-L1 adipocytes (Fig. 2), an expression pattern shared by CHOP/gadd153 (Ref. 9 and Fig. 2). gas1 levels also fluctuate during this period (Fig. 2). It has been shown that maximal expression of gadd153 coincides with medium replenishment of cultured adipocytes and is likely a consequence of nutrient deprivation (9). Carlson et al. (9) demonstrated that frequent medium replenishment throughout phenotypic differentiation abrogates the alternate day fluctuations in gadd153 expression. We wondered whether gas3 and gas1 are also regulated by nutrient changes in post-mitotic adipocytes. To test this possibility, we differentiated 3T3-L1 cells with frequent medium replenishment according to Carlson et al. (9). Specifically, 3T3-L1 cells were differentiated as above except, after switching cells to differentiation medium on day 2, the medium was replenished every 8 h instead of every 48 h as in the standard protocol. RNA was prepared for Northern analysis from cells throughout the modified differentiation. A Northern blot analysis of gas1 and gas3, along with gadd153, throughout adipose conversion with frequent medium replenishment is shown in Fig. 4A. Quantification of the mRNA levels after normalization to pAL15 reveals that CHOP (gadd153) is not as abundant in these adipocytes (compare day 8 in Fig. 4B versus Fig. 2B) nor do the levels fluctuate throughout phenotypic differentiation (Fig. 4B), in agreement with Carlson et al. (9). Furthermore, a coordinate change in the expression of gas3 and gas1 along with CHOP is evident in the cells differentiated with frequent medium replenishments (Fig. 4). Thus, gas3 and gas1
Expression during Adipose Conversion

Fig. 3. Flow cytometric analysis of DNA content during adipogenesis. Nuclei were prepared from 3T3-L1 cells at the time points described in Fig. 2 and additionally at half-day time points from days 0.5 to 2.5. Becton Dickinson Cell Fit software was used to calculate the percentage of cells in G1, S, and G2-M phases of the cell cycle, and the resulting data were plotted using the Cricket Graph. The initial exit from the cell cycle in response to contact inhibition (day 0), reentry in response to the presence of adipogenic hormones (days 0–2), and the final exit from the cell cycle after hormone withdrawal (day 3) are all evident in the profile. The profile is unchanged from days 4–8 (data not shown). The software assigns any cells with a staining intensity between the 2N and 4N median as S-phase cells. The 3T3-L1 line has variable ploidy, with the reported chromosome number ranging from 28–144, and concentrated from 30–70 (American Type Culture Collection). The small population of cells reported as S phase on days 0 and 3–8 are likely cells in G0 with large chromosomal content.

appear to be regulated both in response to nutrient deprivation in post-mitotic adipocytes (Fig. 3) and in response to exit from the mitotic division cycle through serum starvation (Fig. 1) or contact inhibition (Fig. 2) of adipoblasts.

We noticed a slight lag in the kinetics of differentiation by morphological criteria in cultures that were differentiated with frequent medium replenishment compared to the standard protocol (data not shown). The lag is evident in the Northern blot analysis in that plateau of the 422 mRNA levels by day 3 in the standard protocol (Fig. 2A) but not until day 4 with frequent medium replenishment (Fig. 4A). The finding prompted us to determine whether there are differences in the final exit from the cell division cycle for adipocytes differentiated with frequent medium replenishments compared to the standard protocol. To evaluate that possibility, we differentiated 3T3-L1 cells in parallel by the standard protocol or with frequent medium replenishments and monitored DNA content by flow cytometry throughout the differentiation process. The result of that analysis are presented in Fig. 5. Medium replenishments began on day 2 when the differentiation inducers were removed (1) and continued at 48-h (standard, S) or 8-h intervals (frequent, FMR). Comparison of the area graphs in Fig. 5 reveals no significant differences in DNA content at various times throughout adipogenesis. Specifically, by day 3, 24 h after the removal of differentiation inducers, both populations are comprised almost exclusively of cells with a G1 DNA content, and both remain so throughout phenotypic differentiation.

Fig. 4. Coordinate regulation CHOP (gadd153) gas3 and gas1 in post-mitotic adipocytes. 3T3-L1 cells were differentiated to adipocytes with frequent media replenishments after day 2 to maintain elevated nutrient levels in the media (9). A, total RNA was harvested from cells throughout adipogenesis, and 10 µg were analyzed by Northern blot as described in Fig. 1. The accumulation of 422 mRNA indicates the progression of phenotypic differentiation. Note that the tubulin probe reveals that the semiconfluent (sc) and day 1 samples are overloaded with respect to other samples. B, relative abundance was determined and plotted as in Fig. 2B.

gas/gadd Genes Examined Are Not Targets of C/EBPα in Differentiated Adipocytes. C/EBPα transactivates numerous adipocyte-specific genes (3) and is essential for adipose conversion of 3T3-L1 (2). We have shown previously that temporal misexpression of C/EBPα in undifferentiated adipoblasts results in a cessation of mitotic growth (4). Specifically, we expressed a steroid hormone-regulated fusion protein of C/EBPα and the estrogen receptor hormone-binding domain, C/EBP-ER, in 3T3-L1 cells and observed estrogen-dependent growth arrest. We have used this cell line to ask whether any of the gas or gadd genes expressed in differentiated adipocytes are regulated by C/EBPα. The C/EBP-ER-expressing 3T3-L1 cell line was differentiated in
Standard Differentiation

Frequent Media Replenishment

Days

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Fig. 5. Frequent media replenishment during phenotypic differentiation does not alter the exit from the mitotic division cycle. Nuclei were prepared from cells differentiated using the standard protocol or with frequent media replenishment, stained with propidium iodide, and analyzed by flow cytometry; the resulting data were plotted as described in Fig. 3. The profiles of both samples are levels from days 4–8 (data not shown). Note that 0.5, 1.5, and 2.5 of the standard differentiation are the same data shown in Fig. 3.

the presence or absence of estrogen throughout phenotypic differentiation. RNA was harvested throughout differentiation, and the expression of the gas and gadd genes was quantified by Northern blot analysis. The results are presented in Fig. 6. In agreement with our previous findings (4), 422, a phenotype-associated target gene of C/EBPα, is elevated on days 4 and 6 in the presence of estrogen as compared to its absence. However, none of the gas/gadd genes expressed in post-mitotic adipocytes (Fig. 2) accumulate to greater levels in response to estrogen stimulation. We conclude that the gas/gadd genes examined are not targets of C/EBPα regulation in post-mitotic adipocytes. The expression profiles of the gas and gadd genes are notably different in the C/EBP-ER cells compared to the parental 3T3-L1 cells (Fig. 2). These differences can most likely be attributed to the low efficiency of differentiation for the C/EBP-ER cells as determined by the number of cells in the population containing fat droplets visible by phase contrast microscopy (2–5%; data not shown). For example, these cells do not exhibit reduced tubulin levels as seen for the parental 3T3-L1 cells (compare Figs. 6 and 2). We reason that the cells do not significantly deplete the media and thus do not significantly induce CHOP (gadd153). Furthermore, the majority of the cells on the plate are not phenotypically differentiated and may be in a growth-arrest state comparable to day 0 or 3 of 3T3-L1 differentiation, characterized by elevated levels of gas1 and gas3 (Fig. 2).

**Discussion**

The results presented here demonstrate the uncoordinated expression of growth arrest-associated genes during a discrete developmental transition: adipose conversion of 3T3-L1 cells. Differential induction during multiple exits from the mitotic division cycle combined with differential expression in post-mitotic adipocytes accounts for most of the variance. gas genes were discovered by virtue of their coordinate accumulation in serum-starved NIH-3T3 cells (5). In adipoblasts, the gas genes examined are coordinately induced in response to serum deprivation, contact inhibition, and the initiation of differentiation (with the exception of gas6). However, in post-mitotic adipocytes, expression of the gas genes varies. gas1 and gas5 levels decline relative to contact-inhibited adipoblasts, similar to gas5 expression in differentiated Friend leukemic cells (15), whereas gas3 is
expressed at high levels in response to nutrient deprivation. *gadd* is also induced by nutrient deprivation of adipocytes (9). To our knowledge, this is the first observation of coordinate regulation of a *gadd* gene and a gas gene independent of growth control. Perhaps these genes cooperate to inhibit adipogenesis. *gadd* (CHOP) can heterodimerize with C/EBPα and act as a repressor of C/EBPα-driven gene transcription (16). It has been suggested that *gadd* accumulation in response to nutrient deprivation opposes adipogenesis by inhibiting C/EBPα stimulation of adipocyte-specific genes (6). We speculate that the coordinate regulation of *gas3* and gas1, which encode plasma membrane proteins, along with *gadd* (CHOP), reflects a need to coordinate activities within the nucleus and at the cell membrane to inhibit adipogenesis. The regulation of *gadd* and *gas3*, and gas1 in response to nutrient deprivation (Fig. 4) seems to be distinct from *gadd* stimulation in response to genomic stress (7) or *gas1* and *gas3* induction in response to serum deprivation and contact inhibition (Figs. 1 and 2; Ref. 5). These regulatory properties suggest that *gadd* and *gas3* may be targets of distinct signaling mechanisms in post-mitotic cells versus growing cells.

*gas6* expression is a notable exception to the general finding that *gas* and *gadd* genes are expressed at growth-arrest states during adipogenesis. *gas6* accumulates to maximal levels during the time that adipoblasts reenter the mitotic division cycle in response to differentiation inducers. Recent characterization of the *gas6* gene product suggests potential roles for this protein in overcoming contact-inhibited growth arrest. The *gas6* protein is a ligand for the axl tyrosine kinase growth factor receptor (17). In addition, *gas6* is a secreted protein related to human protein S (18), a component of a protease cascade that negatively regulates blood coagulation. Our preliminary results reveal that in postconfluent adipoblasts, *gas6* is regulated by dexamethasone (19). *gas6* and CHOP (gadd) are expressed in differentiated adipocytes (Fig. 2), the time that C/EBPα accumulates (19). *gas3* and CHOP (*gadd*) are expressed in differentiated adipocytes (Fig. 2) but likely antagonize adipogenesis (Ref. 9 and this report), whereas C/EBPα stimulates adipogenesis (3, 19). It seems unlikely that C/EBPα would stimulate the transcription of genes involved in an opposing metabolic response. We are conducting molecular screens to identify novel growth arrest-associated genes that are targets of C/EBPα stimulation in post-mitotic adipocytes.

**Materials and Methods**

**Tissue Culture.** 3T3-L1 cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% calf serum. For serum starvation, cells were seeded at a density of 10^5^ cells/ml on a 10-cm dish with DMEM plus 10% calf serum (GIBCO) for 24 h, then the media was changed to DMEM plus 0.2% calf serum. Differentiation of 3T3-L1 cells was as described previously (10). The stable cell line expressing C/EBP-ER was described previously (4). For differentiation, C/EBP-ER cells were incubated to 2 days postconfluence in DMEM with 15% charcoal-stripped calf serum (4), then switched to DMEM without phenol red (GIBCO) and 10% fetal bovine serum (Hyclone) containing dexamethasone (1 x 10^-6 M), isobutylmethylxanthine (115 ng/ml), and insulin (100 ng/ml). After 2 days, the media was changed to DMEM without phenol red plus 15% charcoal-stripped fetal bovine serum, insulin (100 ng/ml), and estrogen (1 x 10^-6 M), or ethanol vehicle. The frequent media replenishment differentiation was conducted according to the modified 3T3-L1 differentiation described by Carlson et al. (9). RNA was collected as described previously (20).

**DNA Probes.** An 108-bp fragment of *gas3* (21) was amplified by reverse transcription-PCR using a sense primer 5'-GGGAATTCCAGAAGGAAGTCAT-3' (nucleotides 79 to 99) and an antisense primer 5'-GTCGAGACTCGAGGTGA3' (nucleotides 1167 to 1188). cDNA was initially denatured at 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 5°C for 45 s, and 72°C for 2 min. pBSgas3 was constructed by subcloning the gas3 fragment into the Xhol site of the Bluescript KS plasmid (pBS). *gas5* (15) was amplified by PCR using a sense primer 5'-AGCCTTTCGGATCCTGTGCGGCAT-3' (nucleotides 1 to 23) and an antisense primer 5'-ATTTGAGCCTGGATCCAGGCAC-3' (nucleotides 425 to 446), which yielded a 446-bp fragment. The gas5 fragment was subcloned into pBS at the BamHI site to give the plasmid pBSgas5. A 482-bp fragment of *gas6* (18) was amplified by PCR using a sense primer 5'-GGTC-CTGAGCTCGAGGTGA3' (nucleotides 79 to 26) and an antisense primer 5'-AGCCTTTCGGATCCTGGAGAAGTGA3' (nucleotides 1600 to 1632) and an antisense primer 5'-ACGCTGCTCGAGGTGA3' (nucleotides 2771 to 2929) with the annealing temperature at 55°C. pBSgas6 was constructed by subcloning the gas5 fragment into the Xhol site of pBS. The gas fragments were amplified from cDNA that was synthesized using the cDNA cycle Kit (Invitrogen) from mRNA isolated from 3T3-L1 cells grown in DMEM with 0.2% calf serum for 72 h. A fragment of CHOP cDNA (16) corresponding to bp 2 to 772 was amplified by PCR using a sense primer 5'-GGGAATTCCAGAAGGAAGTGA3' (nucleotides 3 to 26) and an antisense primer 5'-GGGAATTCCACTTTACGATCATG3' (nucleotides 751 to 772) from cDNA synthesized from mRNA pooled from days 6, 8, 9, and 10 of a standard differentiation of 3T3-L1 cells. pBSCH-10full was constructed by subcloning the CHOP fragment into the EcoRI site in pBS. *gas1* (5) cDNA was provided by L. Phillipson (European Molecular Biology Laboratory, Heidelberg, Germany). *gadd45* cDNA was provided by N. Holfbrook (National Institute on Aging, Baltimore, MD). The 636-bp 422 cDNA (11) was provided by M. D. Lane (Johns Hopkins University, Baltimore, MD), and the 1600-bp tubulin cDNA (13) was provided by D. Cleveland (John Hopkins University, Baltimore, MD). P. Cornelius (John Hopkins University, Baltimore, MD) and M. D. Lane provided the pAL5 cDNA (12). cDNA probes were labeled with [32P]dCTP according to manufacturer's recommendations using a random-primed labeling kit (Pharmacia).

**Northern Blot Imaging.** Images were obtained with a phosphorimager (Molecular Dynamics or Fuji) and exported to Photoshop (Adobe) as 8-bit TIFF files. The im-
ported TIFF files were adjusted for brightness and contrast automatically, despeckled once, and blurred once. The enhanced images were saved as TIFF files, imported into Canvas (Deneba) to be decorated with text, and printed on a dye-sublimation printer. Original unmolested 8-bit TIFF files were archived.

Flow Cytometry Analysis. Cells from a 10-cm dish were scraped into 10 ml of PBS, pelleted in a centrifuge (1500 × g for 3 min), resuspended in 1.5 ml PBS, and stored in liquid nitrogen. For analysis, cells were thawed, pelleted at room temperature (7000 × g 30 s), and resuspended in 1.5 ml propidium iodide staining solution (0.1% sodium citrate, 0.3% NP40, 100 µg/ml RNase A, and 50 µg/ml propidium iodide). The nuclei were incubated in staining solution for 30 min on ice. The stained cells were analyzed in a Becton Dickinson FACScan analyzer using Cell Fit software. A particle size gate was defined using area versus width, and 2 × 10^4 gated events were scored for each sample.

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References